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Immunology

Biochemical signal transmitted by Fcγ receptors: Phospholipase A_2 activity of Fcγ2b receptor of murine macrophage cell line $P388D_1$

(arachidonic acid/prostaglandin/immunoregulation)

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ABSTRACT **The detergent lysate of the $P388D_1$ macrophage cell line was subjected to affinity chromatography on two different media, Sepharose coupled to heat-aggregated human IgG (IgG-Sepharose) and Sepharose coupled to the phosphatidylcholine analog *rac*-1-(9-carboxyl)nonyl-2-hexadecylglycero-3-phosphocholine (PC-Sepharose). Both IgG- and phosphatidylcholine-binding proteins were further purified by Sephadex G-100 gel filtration and isoelectric focusing in the presence of 6 M urea. The isolated IgG-binding proteins specifically bound to IgG2a, but not to IgG2b, whereas the isolated phosphatidylcholine-binding proteins specifically bound to IgG2b but not to IgG2a. Phosphatidylcholine-binding proteins possessed a typical phospholipase A_2 activity (phosphatide 2-acylhydrolase, EC 3.1.1.4), which was maximal (10 μmol/min per mg of protein) at pH 9.5, depended on Ca^{2+}, and was specific for cleavage of fatty acid from the C-2 position of the glycerol backbone of phosphatidylcholine. The noted enzymatic activity was augmented 4-fold by preincubating phosphatidylcholine-binding proteins with heat-aggregated murine IgG2b but not with IgG2a. IgG-binding proteins, on the other hand, are devoid of any detectable phospholipase A_2 activity. Thus, the functional significance of Fcγ2b receptor of $P388D_1$ macrophage cell line would be the generation of phospholipase A_2 activity at the cell surface upon specific binding to Fcγ2b fragment.**

Fcγ receptor (FcγR) is an integral membrane phospholipoprotein that specifically binds the Fc portion of IgG proteins at the surface of various cells, including B lymphocytes and macrophages (1–7). The precise function of FcγR at the cell surface in the immune response is not yet clearly defined, with one notable exception. This is FcγRs present on K cells, which were shown to be directly involved in antibody-dependent cell-mediated cytotoxicity (8, 9).

A cell surface receptor is defined as a molecule that transmits, upon specific binding of ligand, a signal that affects cell functions. Immune complexes have been known to suppress humoral immune responses (10) or B cell differentiation (11). Such suppression could result from the increased synthesis of prostaglandins (PGs) of the E series, which are potent inhibitors of cell function (12–14), because a marked increase of PGE_2 synthesis by human as well as murine peritoneal macrophages upon interaction of cell surface FcγR with Fcγ fragment or immune complexes has been reported (15–17). One of the initial rate-limiting steps of PG synthesis is the activation of phospholipase A_2 (EC 3.1.1.4), which catalyzes hydrolysis of the ester bond at the C-2 position of phospholipids to release an unsaturated fatty acid such as arachidonic, the precursor of PGs (18). If the specific binding of the Fcγ portion to FcγR at the cell surface activates phospholipase A_2, FcγR has to be either closely associated with or even identical to phospholipase A_2. Our previous studies (19) have shown that FcγR proteins isolated from human B cells are indeed bifunctional—i.e., endowed with both Fc-binding and phospholipase A_2 activity.

In this study, the question of whether or not FcγR proteins present on macrophages of different species also possess phospholipase A_2 activity is raised. The data in this paper will demonstrate that the proteins isolated from the detergent lysate of $P388D_1$ murine macrophage cell line by affinity chromatography on PC-Sepharose 4B [which is *rac*-1-(9-carboxyl)nonyl-2-hexadecylglycero-3-phosphocholine, a phosphatidylcholine (PtdCho) analog, coupled to Sepharose 4B] bind specifically to murine IgG2b and also possess phospholipase A_2 activity, which is augmented by the binding of heat-aggregated IgG2b. The materials isolated by affinity chromatography on IgG-Sepharose 4B bind specifically to murine IgG2a and are devoid of phospholipase A_2 activity.

