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Biochemical Signal Transmitted by Fc Receptor for Immunoglobulin G_{2a} of a Murine Macrophage-like Cell Line, $P388D_1$: Mode of Activation of Adenylate Cyclase Mediated by Immunoglobulin G_{2a} Binding Proteins†

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Received November 13, 1985; Revised Manuscript Received April 7, 1986

ABSTRACT: The effects of immunoglobulin G_{2a} binding proteins isolated from $P388D_1$ cells on adenylate cyclase of cyc^- cells were investigated to explore a potential role of $Fc\gamma_{2a}$ receptor in the activation of the adenylate cyclase system. Immunoglobulin G (IgG) binding proteins obtained from the detergent lysate of $P388D_1$ cells by affinity chromatography on IgG–Sepharose were separated into two fractions (denoted as IgG-B1 and IgG-B2) by Sephadex G-100 gel filtration in the presence of 6 M urea. Polyacrylamide gel electrophoretic analysis in the presence of sodium dodecyl sulfate revealed that the major component in the IgG-B1 fraction was a protein of molecular weight near 50000, whereas the IgG-B2 fraction contained two major components of molecular weight near 25000 and 17000. Both IgG-B1 and -B2 proteins can be inserted into liposome consisting of phosphatidylcholine and phosphatidylethanolamine. Liposomes containing IgG-B1 proteins effectively inhibited EA_{2a}, but not EA_{2b}, rosetting by either S49 or $P388D_1$ cells, suggesting their proper orientation within liposome, whereas IgG-B2-containing liposome failed to do so. Simultaneous fusion of the liposomes containing IgG-B1 and -B2 proteins with guanine nucleotide binding stimulatory (G/F) protein/$Fc\gamma_{2a}$R-deficient cyc^- cells resulted in the formation of the hybrid membrane whose adenylate cyclase responds to immune complex formed with IgG_{2a}-subclass antibody (IC_{2a}) by about a 2.7-fold increase in the activity over the control (hybrid membrane between cyc^- cells and liposome containing no protein). The response appeared to be specific, since IC_{2b} failed to stimulate the enzymatic activity of this hybrid membrane. Furthermore, IgG-B1 and -B2 proteins were able to confer their activating effects on the enzyme only in concert, since the fusion of liposomes containing either type of protein alone with cyc^- cells did not result in the activation of adenylate cyclase of cyc^- membrane. IgG-B1 and -B2 proteins could also confer their activating effects in concert to the enzyme in cholate-solubilized forms. Such activation was dependent on the concentration of IC_{2a}, suppressed by the chelating agent ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and significantly inhibited by trifluoperazine, suggesting potential involvement of Ca^{2+} and calmodulin in the activating process. Since the activation of adenylate cyclase of cyc^- cells mediated by IgG-B1 and -B2 proteins was not augmented in vitro by either addition of NaF or deletion of GTP and since IgG-B1 and -B2 proteins inserted into liposome or in cholate-solubilized form had neither detectable GTPase activity nor a cholera toxin sensitive ADP-ribosylation site, the mechanism by which liposome-inserted or cholate-solubilized IgG-binding proteins confer their activating effect on cyc^- cell adenylate cyclase may be different from that mediated by G/F protein in a classical manner.

Murine macrophages and macrophage-like 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 antibody-dependent cell-mediated cytotoxicity (Perlman et al., 1972), suppression of humoral immune response (Uhr & Moller, 1968) or B cell differentiation (Kolsch et al., 1980) by circulating immune complexes, or 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 should transmit, upon binding of specific ligands, signals unique to each type that trigger and maintain a complex program of biochemical events, involving the generation, amplification, and propagation of a series of signals, leading to

† This work was supported in part by a grant (CA 35977) from the National Cancer Institute.
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[1] Abbreviations: EA, SRBC coated with IgG-class monoclonal anti-SRBC antibody; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $Fc\gamma R$, receptor for the Fc portion of IgG; G/F protein, guanine nucleotide binding stimulatory protein; Gpp(NH)p, 5′-guanylyl imidodiphosphate; HBSS, Hank's balanced salt solution without Mg^{2+} and Ca^{2+}; IC_{2a} and IC_{2b}, immune complexes formed with IgG_{2a}- and IgG_{2b}-subclass monoclonal antibodies, respectively; IgG, immunoglobulin G; Ni, guanine nucleotide binding inhibitory protein; PBS, phosphate-buffered saline (0.15 M, pH 7.4); PC, *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphocholine; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes; TBS, Tris-HCl-buffered saline (0.15 M, pH 8); TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/86/0425-4388$01.50/0

the modulation of cellular functions. We have previously shown that a signal to trigger the arachidonic acid metabolic cascade through 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). Prostaglandins, which are newly synthesized by $P388D_1$ cells, then activate the membrane adenylate cyclase via prostaglandin receptors. On the other hand, the binding of antigen-complexed IgG_{2a} (IC_{2a}) to cell surface $Fc\gamma_{2a}R$ appears to directly cause the intracellular accumulation of cAMP by a process that is not affected by a stimulator of guanine nucleotide binding stimulatory (G/F) protein (Nitta & Suzuki, 1982b). The latter findings led us to a question of whether or not $Fc\gamma_{2a}R$ confers its effect directly on the catalytic subunit of the membrane adenylate cyclase system. We have examined this question by testing the ability of IgG_{2a}-binding proteins, isolated from $P388D_1$ cells and inserted into liposome, to activate adenylate cyclase of G/F protein deficient cyc^- cells, particularly because cyc^- cells were found not to carry $Fc\gamma_{2a}R$ on their surface. The data presented in this paper will demonstrate that such liposome-inserted or cholate-solubilized $Fc\gamma_{2a}R$ proteins indeed activate the adenylate cyclase of cyc^- membrane only in the presence of IC_{2a} and a second protein component(s) that copurifies (copurify) with $Fc\gamma_{2a}R$ materials during the affinity chromatography step.

Materials and Methods

Cells. The murine macrophage-like 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 US 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 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 RPM1 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 at 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 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, G/F 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 an intact catalytic unit of adenylate cyclase but lacks G/F protein (Bourne et al., 1975; Ross & Gilman, 1977; Naya-Vigne et al., 1978). 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 [^{14}C]leucine (0.33 μCi/mL, Schwarz/Mann, Spring Valley, NY) for 16 h in the RPM1 1640 culture medium from which leucine was deleted, as described (Fernandez-Botran & Suzuki, 1985). This procedure usually yielded IgG-binding proteins of the specific radioactivity of 3000 cpm/10 μg of protein.

Radioiodination of $P388D_1$ Cells. Cells (5×10^8) were radioiodinated at 4 °C with 1 mCi of ^{125}I (carrier free, NaOH solution, pH 7–11, 100 mCi/mL; Amersham, Arlington Heights, IL) by the lactoperoxidase-catalyzed method (Morrison & Bayse, 1970) with the use of Enzymobeads (Bio-Rad, Richmond, CA) (Thorell & Johansson, 1971) in the presence of cytochalasin D (10 μg/mL) as described (Suzuki et al., 1980, 1982).

Preparation of Lysate. Cells that were metabolically and/or externally radiolabeled were washed 3 times with cold Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS; Flow Lab, 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. Clear supernatants obtained were 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-hexadecylglycero-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 equilibrated against buffer I. Isolation and purification of PC- and IgG-binding proteins were carried out as described (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).