MATERIALS AND METHODS

Cells. Murine macrophage cell line ($P388D_1$) was a gift of H. Koren of Duke University. Cells were cultured in a spinner flask at 37°C in an atmosphere containing 5% CO_2 in RPMI 1640 medium supplemented with fetal calf serum (10%), streptomycin (100 mg/ml) and penicillin (100 units/ml). Cell density was maintained at approximately 5×10^5 per ml. About 90% of these cells were $Fc\gamma R^+$, as determined by the rosette assay using the system of human or sheep erythrocytes coated with IgG antibody (EAγ system). Normal human peripheral blood mononuclear cells were obtained from heparin-treated blood by Ficoll/Hypaque centrifugation (20). Hybridoma cell lines (N-S 8.1 and S-S.1, which secrete anti-sheep erythrocyte antibody of IgG2b and IgG2a subclasses, respectively) were obtained from the Cell Distribution Center of the Salk Institute (San Diego, CA) and cultured in the medium described above.

Surface Radioiodination and Detergent Lysis of Cells. Cultured cells were radioiodinated with 2 mCi (1 Ci = 3.7×10^{10} becquerels) of ^{125}I (Amersham) by the lactoperoxidase-catalyzed method (21), using Enzymobeads (Bio-Rad) (22) as described (19). Cells including 40–50% radioiodinated cells were suspended in phosphate-buffered saline (P_i/NaCl) containing 25 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) at 0°C and were lysed with 1% Triton X-100 (Amersham). After stirring for 30 min, nuclear materials, unlysed cells, and debris were removed by centrifugation at 22,370 × *g* for 60 min at 5°C. The supernatant solution, designated as cell lysate, was immediately subjected to affinity chromatography.

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Abbreviations: EAγ, IgG class antibody-coated erythrocytes; FcγR, Fcγ receptor; IgG-Sepharose, heat-aggregated human IgG-Sepharose 4B conjugate; PC-Sepharose 4B, *rac*-1-(9-carboxyl)nonyl-2-hexadecylglycero-3-phosphocholine coupled to Sepharose 4B; PG, prostaglandin; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; P_i/NaCl, phosphate-buffered saline; PtdCho, phosphatidylcholine; sRBC, sheep erythrocytes; Tris/NaCl, Tris·HCl-buffered saline.

Affinity Chromatography. PC-Sepharose 4B, the medium used to extract phospholipase A_2, was prepared as described (19). Normal human IgG proteins, Fabγ and Fcγ fragments, heat-aggretated IgG, and heat-aggregated IgG-Sepharose 4B conjugate were prepared as described (6, 7). Staphylococcal protein A-Sepharose CL-4B conjugate was purchased from Pharmacia (Uppsala, Sweden). All affinity chromatography media were packed in glass columns and equilibrated with Tris·HCl-buffered saline (Tris/NaCl) containing Triton X-100 (0.5%) and $PhMeSO_2F$ (1 mM). After thorough washing with the same buffer, the bound materials were eluted with deionized 6 M urea made 0.2 in ionic strength, pH 8 Tris·HCl buffer containing $PhMeSO_2F$ (1 mM).

Other Physicochemical Methods. The method of isoelectric focusing in the presence of 6 M urea has been described (6, 7, 19). Approximate estimations of protein concentrations in Triton X-100-containing buffer was made by Coomassie blue colorimetry (23) using a Bio-Rad protein assay kit. The more precise determination of protein concentration was carried out by nitrogen analysis (Kjeldahl), assuming the nitrogen contents of the protein samples to be 16%. Polyacrylamide gel electrophoresis in the presence of $NaDodSO_4$ followed the method of Weber and Osborne (24).