Preparation of Liposome. Phosphatidylcholine and phosphatidylethanolamine (both from Sigma, St. Louis, MO) were dissolved in a tube with 15 mL of chloroform/methanol (2/1 v/v) to a final concentration of 2.5 mM each and dried with the use of a rotary evaporator. Residual solvent was removed further by evacuating a tube for 5 min with the use of a vacuum pump. Fifteen milliliters of Tris-HCl buffer (10 mM, pH 7.4) containing 100 mM NaCl was added to the dried sample. The sample was then sonicated for 5 min and cooled. The sonication was repeated 6 more times. The sample was then centrifuged for 5 min at 100000*g* to remove lipids that were not converted to unilamellar liposome. The resultant liposome was stored at 4 °C in 15 mL of Tris-HCl buffer (10 mM, pH 7.4) containing 100 mM NaCl.

Insertion of IgG- or PC-Binding Proteins into Liposome. This was carried out by a modification of the method described by Cerione et al. (1983a,b). Briefly, IgG- or PC-binding proteins obtained from the detergent lysate of biosynthetically radiolabeled P388D$_1$ cells were first dialyzed against 9 mM Tris-HCl buffer, pH 7.4, containing NaCl (90 mM), Triton X-100 (0.5%), and bovine serum albumin (BSA, 4 mg/mL) (buffer IV). The dialyzed proteins (14–30 μg in 50 μL) were mixed with 600 μL of liposome suspension in buffer IV and were incubated for 45 min at 4 °C. SM-2 resin (~0.4 g wet weight; Bio-Rad, Richmond, CA) was then added, and the mixtures were stirred for 30 min at 4 °C. The resin was removed by centrifugation for 5 min at 1000 rpm. The removal of Triton X-100 by SM-2 resin was repeated 2 more times. As judged by the radioactivity due to a trace amount of [^{3}H]Triton X-100 (Amersham) included in buffer IV, this procedure removed >99.5% of the detergent in the original sample. Poly(ethylene glycol) (PEG) was added to the detergent-free supernatants to a final concentration of 30%. After being incubated for 10 min at 25 °C, the mixtures were diluted 10–20-fold with 10 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl and centrifuged for 90 min at 250000*g* at 4 °C. The resultant sediments were resuspended in 15 mL of the same buffer and stored at 4 °C. The efficiency of the insertion of proteins into liposome ranged from 15 to 37%.

Fusion of cyc$^-$ Cells with Liposome Containing IgG- or PC-Binding Proteins. This was carried out by a modification of the methods described by Schramm (1979) and Strulovici et al. (1983). Briefly, cyc$^-$ cells grown in culture (2×10^7) were harvested, washed 3 times with HBSS, and mixed with 200 μL of liposome preparations. The mixtures were cooled to 0 °C, added to 15 μL of lipids [10 mg of lecithin, 0.5 mg of lysolecithin, and 1 mg of stearylamine (all from Sigma) sonicated in 1 mL of 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA], and incubated for 5 min at 10 °C. Fifteen microliters of 10 mM $MgCl_2$ was then added to the mixture. Five minutes later, 500 μL of PEG solution (520 mg of PEG in 480 μL of buffer V consisting of 10 mM Tris-HCl buffer, pH 7.5, containing 95 mM NaCl, 5 mM KCl, 4.8 mM $MgCl_2$, 5 mM glucose, and 2 mM ATP) was added to the mixtures at 30 °C. One hundred seconds later, 0.2, 0.3, 0.5, 1.5, 3, 5, and 7 mL of buffer V were added sequentially at 2-min intervals. The mixtures were then cooled to 0 °C and centrifuged for 10 min at 1500 rpm at 4 °C. The cells fused with liposome (hybrid) were homogenized in 25 mM Tris-HCl buffer, pH 7.5, containing 2.5 mM $MgCl_2$ and 25 μM $AlCl_3$ and centrifuged for 5 min at 12 000 rpm. The supernatants obtained were assayed for adenylate cyclase.

Preparation of Cholate Extracts. These were prepared by the method of Sternweiss and Gilman (1979). Briefly, membranes (5 mg/mL) of P388D$_1$, S49, or cyc$^-$ cells were solubilized with 25 mM sodium cholate in buffer III. After being shaken for 1 h at 0 °C, insoluble materials were removed by centrifugation at 200000*g* for 1 h. Extracts containing approximately 2 mg of protein/mL were then heated at 25 °C for 20 min to inactivate adenylate cyclase activity in these preparations.

Adenylate Cyclase Assay. This was performed by the method of Salomon (1979) as described (Fernandez-Botran & Suzuki, 1984). Briefly, assay was initiated by the addition of either partially purified membranes (10 μg of protein), cyc$^-$ cell homogenate, hybrid homogenate, or cholate extracts 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), BSA (0.1 mg/mL), 3-isobutyl-1-methylxanthine (1 mM), and [α-^{32}P]ATP [(2–6) $\times 10^6$ cpm]. All of the chemicals except [α-^{32}P]ATP in assay mixture were obtained from Sigma (St. Louis, MO). [α-^{32}P]ATP (>400 Ci/mmol) was from ICN (Irvine, CA). After incubation for 30 min at 37 °C, the reaction was stopped by adding 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 the internal standard [^{3}H]cAMP thus obtained was processed for liquid scintillation counting. Adenylate cyclase activity was expressed as picomole of cAMP formed per milligram 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, hybrid membranes containing either IgG- or PC-binding proteins or membranes of P388D$_1$, S49, or cyc$^-$ cells were incubated for 15 min at 37 °C in a final volume of 100 μL of 50 mM imidazole–HCl 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 adenylylimidodiphosphate, 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 reactions were 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.

Protein Kinase Assay. Protein kinase activity potentially associated with IgG- and PC-binding proteins was assayed by the method described by Kikkawa et al. (1983). Briefly, IgG-B1, IgG-B2, or PC-binding proteins (5–20 μg/assay) previously dialyzed against 25 mM Tris-HCl buffer, pH 7.5, containing Lubrol PX (1%) were incubated for 30 min at 30 °C with calf thymus histone (250 μg/assay) (Sigma) and [γ-^{32}P]ATP [0.25 mM, sp act. of (5–15) $\times 10^4$ cpm/nmol] in the final volume of 250 μL of 20 mM Tris-HCl buffer, pH 7.5, containing magnesium acetate (5 mM) and calcium chloride (0.5 mM) or EGTA (0.5 mM). At the end of incubation period, the reaction was stopped by addition of 3 mL of 25% trichloroacetic acid (TCA). The precipitate was collected by filtering the assay mixture through a 0.45-μm Amicon HA filter and washed with an additional 12 mL of

25% TCA. The filter was then processed for counting in a scintillation counter.