Assay of Phospholipase A_2 Activity. The rate of hydrolysis of PtdCho (Sigma) by various preparations in the assay solution (5 mM $CaCl_2$/20 mM KCl/0.5% Triton X-100) was followed by titration with 5 mM NaOH in a pH-stat titrator (19).

The positional specificity of phospholipase A_2 activity was assessed by measuring the levels of the radioactive oleic acid cleaved from 2-[^{3}H]oleoyl PtdCho by FcγR materials in the presence or absence of various IgG preparations as described (19). Preparation of 2-[^{3}H]oleoyl PtdCho followed the method of Robertson and Lands (25).

EAγ Rosette Assay. Human EAγ was prepared as described (6, 7, 19). Murine EAγ was prepared by sensitizing sheep erythrocytes (sRBC) with subagglutinating dose of murine monoclonal anti-sRBC antibodies (IgG2a or IgG2b). EAγ rosetting with human peripheral mononuclear cells or $P388D_1$ cells suspended in P_i/NaCl (1×10^6 cells per ml) and inhibition of EAγ rosetting systems with various IgG preparations were examined as described (6, 7, 19). Individual assays performed in triplicate had a standard error of less than 10%.

RESULTS

Isolation of IgG- and PtdCho-Binding Proteins from $P388D_1$ Cell Lysate. The presence of separate FcγR for IgG2a and IgG2b on the plasma membrane of murine macrophages and of macrophage cell lines has been suggested by several laboratories (26–32). If these FcγRs possess phospholipase A_2 activity as do human B cell FcγRs (19), they should bind specifically not only to IgG, but also to PC-Sepharose, an affinity chromatography medium made with a specific substrate analog.

In the first experiment, the lysate of 3×10^9 cells (40% of cells surface radioiodinated) was first adsorbed on IgG-Sepharose. The unbound material was then passed through a PC-Sepharose column in the presence of 25 mM Ca^{2+}. On the basis of the trichloroacetic acid-precipitable radioactivity, about 0.4% and 1.3% of the original material were obtained as IgG- and PtdCho-binding proteins, respectively. In the second experiment, the order of the affinity chromatography was reversed with the lysate of the same number of cells (53% of cells surface radioiodinated). The yields of the materials that could be eluted from PC- and IgG-Sepharose were equivalent to the first experiment. The IgG- and the PtdCho-binding proteins obtained from the above two experiments were pooled separately and were subjected to gel filtration using a column (5×60 cm) of Sephadex G-100 that was previously equilibrated with the deionized 6 M urea made 0.2 in ionic strength, pH 8 Tris·HCl buffer with 1 mM $PhMeSO_2F$. About 90% of PtdCho-binding proteins applied to this column were eluted in the void volume. About 85% of IgG-binding proteins were similarly excluded from Sephadex G-100 gel beads.

PtdCho- and IgG-binding proteins obtained as the excluded fractions from Sephadex G-100 gel were separately dialyzed against deionized water and electrofocused in a pH gradient formed with carrier ampholyte pH 5–10 in the presence of 6 M urea. As illustrated by Fig. 1A, the PtdCho-binding proteins