ADP-Ribosylation of Membranes and of IgG- and PC-Binding Proteins. This was carried out as described by Hildebrandt et al. (1983). Briefly, membranes (2–3 mg of protein/mL) of hybrids, S49, P388D$_1$, or cyc$^-$ cells, or cholate-solubilized IgG- or PC-binding proteins (10 μg/mL) were incubated at 32 °C for 30 min in a final volume of 100 μL in 0.1 M potassium phosphate buffer, pH 7.5, containing 20 μM [^{32}P]NAD (500 mCi/mmol, New England Nuclear), 1 mM ATP, 0.5 mM GTP, 15 mM thymidine, 5 mM DTT, and 20 μg/mL cholera toxin previously activated for 15 min at 32 °C in the presence of 40 mM DTT. The reactions were terminated by addition of cold buffer, membranes were collected by centrifugation, washed twice with the buffer, and solubilized in 2% SDS. The solubilized membrane proteins and cholate-solubilized IgG- or PC-binding proteins were subjected to polyacrylamide gel (7.5%) electrophoresis in the presence of SDS by the method of Laemmli (1970). The gels were stained with Coomassie blue and analyzed for incorporation of ^{32}P by liquid scintillation counting of 2-mm slices. The association of ^{32}P with the protein of M_r 42 000–45 000 was regarded as a positive ADP-ribosylation of G/F protein.

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 IgG-binding proteins.

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-S.1 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. SRBC coated with antibodies (EA$_{2a}$ or EA$_{2b}$) were prepared by incubating SRBC (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. EA rosette formation by P388D$_1$, S49, or cyc$^-$ cells was assessed under a light microscope by enumerating the percentages of cells binding three or more EA among a minimum of 200 cells following the incubation of cells (5 × 10^5/mL) for 2 h at 37 °C and for additional 16 h at 4 °C with either EA$_{2a}$ or EA$_{2b}$ (2 × 10^7 sensitized SRBC).

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 method (1951).

Results

Our previous studies with P388D$_1$ cells demonstrated that the binding of IC$_{2a}$ to Fcγ_{2a}R on the cell surface leads to the increased accumulation of intracellular cAMP (Nitta & Suzuki, 1982b). This process appears to require no participation of G/F protein, since Fcγ_{2a}R-triggered elevation of cAMP level was (1) not affected by the known stimulator 5'-guanylyl imidodiphosphate [Gpp(NH)p] (Rodbell et al., 1972; Pfeuffer & Helnreich, 1975; Ross & Gilman, 1980) or the uncoupler Mn^{2+} (Linbird et al., 1979) of G/F protein and (2) synergistically increased with the β-adrenergic receptor mediated stimulation of adenylate cyclase, which requires the participation of G/F protein. These observations thus raised a question whether or not the IC$_{2a}$–Fcγ_{2a}R interaction directly activates the catalytic subunit of adenylate cyclase. This possibility can be examined by investigating the effect of Fcγ_{2a}R material isolated from P388D$_1$ cells on the adenylate cyclase activity of the cyc$^-$ cell membrane, which have been shown to lack G/F protein (Bourne et al., 1975; Ross & Gilman, 1977; Naya-Vigne et al., 1978; Ross et al., 1978), only if cyc$^-$ cells do not possess Fcγ_{2a}R on their surface. Therefore, the potential presence of Fc$_\gamma$Rs on the surface of cyc$^-$ cells and their parent cell line S49 was first investigated by EA rosetting technique. Results showed that about 32 and 44% of S49 cells, which possess a complete adenylate cyclase system, formed rosettes with EA$_{2a}$ and EA$_{2b}$, respectively. cyc$^-$ cells, which lack G/F protein, were found to form rosettes with only EA$_{2b}$ (about 39% of the cells examined) but not at all with EA$_{2a}$. Since neither S49 nor cyc$^-$ cells bound unsensitized SRBC, these results suggest that (1) G/F protein deficient cyc$^-$ cells do not express Fcγ_{2a}R on their surface, whereas S49 cells do so, and (2) both cell types possess Fcγ_{2b}R to an approximately same proportion. A potential direct effect of Fcγ_{2a}R on the catalytic subunit of adenylate cyclase was then examined by assaying for the enzymatic activities of cyc$^-$ membranes fused with liposome containing the IgG-binding proteins isolated from P388D$_1$ cells as follows.

Isolation and Fractionation of IgG-Binding Proteins. In order to obtain a sufficient quantity of IgG-binding proteins, the detergent lysate of 5 × 10^9 P388D$_1$ cells (containing 6% of cells previously biosynthetically radiolabeled with [^{14}C]-leucine and another 6% of cells externally radioiodinated with ^{125}I) was passed through a tandem column consisting of Fab–, PC–, and IgG–Sepharose connected in this order as described under Materials and Methods. After thorough washing with buffer I, the columns were disconnected, and the materials bound to IgG– and PC–Sepharose were separately eluted with buffer II. Keeping with the previously reported results (Suzuki et al., 1982), on the basis of trichloroacetic acid (TCA) precipitable ^{125}I counts, about 0.4 and 1.2% of the original material were obtained as IgG- and PC-binding proteins, respectively. They were then separately passed through a column (2.5 × 40 cm) of Sephadex G-100 that was previously equilibrated against buffer II. As depicted by Figure 1, about 55% of IgG-binding proteins applied to this column were eluted in the void volume, whereas about 45% were eluted as a broad shoulder material that followed the first peak. These two fractions were denoted as IgG-B1 and IgG-B2 proteins, respectively, and separately subjected to a second gel filtration over the same column to minimize cross-contamination with each other. IgG-B1 proteins were then dialyzed against deionized water and subjected to isoelectric focusing in a pH gradient formed with carrier ampholyte pH 5–10 in the presence of 6 M urea. As described earlier (Suzuki et al., 1982), IgG-B1 proteins focused sharply at pH near 4.5, which is the interface between the lower range of the pH gradient and the anode solution. About 90% of PC-binding proteins that were eluted as a sharp peak from Sephadex G-100 in the void volume were pooled, dialyzed against deionized water, and also electrofocused. PC-binding proteins focused sharply at pH 5.8 as reported earlier (Suzuki et al., 1982). The electrofocused IgG-B1 and PC-binding proteins were separately extensively dialyzed at 4 °C against buffer I to remove sucrose, urea, and ampholyte. As shown by Figure 2, SDS–PAGE analysis in the presence of 2-mercaptoethanol revealed

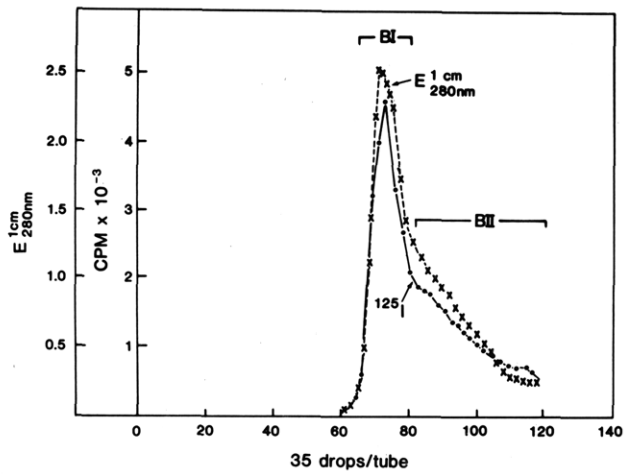

FIGURE 1: Sephadex G-100 gel filtration pattern of the IgG-binding proteins obtained from the detergent lysate of P388D$_1$ cells (5×10^9) by affinity chromatography with the use of IgG–Sepharose. The column (2.5 × 45 cm) of Sephadex G-100 was previously equilibrated against buffer II.