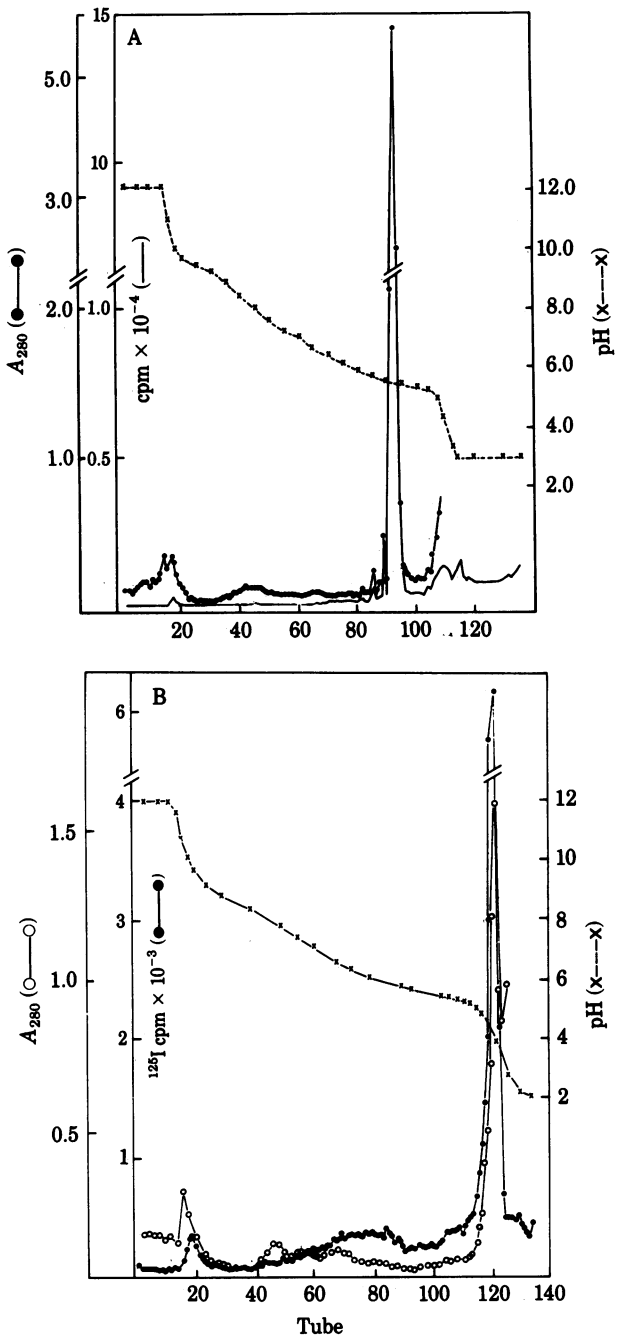

FIG. 1. Isoelectric focusing patterns of the PtdCho-binding proteins originated from the lysate of 6×10^9 $P388D_1$ cells (*A*) and of the IgG-binding proteins originated from the lysate of 1.2×10^9 cells (*B*). Electrofocusing was carried out in an LKB Ampholine column (440 ml) at 1200 V for 72 hr at 10°C in the pH gradient 5–10 in the presence of 6 M urea. Each tube contained 120 drops.

focused sharply at pH 5.8 as a single peak (in tubes 87–94), indicating their charge homogeneity. The IgG-binding proteins were apparently more acidic, because they focused (in tubes 112–122) at approximately pH 4.5, which is in the interphase region between the lower range of the pH gradient and the anode solution (Fig. 1*B*). IgG-binding proteins used in the subsequent experiments were not electrofocused to avoid possible damage at this low pH. The PtdCho-binding proteins separated by electrofocusing and the IgG-binding proteins fractionated by gel filtration were each extensively dialyzed against deionized water, and then lyophilized. On the basis of the dry weight, the yields of PtdCho- and IgG-binding proteins were approximately 7 and 5 mg per 10^9 cells, respectively.