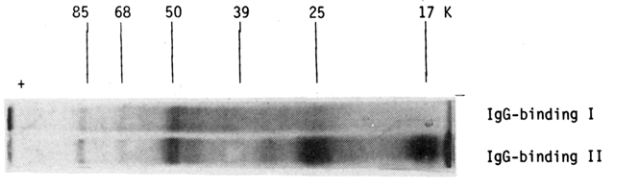

FIGURE 2: SDS–PAGE pattern of IgG-B1 and -B2 proteins. About 5 μg each of IgG-B1 and -B2 proteins was electrophoresed in a 10% slab gel by the method of Laemmli (1970). Proteins were visualized by silver staining. Numbers indicate the molecular weight of marker proteins.

Table I: Efficiency of Insertion of IgG- and PC-Binding Proteins into PE–PC Liposomes[a]

proteins	% proteins inserted into liposomes in expt I	II	III
PC-B	37	37	35
IgG-B1	20	17	19
IgG-B2	20	15	14

[a] IgG-B1 (24 μg), IgG-B2 (14 μg), and PC-B (24 μg) proteins obtained from metabolically radiolabeled P388D$_1$ cells were inserted into PE–PC (1/1 w/w) liposomes as described under Materials and Methods. The degree of protein incorporation was assessed by measuring the radioactivity (^{14}C) associated with liposomes.

that IgG-B1 proteins contain a major band migrating as a protein of molecular weight of about 50 000 and two minor bands corresponding to molecular weight of about 85 000 and 68 000, respectively. IgG-B2 proteins contained two major bands migrating as proteins of M_r of 25 000 and 17 000, respectively, and a minor band migrating as protein of M_r near 50 000.

Insertion of IgG-B1, IgG-B2, and PC-Binding Proteins into Liposomes. In order to assess the capability of IgG-B1 and IgG-B2 proteins to stimulate the adenylate cyclase activity of cyc$^-$ cells, 24 μg of IgG-B1 and 14 μg of IgG-B2 proteins were separately inserted into liposomes consisting of phosphatidylcholine and phosphatidylethanolamine (1/1 w/w) as described under Materials and Methods. As control, PC-binding proteins (24 μg) were also separately inserted into liposomes by the same method. As summarized in Table I, on the basis of ^{14}C radioactivity measurement, the incorporation of IgG-B1 and IgG-B2 proteins in three separate experiments varied from 20 to 17% and from 20 to 14%, respectively, whereas that of PC-binding proteins varied from 37 to 35%. If these proteins

Table II: Inhibition of EA Rosette Formation by Liposomes Containing IgG-B1, IgG-B2, and PC-B Proteins[a]

proteins in liposomes	% inhibition of EA rosette formation: S49 EA_{2a}	S49 EA_{2b}	P388D$_1$ EA_{2a}	P388D$_1$ EA_{2b}
none	0	0	0	0
IgG-B1				
2.5 μg	0	0	10	0
5.0 μg	9	0	ND	ND
10.0 μg	43	0	50	0
IgG-B2				
10.0 μg	0	0	0	0
PC-B				
2.5 μg	3	29	2	15
5.0 μg	3	50	4	20
10.0 μg	0	32	7	25

[a] P388D$_1$ or S49 cells (5×10^6) were preincubated at 4 °C for 30 min with liposome preparations (200 μL) containing various amounts of various proteins or no protein in the total volume of 400 μL. EA_{2a} or EA_{2b} (100 μL of 1% suspension) was added to the mixtures, which were then incubated at 4 °C for additional 16 h before the microscopic examination for the rosette formation. About 30% of S49 cells and 90% of P388D$_1$ cells formed rosettes with either EA_{2a} or EA_{2b} in the absence of liposome preparations.

Table III: Efficiency of Fusion of IgG-B1-Containing Liposomes with cyc$^-$ Cells[a]

proteins in liposomes (μg)	proteins inserted (μg)	% fused
19.8	7.2	36.4
9.9	5.9	59.3
5.0	3.4	68.6
2.5	1.7	69.9
1.25	1.25	100

[a] Liposomes containing various amounts (1–20 μg) of IgG-B1 proteins metabolically radiolabeled with [^{14}C]leucine were fused with 2×10^7 cyc$^-$ cells as described under Materials and Methods. The degree of the insertion was estimated by measuring the radioactivity associated with the cells, using the specific radioactivity of 3000 cpm/10 μg of IgG-B1 proteins.

were inserted into liposome in a proper orientation, they should be able to inhibit the rosette formation between P388D$_1$ or S49 cells and EA preparations, since our previous data showed that IgG-B1 and PC-binding proteins specifically bind to the Fc portion of IgG_{2a} and IgG_{2b}, respectively (Suzuki et al., 1982). As shown by Table II, IgG-B1 protein inserted liposomes were found to inhibit at 10 μg about 50 and 43% of EA_{2a} rosetting by P388D$_1$ and S49 cells, respectively, but none of EA_{2b} rosetting by either type of cells. PC-binding protein inserted liposomes inhibited at 10 μg about 32 and 25% of EA_{2b} rosetting by S49 and P388D$_1$ cells, respectively. This liposome preparation failed to inhibit EA_{2a} rosetting by S49 cells but did inhibit about 7% of EA_{2a} rosetting by P388D$_1$ cells. IgG-B2 protein inserted liposomes did not affect either EA_{2a} or EA_{2b} rosetting by S49 or P388D$_1$ cells. This is probably due either to the presence of relatively low concentration of contaminating IgG-B1 proteins in IgG-B2 proteins or to the difference in the biological functions of M_r 50 000 proteins present in both IgG-B1 and -B2 proteins.

Activation of Adenylate Cyclase of cyc$^-$ Cells with Liposome-Inserted IgG-Binding Proteins. The ability of IgG-B1 and -B2 proteins to activate adenylate cyclase of cyc$^-$ cells was then investigated by assaying for the enzymatic activities of the hybrid membranes obtained from cyc$^-$ cells fused with liposome preparations. To this end, the efficiency of the fusion carried out by the method of Schramm (1979) was first estimated by measuring the radioactivities associated with hybrid cells after fusion of cyc$^-$ cells with [^{14}C]leucine-labeled IgG-B1

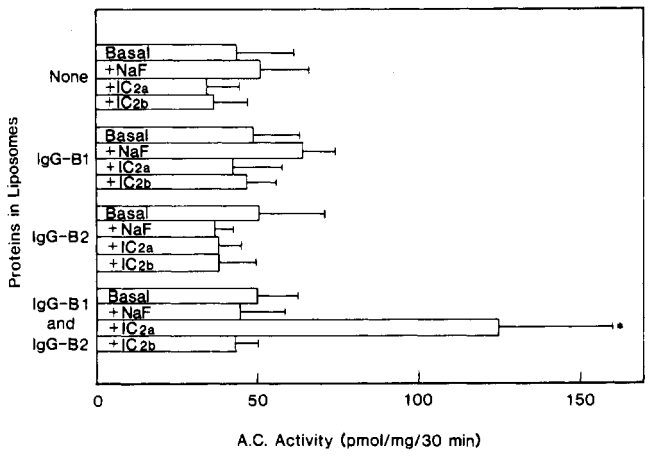

FIGURE 3: Changes in the adenylate cyclase activities of cyc⁻ membranes fused with liposome containing no (control) or IgG-binding proteins in response to NaF (10 mM), IC_{2a} (10 μg/mL), or IC_{2b} (10 μg/mL). Preparation of liposomes and fusion with cyc⁻ cells was carried out as described under Materials and Methods. The amount of IgG-B1 and -B2 proteins in the resulting hybrid membranes were about 1.5 μg/2 × 10^7 cells. Basal adenylate cyclase activity of cyc⁻ membranes reconstitued with the liposome containing the cholate extracts (40 μg of protein) of S49 cells was 1387 ± 235 pmol mg^{-1} $(30\ min)^{-1}$ *. (*) indicates $p < 0.02$.