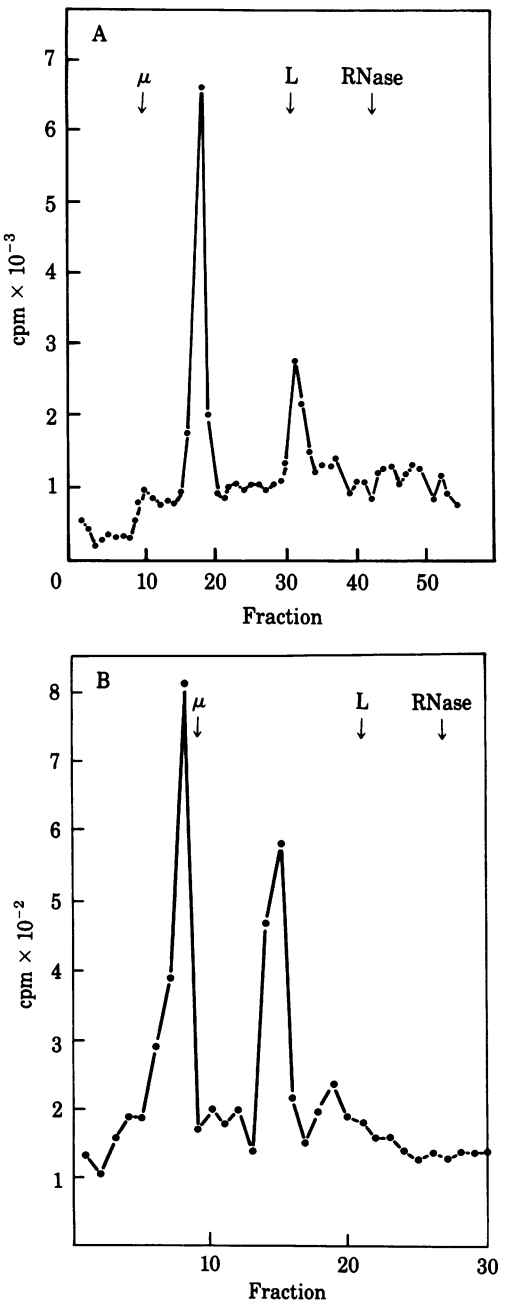

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis patterns of IgG-binding (*A*) and PtdCho-binding (*B*) proteins. Electrophoreses were carried out in 7.5% polyacrylamide gels for 6.5 hr (*A*) and 4 hr (*B*) at about 8 mA/gel. Fractions are 2-mm gel slices. Positions of IgG μ chain, light chain (L), and RNase markers are shown.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis Patterns. Both IgG- and PtdCho-binding proteins form polymeric complexes in the presence of 6 M urea, as was concluded from the observation that they were excluded from Sephadex G-100 gel beads. On $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing condition, IgG-binding proteins were separated into a major and a minor band, corresponding to molecular weights of 50,000 and 25,000 (Fig. 2*A*), whereas PtdCho-binding proteins gave rise to two major bands (corresponding to molecular weights of 40,000 and 80,000) (Fig. 2*B*). Size heterogeneity revealed by $NaDodSO_4$/polyacrylamide gel electrophoresis of both types of FcγR materials isolated from $P388D_1$ cells has been noted by a number of investigators (31–35). The ability of both IgG- and PtdCho-binding proteins to aggregate is probably an intrinsic property of membrane glycoprotein and may be due to strong noncovalent association between hydrophobic segments of their polypeptide chains (36). The association of lipids (7) and carbohydrates (35) with FcγR protein moiety may also contribute to size heterogeneity as a result of anomalous binding of $NaDodSO_4$ (24).

IgG-Binding Properties. The lyophilized IgG- and PtdCho-binding proteins were suspended in P_i/NaCl and examined for their capability to inhibit the murine EAγ rosetting system. As shown by Fig. 3, the IgG-binding proteins inhibited in a dose-dependent manner the rosette formation only between $P388D_1$ cells and EAγ2a systems. On the other hand, the EAγ2b rosette formation was inhibited in a dose-dependent manner by the PtdCho-binding proteins. In addition, as shown by Fig. 4, the EAγ2a rosette inhibition by IgG-binding proteins could be reversed in a dose-dependent manner by preincubating IgG-binding proteins with monomeric IgG2a but not with monomeric IgG2a. The EAγ2b rosette inhibition by PtdCho-binding proteins could be reversed in a dose-dependent manner by preincubating PtdCho-binding proteins with heat-aggregated IgG2b but not with aggregated IgG2a (Fig. 4).

Thus, the isolated IgG- and PtdCho-binding proteins appear to represent the IgG2a and the IgG2b receptors, respectively. Furthermore, at 25 μg/ml both proteins could inhibit 90% of the EAγ rosette formation between human mononuclear cells and human anti-Rh antibody-coated erythrocytes, confirming the lack of species specificity noted previously (33). The inhi-

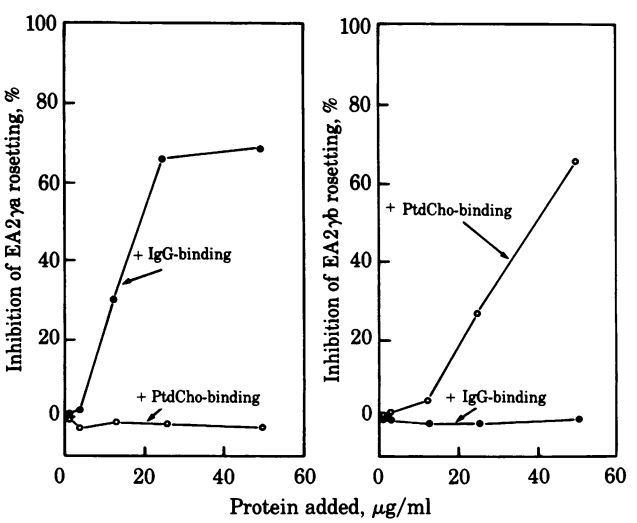

FIG. 3. Inhibition of EAγ rosette formation by the IgG-binding (●) and PtdCho-binding (○) proteins. EAγ was prepared by sensitizing sRBC with the subagglutinating doses of monoclonal anti-sRBC antibodies of IgG2a or IgG2b subclasses. Rosetting cells were $P388D_1$ cells.

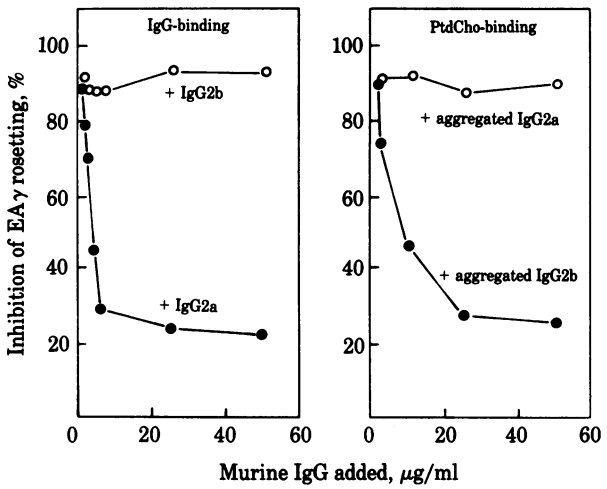

FIG. 4. Reversal of the EAγ rosette inhibitory capacity of the IgG-binding (*Left*) and PtdCho-binding (*Right*) materials by murine IgG preparations. Various amounts of IgG2a or IgG2b (monomeric and heat-aggregated) were preincubated with IgG- or PtdCho-binding proteins (25 μg/ml) before they were added to a EAγ rosette system consisting of monoclonal anti-sRBC-coated sRBC and P388D_1 cells. Aggregated IgG2a or IgG2b did not reverse the inhibition by IgG-binding protein. Likewise, monomeric IgG2a and IgG2b failed to reverse the inhibition by PtdCho-binding protein. Data relative to these effects are omitted from this figure for simplification.

bition of the human EAγ rosetting system by IgG-binding proteins was reversed again only by the preincubation with monomeric murine IgG2a. Likewise, the inhibitory capacity of PtdCho-binding proteins was abolished only by the preincubation with heat-aggregated IgG2b proteins.