protein. As summarized by Table III, the fusion efficiency increased by lowering the amount of the protein incorporated into liposome, ranging from 36.4% for 19.8 μg to 100% for 1.24 μg per 2 × 10^7 cyc⁻ cells. However, when 10–20 μg of proteins was used, a larger amount of the protein could be incorporated into the cells, despite the relatively low efficiency. Therefore, these amounts of proteins were routinely used for fusion with 2 × 10^7 cyc⁻ cells in the following experiments. The efficiency of the fusion of liposomes containing PC-binding or IgG-B2 proteins ranged also about 35–60%, when 10–20 μg of proteins was used.

Adenylate cyclase activities of the hybrid membranes containing 2–3.5 μg of IgG-B1, IgG-B2, or PC-binding proteins per 2 × 10^7 cells were then assayed in the presence or absence of 10 mM NaF or of the soluble immune complexes (10 μg/mL) consisting of $F(ab')_2$ of bovine IgG and murine monoclonal anti-bovine IgG of either IgG_{2a} or IgG_{2a} subclasses (referred to as IC_{2a} and IC_{2b}, respectively). Results illustrated by Figure 3 show that the fusion of cyc⁻ cells with liposomes containing no protein or IgG-B1 or IgG-B2 proteins resulted in no significant alteration in the adenylate cyclase activity under all conditions examined. However, the fusion of cyc⁻ cells with the 1:1 mixture of the liposomes containing IgG-B1 and those containing IgG-B2 proteins resulted in about a 2.7-fold increase in the enzymatic activity over the control (enzymatic activity of the hybrid membrane with liposome containing no protein), when assayed in the presence of IC_{2a} ($p < 0.02$). The activating effect confered by IgG-B1 and IgG-B2 proteins was independent of GTP, as the removal of GTP from the assay mixture did not alter the degree of the activation. Furthermore, the inclusion of NaF did not stimulate the enzymatic activity of this hybrid membrane. The fusion of PC-binding protein containing liposome with cyc⁻ cells was found, on the other hand, to result in a significant suppression (>80%) of adenylate cyclase activity, particularly in the presence of IC_{2b} (data not shown).

The noted combined effects of IgG-B1 and -B2 proteins on cyc⁻ cell adenylate cyclase in the presence of IC_{2a} were, as illustrated by Table IV, found to be dependent on the amounts of the proteins used in fusion. Thus, a significant activation could be observed when 2 × 10^7 cyc⁻ cells were fused with

Table IV: Effects of Amounts of IgG-B1 and -B2 Proteins in Liposomes on cyc⁻ Membrane Adenylate Cyclase[a]

IgG-B proteins in liposomes (μg)	adenylate cyclase [pmol mg^{-1} (30 $min)^{-1}$]	x-fold increase
0	44.1 ± 3.7	1.0
0.17	52.5 ± 5.1	1.2
0.33	58.9 ± 4.2	1.3
1.0	67.1 ± 8.7	1.5
1.5	125.3 ± 15.2	2.8

[a] Liposomes containing increasing amounts of IgG-B1 and -B2 proteins (as 1:1 w/w mixture) were fused with 2 × 10^7 cyc⁻ cells. Results represent the mean ± SEM of triplicate assays for adenylate cyclase of the hybrid membranes carried out in the presence of IC_{2a} (10 μg/mL) as described under Materials and Methods.

liposomes containing proteins as low as 0.33 μg. The prior boiling for 2 min or Pronase treatment for 3 h at 37 °C (at enzyme to substrate ratio of 1:100) totally eliminated the capability of IgG-B1 and -B2 proteins to activate adenylate cyclase of cyc⁻ cells.

Activation of cyc⁻ Membrane Adenylate Cyclase by IgG-Binding Proteins in Soluble Form. Since adenylate cyclase of cyc⁻ cells has been shown to be reconstituted by cholate extracts of S49 cells (Sternweiss & Gilman, 1979), the effects of IgG-B1 and -B2 proteins in cholate-solubilized form on the enzymatic activity of cyc⁻ cells were also investigated as follows. IgG-B1 and -B2 proteins in buffer I were treated with SM2 resin to make them free of Triton X-100. They were then exhaustively dialyzed against 25 mM sodium cholate in buffer III. The aliquots containing about 5 μg of the dialyzed IgG-B1 and -B2 proteins were mixed in a weight ratio of 1:1 and were incubated for 60 min at 10 °C with cyc⁻ membranes (100 μg of protein/assay). Adenylate cyclase activities of the mixtures were then assayed in the presence or absence of IC_{2a} or IC_{2b} (10 μg/mL). Results showed, as illustrated by Figure 4, that the mixtures of IgG-B1 and -B2 proteins in cholate-solubilized form enhanced, in the presence of IC_{2a}, adenylate cyclase activities of cyc⁻ membrane about 2.4 and 2.3-fold over the control. In the absence of IC_{2a}, these proteins appeared to show the tendency also to stimulate the enzymatic activity of cyc⁻ membrane, although the effects were statistically insignificant. The combined activating effects of IgG-B1 and -B2 proteins were neither augmented by addition of NaF (10 mM) nor reduced by removal of GTP from the assay mixture.

Mode of IgG-Binding Protein Mediated Activation of cyc⁻ Membrane Adenylate Cyclase. Thus, the above results show that the IgG-binding proteins isolated from the detergent lysate of $P388D_1$ cells activate adenylate cyclase of G/F protein-deficient cyc⁻ cells. The noted activation of the enzymatic activity appears to require not only the binding of IC_{2a} to $Fc\gamma_{2a}R$ (isolated as IgG-B1 protein) but also the participation of a second protein component(s) that copurifies (copurify) with $Fc\gamma_{2a}R$ under materials during the affinity chromatography step but could be separated as IgG-B2 protein from $Fc\gamma_{2a}R$ (IgG-B1) protein by Sephadex G-100 gel filtration in the presence of 6 M urea. Fusion of liposomes containing IgG-B1 and -B2 proteins with cyc⁻ cells or mixing of cholate-solubilized proteins with cyc⁻ membranes results in stimulation of adenylate cyclase activity of cyc⁻ membranes. In order to further characterize the mode of IgG-binding protein mediated activation of cyc⁻ membrane adenylate cyclase, the effects of IC_{2a} concentration, chelating agent, and calmodulin inhibitor were investigated as follows.

Effects of IC_{2a} Concentration. The mixture of IgG-B1 and -B2 proteins (1:1 w/w) in 25 mM sodium cholate in 20 mM HEPES buffer, pH 8.0, containing 1 mM EDTA and 2 mM

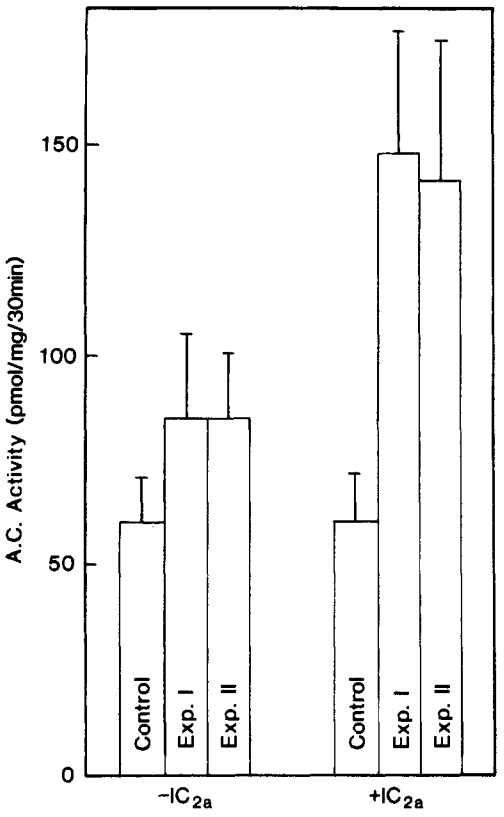

FIGURE 4: Combined effects of cholate-solubilized IgG-B1 and -B2 proteins on the adenylate cyclase activities of cyc⁻ cell membranes in the presence or absence of IC_{2a} (10 μg/mL). IgG-B1 and -B2 proteins (5–10 μg) in 25 mM cholate–HMe buffer, pH 8, were incubated at 10 °C for 60 min with cyc⁻ membrane (100 μg). The mixtures were then assayed for adenylate cyclase in triplicate. Adenylate cyclase activities of cyc⁻ cell membrane reconstituted with cholate extracts (40 μg) of S49 cells were 345.5 ± 32.3 and 617.1 ± 23.4 pmol mg^{-1} (30 $min)^{-1}$ for basal and NaF response, respectively.

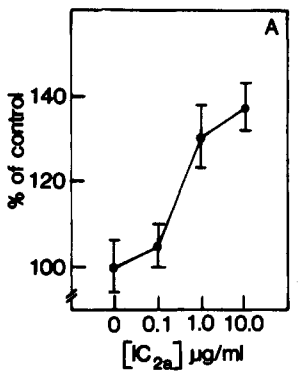

FIGURE 5: Effects of the concentration of soluble immune complex, IC_{2a}, on the adenylate cyclase activities of the mixtures of cholate-solubilized IgG-B1 and -B2 proteins and cyc⁻ membrane. The mixture of IgG-B1 and -B2 proteins (1:1 w/w) (10 μg/assay) was incubated for 60 min at 10 °C with cyc⁻ membranes (100 μg/assay). The adenylate cyclase activities were then assayed in the presence of various amounts of IC_{2a}. IC_{2a} were prepared with bovine IgG- and IgG_{2a}-subclass monoclonal anti-bovine IgG light-chain antibody as described under Materials and Methods. The levels of adenylate cyclase of the mixture of cyc⁻ membrane and IgG-B1 and -B2 proteins in the absence of IC_{2a} (control) were 90.8 ± 4.3 pmol mg^{-1} (30 $min)^{-1}$. Each point represents the mean ± SEM of triplicate determination.

Table V: Effects of Trifluoperazine on Adenylate Cyclase Activities of cyc⁻ Membranes Incubated with Cholate-Solubilized IgG-B1 and IgG-B2 Proteins in the Presence of IC_{2a}[a]

trifluoperazine (μg/mL)	adenylate cyclase activity [pmol mg^{-1} (30 $min)^{-1}$]	% inhibition
0	124 ± 24	0
0.1	76 ± 16	39
1.0	69 ± 14	44
10.0	47 ± 6	62

[a] IgG-B1 and -B2 proteins (5 μg each) in 25 mM cholate–HME buffer, pH 8, were incubated for 60 min at 10 °C with cyc⁻ membrane (100 μg) in each assay. The mixtures were then assayed for adenylate cyclase in triplicate in the presence of IC_{2a} (10 μg/mL) and various concentrations of trifluoperazine.

$MgCl_2$ was incubated for 60 min at 10 °C with cyc⁻ membrane in a weight ratio of 10 μg/100 μg of membrane protein per assay. After the incubation period, adenylate cyclase activities of the mixtures were assayed in the presence of various doses of IC_{2a} as described under Materials and Methods. The results illustrated by Figure 5 clearly show that the increase in the activating effect of IgG-binding proteins on cyc⁻ membrane adenylate cyclase is dependent on the concentration of IC_{2a} and approaches the maximum at 10 μg/mL. Further increase in IC_{2a} concentration resulted in gradual loss of the activating effects (data not shown).

Effects of EGTA. Next, the question of whether or not IgG-binding protein mediated activation of cyc⁻ membrane adenylate cyclase requires the participation of divalent cations, particularly Ca^{2+}, was investigated by examining the effects of the chelating agent EGTA. To this end, adenylate cyclase activity of the mixture of IgG-B1 and -B2 proteins and cyc⁻ membrane in the presence of IC_{2a} (10 μg/mL) was assayed in the presence or absence of EGTA (0.1–1 mM). Results showed that simultaneous addition of 0.1 mM EGTA caused about 40% reduction in the IgG-binding protein mediated activation of the enzymatic activity. Addition of 1 mM EGTA to the system further reduced the enzymatic activity to about 60% of the control value, although the inhibitory effects of two different concentrations of EGTA were not statistically significantly different. Since the assay was performed in the presence of the excess Mg^{2+} (10 mM), the noted inhibitory effect of EGTA is probably not due to depletion of Mg^{2+} but of some other divalent cation such as Ca^{2+}, which may be associated with cyc⁻ membranes.

Effects of Trifluoperazine. Since calmodulin has been implicated in the activation of adenylate cyclase of some cell types (Westcott et al., 1979; Wolff et al., 1980), the above results prompted us to investigate next the potential involvement of calmodulin in IgG-binding protein mediated activation of cyc⁻ membrane adenylate cyclase by examining the effects of the calmodulin inhibitor trifluoperazine (Weiss, 1983). As summarized by Table V, adenylate cyclase activated by IgG-binding proteins in the presence of IC_{2a} was indeed found to be inhibited in a dose-dependent manner by trifluoperazine.

GTPase Activity and ADP-Ribosylation. Since IgG-binding protein mediated activation of cyc⁻ adenylate cyclase was not affected by the presence of NaF or by the absence of GTP, IgG-binding proteins probably mediate their effects directly to the catalytic subunit of the enzyme rather than indirectly by G/F protein-like activity, which may be associated with $Fc\gamma_{2a}R$. To substantiate this notion, potential association of GTPase activity with IgG-B1 and -B2 proteins was examined as described under Materials and Methods, since such an enzymatic activity has been shown to be an inherent property of G/F protein (Cassel & Selinger, 1976, 1977). Results showed that both IgG-B1 and -B2 proteins failed to release $^{32}PO_4^{3-}$ from [γ-^{32}P]GTP, whereas cholate extracts of S49 or $P388D_1$ cells used as control clearly showed GTPase activity (S49 and $P388D_1$ extracts released 28.6 and 30.9% of [^{32}P]P_i in 15 min, respectively).