Phospholipase A_2 Activity. In order to determine whether or not PtdCho- and IgG-binding proteins possess phospholipase A_2 activity, they were first extensively dialyzed against 0.15 M KCl containing 25 mM $CaCl_2$ and 0.5% Triton X-100. By using a pH-stat assay (19), it was found that PtdCho-binding proteins were capable of catalyzing the hydrolysis of PtdCho between

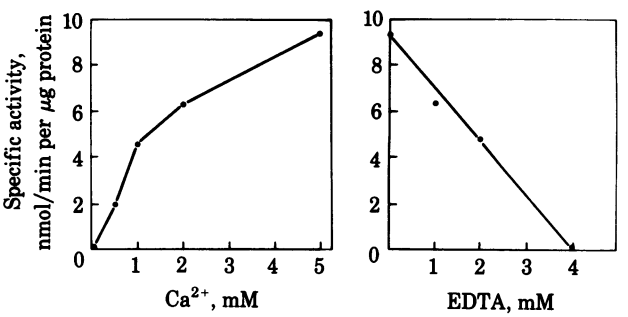

FIG. 5. Activation by Ca^{2+} (*Left*) and inhibition by EDTA (*Right*) of phospholipase A_2 activity of the PtdCho-binding proteins.

pH 7.5 and 10, the optimal pH being near 9.5. IgG-binding proteins, however, showed no activity over the pH 4–10 range. As shown in Fig. 5, the maximal enzymatic activity of PtdCho-binding proteins in the presence of 5 mM Ca^{2+} was about 10 μmol/min per mg. Higher levels of Ca^{2+} resulted in a gradual diminution of enzymatic activity. EDTA at 4 mM totally suppressed the noted enzymatic activity. Mg^{2+} or other divalent cations could not substitute for Ca^{2+}.

Positional Specificity of Phospholipase Activity of the PtdCho-Binding Proteins. The results summarized in Table 1 demonstrate that the PtdCho-binding proteins were capable of hydrolyzing specifically the ester bond at the C-2 position of the glycerol backbone of this substrate, releasing about 20% of the radioactivity as free oleic acid. Preincubation of the PtdCho-binding proteins with heat-aggregated murine IgG2b resulted in the augmentation of the phospholipase A_2 activity, with liberation of about 88% of the radioactivity as oleic acid. This level of enzymatic activity was found to be equivalent to that obtained when bee venom phospholipase A_2 was used as control. On the other hand, neither IgG2b (monomeric) nor IgG2a (monomeric or heat-aggregated) activated this enzymatic activity. A small amount of the radioactivity found in lyso-PtdCho fractions may have arisen from 1-[^{3}H]oleoyl PtdCho, which is usually present in small quantity (about 1%) in 2-[^{3}H]oleoyl PtdCho.

Table 1. Positional specificity of phospholipase activity of the PtdCho-binding proteins in the presence and absence of two different subclasses (2a and 2b) of murine IgG*

Exp.	Enzymes	IgG added	cpm × 10^{-4} (%)† found in Free fatty acid	PtdCho	Lyso-PtdCho
1	Bee venom phospholipase A_2	—	162.0 (93.9%)	10.0 (5.8%)	0.6 (0.3%)
2	PtdCho-binding protein	—	34.2 (20.0%)	136.0 (79.5%)	0.9 (0.5%)
3	PtdCho-binding protein	Aggregated 2b	150.0 (88.2%)	19.0 (11.2%)	1.0 (0.6%)
4	PtdCho-binding protein	Monomeric 2b	33.8 (19.9%)	135.0 (79.6%)	0.7 (0.4%)
5	PtdCho-binding protein	Aggregated 2a	32.0 (19.0%)	136.0 (80.7%)	0.5 (0.3%)
6	PtdCho-binding protein	Monomeric 2a	35.0 (21.0%)	131.5 (78.8%)	0.3 (0.2%)
7	—	Aggregated 2b	0.5 (0.3%)	162.0 (99.5%)	0.3 (0.2%)
8	—	Monomeric 2b	0.5 (0.3%)	167.0 (99.3%)	0.6 (0.4%)
9	—	Aggregated 2a	0.4 (0.2%)	162.0 (99.7%)	0.1 (0.1%)
10	—	Monomeric 2a	0.7 (0.4%)	168.0 (99.6%)	0.08 (0.05%)
11	—	—	0.4 (0.2%)	168.0 (99.6%)	0.17 (0.1%)