An additional evidence for nonidentity of IgG-B1 and -B2 proteins with G/F protein was sought by examining potential cholera toxin dependent ADP-ribosylation of IgG-B1 and -B2 proteins, since G/F proteins from several different cell sources have been shown to be ADP-ribosylated by activated cholera toxin (Cassel & Selinger, 1977; Hanski et al., 1981; Schleifer et al., 1982). To this end, the membranes of cyc$^-$ cells mixed with IgG-B1 and -B2 proteins or reconstituted with S49 or P388D$_1$ cell extracts were incubated with [^{32}P]NAD in the presence of preactivated cholera toxin as described under Materials and Methods. At the end of the incubation period, samples were dissolved in the sample buffer of Laemmli (1970) and subjected to SDS–PAGE analysis. The results indicated that ^{32}P was incorporated into proteins of molecular weight near 50 000 in the membrane reconstituted with S49 or P388D$_1$ cell extracts but not at all in the membrane mixed with cholate-solubilized IgG-B1 and -B2 proteins (data not shown).

Discussion

The data presented in this paper demonstrate that IgG$_{2a}$-binding (IgG-B1) proteins copurify with the second protein component(s) (IgG-B2) during the affinity chromatography of the detergent lysate of P388D$_1$ cells over a column of IgG–Sepharose, which can be separated, although poorly, by Sephadex G-100 gel filtration in the presence of 6 M urea (Figure 1). Both IgG-B1 and -B2 proteins can be inserted into liposome consisting of phosphatidylcholine and phosphatidylethanolamine with about the same efficiency as the incorporation of purified β-adrenergic receptors into a similar liposome preparation (Cerione et al., 1983a). The data of Table II then demonstrated a proper orientation of IgG-B1 proteins as well as PC-binding proteins (used as control) within liposome as seen by the effective inhibition of the EA$_{2a}$ or EA$_{2b}$ rosetting, respectively, by either S49 or P388D$_1$ cells as predicted from our previous results (Suzuki et al., 1982). A reason that IgG-B2 protein containing liposome did not inhibit EA$_{2a}$ rosetting is not clear but may be due either to a presence of relatively low concentration of IgG-B1 proteins in the preparation or the functional differences between proteins of M_r of 50 000 present in IgG-B1 and -B2 proteins. The data of Figure 3 show that simultaneous fusion of the liposomes containing IgG-B1 and -B2 proteins with G/F protein/Fcγ_{2a}R-deficient cyc$^-$ cells resulted in the formation of the hybrid membrane whose adenylate cyclase responds to IC$_{2a}$ by about a 2.7-fold increase in the activity over the control (fusion product between cyc$^-$ cells and liposomes containing no protein). This response appeared to be specific, since IC$_{2b}$ failed to stimulate adenylate cyclase of this hybrid membrane. Furthermore, IgG-B1 and -B2 proteins were able to confer their activating effects on the enzyme only in concert, since the fusion of liposomes containing either type of protein alone with cyc$^-$ cells did not result in the activation of adenylate cyclase of cyc$^-$ membrane. The data of Figure 4 show that IgG-B1 and -B2 proteins together can confer their effects on cyc$^-$ membrane adenylate cyclase in their cholate-solubilized forms as well. Such activation by cholate-solubilized IgG-B1 and -B2 proteins is dependent on the concentration of IC$_{2a}$ (Figure 5), suppressed by the exogenously added Ca^{2+} chelator EGTA and significantly inhibited by trifluoperazine (Table V).

cyc$^-$ cells, a genetic variant of a murine lymphoma cell line (S49), possess receptors for various hormones and the intact catalytic subunit of adenylate cyclase but lack functional G/F protein (Bourne et al., 1975; Ross & Gilman, 1977). An adenylate cyclase system of cyc$^-$ cells that consequently cannot be activated by various hormonal signals has been successfully reconstituted by supplementing functional G/F proteins from other cell sources (Ross & Gilman, 1977; Naya-Vigne et al., 1978; Ross et al., 1978). G/F proteins responsible for the reconstitution of cyc$^-$ adenylate cyclase have been isolated from rabbit liver (Rodbell et al., 1972), pigeon erythrocytes (Pfeuffer & Helnreich, 1975), and human erythrocytes (Ross & Gilman, 1980). In all instances, G/F proteins were found to be a heterodimer consisting of an α subunit of M_r 45 000–50 000 and a β subunit of M_r of 35 000. Recent report by Northup et al. (1982) indicated that the α subunit, which possesses a cholera toxin dependent ADP-ribosylation site and a GTP-binding site, is sufficient to activate adenylate cyclase activity of cyc$^-$ cells. A major component of IgG-B1 proteins that specifically bind to IgG$_{2a}$ has a M_r of 50 000, which is close to that of an α subunit of G/F protein. cyc$^-$ cells, which lack G/F protein, were found to lack also the receptor for IgG$_{2a}$. We therefore originally considered a remote possibility of the association of G/F-like activity with Fcγ_{2a}R, although our previous studies showed that cAMP synthesis by P388D$_1$ cells in response to IgG$_{2a}$ binding to cell surface Fcγ_{2a}R was unaffected by a G/F protein activator, Gpp(NH)p (Nitta & Suzuki, 1982b). However, this was found to be not the case, since the present studies showed that (1) the activation of adenylate cyclase of cyc$^-$ cells mediated by IgG-B1 and -B2 proteins was not augmented by NaF and (2) IgG-B1 and -B2 proteins inserted into liposomes or in cholate-solubilized form had no detectable GTPase activity and could not be ADP-ribosylated by the preactivated cholera toxin.

Thus, the mechanism by which liposome-inserted or cholate-solubilized IgG-binding proteins confer their activating effect on cyc$^-$ cell adenylate cyclase may be different from that mediated by G/F protein in a classical manner and requires further investigation. Of interest in this regard is the finding that the activating effects were exerted only when cyc$^-$ membranes were supplemented with both IgG-B1 and -B2 proteins and only in the presence of IC$_{2a}$. This suggests that IgG-B1 and -B2 proteins represent an Fcγ_{2a}R complex tightly bound at the cell surface and need to interact with each other to activate cyc$^-$ membrane adenylate cyclase. The finding that neither IgG-B1 nor -B2 proteins alone could activate cyc$^-$ membrane adenylate cyclase also suggests that IgG-B2 proteins are a part of Fcγ_{2a}R complex but not some proteins that may be present also in the cyc$^-$ membrane. However, the definitive proof for such notion requires further investigation such as cross-linking studies. Our data thus suggest that the activation may be initiated by specific binding of IC$_{2a}$ to Fcγ_{2a}R (obtained as IgG-B1 proteins), which may promote the interaction of Fcγ_{2a}R with a protein component of M_r of 25 000 and/or 17 000, both of which appear to be tightly associated with the receptor and obtained as IgG-B2 proteins. Possible causes by which the putative interaction between these IgG-binding proteins confers their effects on adenylate cyclase include (1) the direct activation of the catalytic unit, (2) the inhibition of cAMP phosphodiesterase; and (3) the inactivation of inhibitory regulatory (Ni) protein (Northup et al., 1982, 1983). Among these, an inhibition of cAMP phosphodiesterase is unlikely, because the assay used in this study measured the formation of cAMP from ATP catalyzed by adenylate cyclase and was carried out in the presence of the cAMP phosphodiesterase inhibitor 3′-isobutyl-1-methylxanthine. A possibility that IgG-binding proteins inactivate Ni protein function was excluded by the finding that IgG-binding protein mediated activation of cyc$^-$ adenylate cyclase was not affected by the inhibitor of Ni activity, a GDP analogue, guanosine 5′-(β-

thiodiphosphate) (data not shown). The noted activating effects of liposome-inserted or cholate-solubilized IgG-binding proteins are thus probably directly confered upon the catalytic subunit of adenylate cyclase of cyc$^-$ cells.

The findings that IgG-binding protein mediated activation of cyc$^-$ adenylate cyclase is suppressed by EGTA and by trifluoperazine suggest a probable involvement of divalent cation, probably Ca^{2+}, and calmodulin in the activation process confered by IgG-B1 and -B2 proteins. Calmodulin, whose molecular weight is 17000 (Dedman & Ketzel, 1983), has been shown to activate, upon formation of complex with Ca^{2+}, various enzymes such as Ca^{2+}-dependent ATPase (Hinds & Vinccuzi, 1983) or protein kinases (Silver & Stull, 1983; Nishizuka, 1984) through hydrophobic interaction (Cheung, 1970; Means et al., 1982). Calmodulin has been also shown to play a role in the activation of adenylate cyclase of some cell types (Westcott et al., 1979f Wolff et al., 1980). It follows that calmodulin, which may be associated with $Fc\gamma_{2a}R$ and can be isolated as a M_r 17000 protein component present in IgG-B2 proteins, may activate, in the presence of Ca^{2+}, an enzymatic activity associated with $Fc\gamma_{2a}R$, which in turn activates the catalytic subunit of cyc$^-$ membrane adenylate cyclase. However, this hypothesis requires further investigations, particularly because trifluoperazine has been shown to inhibiti not only calmodulin but also other Ca^{2+}-dependent enzymes (Wülfroth & Petzeit, 1985). Alternatively, calmodulin, which may be associated with IgG-B2 proteins, may directly activate adenylate cyclase as shown in bovine brain cortex (Westcott et al., 1979) or in prokaryotic systems (Wolff et al., 1980), irrespective of $Fc\gamma_{2a}R$. The latter hypothesis cannot explain, however, the requirement of IC_{2a} as well as the simultaneous presence of both IgG-B1 and -B2 proteins in mediating the enhancing effect on cyc$^-$ membrane adenylate cyclase. In addition, results of our preliminary studies indicated that both IgG-B1 and -B2 proteins indeed exhibit protein kinase activity, when assayed with the use of [γ-^{32}P]ATP as substrate and calf thymus histone as phosphate acceptor, whereas PC-binding proteins failed to catalyze phosphorylation (unpublished results). Although such results are interesting especially in view of a recent report by Sugden et al. (1985) that demonstrated that α_1-adrenergic potentiation of the β-adrenergic-sensitive adenylate cyclase system of pineal gland involves activation of protein kinase C, further studies are obviously required to delineate the exact nature of protein kinase activity associated with IgG-binding proteins and its relationship to $Fc\gamma_{2a}R$ and the catalytic subunit of adenylate cyclase, because the results of our preliminary studies suggested that the protein kinase activity may be calmodulin dependent.

Registry No. Ca, 7440-70-2; adenylate cyclase, 9012-42-4.

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Rotational Dynamics of the Fc Receptor for Immunoglobulin E on Histamine-Releasing Rat Basophilic Leukemia Cells

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Received January 7, 1986; Revised Manuscript Received March 26, 1986

ABSTRACT: The rotational diffusion of immunoglobulin E (IgE) bound to its specific Fc receptor on the surface of living rat basophilic leukemia cells was determined from time-resolved phosphorescence emission and anisotropy measurements. The IgE–receptor complexes are mobile throughout the range of temperatures of 5–38 °C. The residual anisotropy does not reach zero, indicating that the rotational diffusion is hindered. The values of rotational correlation times for each temperature are consistent with dispersed receptors rotating freely in the cell membrane and rule out any significant aggregation of occupied receptors before cross-linking by antigen or anti-IgE antibodies. The rotational correlation times decrease with increasing temperature from 65 μs at 5.5 °C to 23 μs at 38 °C. However, the degree of orientational constraint experienced by the probe is unchanged. Thus, the temperature dependence can be attributed primarily to a change in the effective viscosity of the cellular plasma membrane. The phosphorescence depolarization technique is very sensitive (our probe concentrations were 10–100 nM) and thus generally applicable to studies of surface receptors and antigens on living cells.

The cell surface receptor for immunoglobulin E (IgE)[1] of mast cells and basophils is an integral membrane protein with a molecular mass of approximately 100 kDa composed of four subunits arranged as an $\alpha\beta\gamma_2$ oligomer (Perez-Montfort et al., 1983; Metzger et al., 1983). The receptor binds the IgE molecule monovalently (Mendoza & Metzger, 1976; Newman et al., 1977) with a dissociation constant in the range 10^{-9}–10^{-12} M for different species (Metzger et al., 1982; Kulczycki & Metzger, 1974; Conrad et al., 1975). A variety of data suggest that the terminal four domains of the Fc region [$(C_\epsilon3–C_\epsilon4)_2$] interact with the receptor [reviewed in Perez-Montfort & Metzger (1982)] and that a bend may occur at the $C_\epsilon2$–$C_\epsilon3$ interface of the IgE molecule when it binds (Holowka & Baird, 1983; Baird & Holowka, 1985; Holowka et al., 1985). No biological effect is observed after binding of the IgE to the cell surface. However, the cross-linking of the IgE–receptor complexes by such agents as multivalent antigens or anti-IgE antibodies or cross-linking of the receptor by antireceptor antibodies initiates the process of cellular degranulation and receptor internalization [reviewed in Metzger et al. (1986)].

Studies of other cell surface receptors suggest that intramembrane aggregation initiated by binding of a specific effector molecule is one of the initial events required to trigger the biological chain of responses [reviewed in Schlessinger (1979) and Schlessinger et al. (1983)]. The state of association and rigidity of surface receptors can be assessed experimentally by the measurement of rotational and lateral diffusion (Jovin & Vaz, 1986). These properties can also reflect the influence of interactions with other intrinsic membrane-bound proteins, as well as with cytoplasmic and cytoskeletal components. The lateral diffusion of proteins in cellular plasma membranes [reviewed in Edidin (1981) and Schlessinger & Elson (1981)] has been determined by techniques sensitive to macroscopic motion in the micron range. The experimental diffusion constants are generally lower than those estimated for unconstrained mobile proteins in a lipid bilayer and also lower than the values determined for molecules reconstituted into synthetic lipid bilayers (Vaz et al., 1982), indicating an influence of physical barriers within the plasma membrane and/or interactions with other membrane-associated components. The IgE system provides no exception to the general

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[1] Abbreviations: Ig, immunoglobulin; kDa, kilodalton; RBL, rat basophilic leukemia; PBS, phosphate-buffered saline; Er–IgE, rat IgE labeled with erythrosin 5′-isothiocyanate.

0006-2960/86/0425-4397$01.50/0