* In the experiments numbered 2–6, 40 μg of PtdCho-binding proteins and 120 μg of various murine IgG proteins were preincubated in 250 μl of the assay solution (20 mM KCl containing 5 mM $CaCl_2$ and 0.5% Triton X-100) at 37°C for 30 min. These were added to substrate (10 mg of PtdCho containing a tracer amount of 2-[^{3}H]oleoyl PtdCho suspended in 6 ml of assay solution) in a thermoregulated vessel. The hydrolysis was followed by a pH-stat titrator at pH 9.5 at 37°C for 2 hr. After this, lipids were extracted from the reaction mixtures with chloroform/methanol (2:1, vol/vol). Lipid extracts were fractionated by silicic acid column chromatography as described (19).

† Percentages given are based on the total radioactivity recovered. The recovery of the radioactivity ranged between 85% and 95%.

DISCUSSION

The data presented in this paper demonstrate the separation of two types of FcγR in a biologically active form from the detergent lysate of P388D_1 cells. The data of Fig. 3 and 4, which clearly support the subclass specificity in their IgG-binding properties, suggest that the IgG- and the PtdCho-binding proteins isolated represent an Fcγ2aR and an Fcγ2bR, respectively. The charge properties of these proteins are quite different, as illustrated by the data of Fig. 1. This may reflect differences in their carbohydrate contents as well as in amino acid compositions. Preliminary results of tryptic peptide mapping also suggested marked differences between IgG- and PtdCho-binding proteins. Further delineation of the two distinct FcγRs of murine macrophages will ultimately depend on thorough biochemical characterization such as amino acid sequence analysis.

The result of human EAγ rosette inhibition indicated that both Fcγ2aR and Fcγ2bR are able to bind to the Fcγ portion of human IgG. The reason why PtdCho-binding proteins (Fcγ2bR) failed to bind IgG-Sepharose is not clear. A possibility is that Fcγ2bR has much lower affinity for human IgG than does Fcγ2aR. Loube *et al.* (31) reported the isolation of Fcγ2aR but not Fcγ2bR from P388D_1 cell lysate by affinity chromatography using human IgG-Sepharose.

The data in this study also demonstrate that phospholipase A_2 activity is an inherent property of PtdCho-binding but not of IgG-binding protein isolated from the P388D_1 cell lysates. The enzymatic activity exhibited by PtdCho-binding protein was essentially identical to that of human B cell FcγR protein (19) in pH optimum (pH 9.5), Ca^{2+} dependency (Fig. 5), specific activity (about 10 μmol of fatty acid released per min per mg of protein) and positional specificity (Table 1). However, the definite positional specificity needs to be confirmed by selectively assaying for phospholipase A_1 activity in these materials. The noted enzymatic activity was shown to be augmented about 4-fold by heat-aggregated murine IgG2b proteins but not by monomeric IgG2b or IgG2a (Table 1). These findings strongly suggest that Fcγ2bRs but not Fcγ2aRs are the surface molecules that, upon specific binding of the immune complexes, transmit a signal for increased synthesis of PGE; this synthesis has been shown by Rouzer *et al.* (17) to be independent of endocytosis or phagocytosis. Indeed, results of our preliminary experiments have demonstrated that P388D_1 cells radiolabeled with [^{3}H]arachidonic acid release [^{3}H]arachidonic acids and convert them into PGs after the interaction with EAγ2b complexes but not with EAγ2a complexes. An interesting question then presents itself as to the biological significance of Fcγ2aRs which lack any demonstrable phospholipase A_2 activity and yet are able to bind to IgG2a proteins.

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