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Mouse macrophage Fc receptor for IgGγ2b/γ1 in artificial and plasma membrane vesicles functions as a ligand-dependent ionophore

(signal transduction/tetraphenylphosphonium ion/cation selectivity/proteoliposome/reconstitution)

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ABSTRACT **We tested the effect of specific ligands to the mouse macrophage IgGγ2b/γ1 Fc fragment receptor (FcR) on ion permeability of plasma membrane vesicles prepared from J774 macrophages by nitrogen cavitation. The monoclonal antibody directed against IgGγ2b/γ1 FcR (γ2b/γ1 FcR), 2.4G2 IgG, and soluble and immobilized immunocomplexes induces a dramatic cation flow through plasma membrane vesicles, as measured by [^{3}H]tetraphenylphosphonium$^+$ accumulation. Challenge with the monovalent 2.4G2 Fab also produces an ion flow but the effect is smaller by a factor of 2, and three other monoclonal antibodies directed against major surface antigens of mouse macrophages produce no net ion flow. Membrane vesicles incubated with FcR ligands do not discriminate between Na^+ and K^+ and show low permeability to Ca^{2+}. γ2b/γ1 FcR was purified by using monoclonal 2.4G2 and reconstituted into proteoliposomes. Under these circumstances, the purified receptor increased the cation permeability of the proteoliposomes in the presence of specific ligands. The data indicate that the γ2b/γ1 FcR of J774 macrophages functions as a ligand-dependent ionophore. The ion influx into macrophages mediated by the FcR may play an important role as a signal for internalization of membranes and stimulus–secretion coupling.**

Phagocytosis of immunocomplexes and the secretion of inflammatory mediators involve specific interactions between the Fc domain of IgGγ2b/γ1 and Fc receptors (FcR) embedded in the plasma membrane. Although the precise sequence of events following receptor–ligand interactions is still unclear, we believe that an early event is membrane depolarization secondary to enhanced sodium influx (1). The initial membrane depolarization is followed by a ouabain-sensitive hyperpolarization. The complexity of the response clearly requires further analysis.

To dissect the system further, we have used tetraphenylphosphonium ion (Ph_4P^+), an indicator of membrane potential, to study plasma membrane vesicles obtained from J774 cells. In addition, we have studied the behavior of proteoliposomes reconstituted with purified IgGγ2b/γ1 FcR (γ2b/γ1 FcR) by detergent dialysis (2). The results with plasma membrane vesicles and proteoliposomes reconstituted with FcR extend and support the hypothesis that the FcR is indeed a ligand-activated ion channel.

MATERIALS AND METHODS

Preparation of Cells and Plasma Membrane Vesicles. J774 cells were maintained as described (3). Cells in logarithmic phase were washed three times in phosphate-buffered saline and suspended at a concentration of 5×10^7/ml in 0.05 M NaCl (or KCl)/0.165 M sucrose/10 mM Hepes/Tris, pH 7.4 (buffer A). Cells were then disrupted in a nitrogen bomb by equilibration with N_2 at 500 psi (1 psi = 6.9 kPa) for 30 min at 4°C. The cell lysate was collected and centrifuged at 1,000 × *g* (10 min, 4°C) to pellet unbroken cells and nuclei. Under these conditions, 70–80% of cells were disrupted with minimal nuclear fragmentation. The supernatant was then centrifuged at 20,000 × *g* (20 min, 4°C) to pellet mitochondria and lysosomes. The final supernatant was centrifuged at 100,000 × *g* (90 min, 4°C); the resulting pellet contained endoplasmic reticulum and plasma membrane vesicles. The resuspended pellet was subjected to differential centrifugation through a dextran gradient (4). The less dense plasma membrane fraction was collected, diluted with buffer A, and centrifuged (100,000 × *g*, 90 min), and the pellet was suspended at a concentration of 5 mg of protein/ml of buffer A (or a different buffer as specified in the text). Assays for alkaline phosphodiesterase (5) and FcR (6) were used as plasma membrane markers.

Purification and Iodination of FcR. γ2b/γ1 FcR was purified by affinity chromatography on 2.4G2 Fab-Sepharose (7). Proteins were labeled with ^{125}I by using IODO-GEN (Pierce) as the oxidant essentially as described (7). Unreacted iodide was removed from the labeled γ2b/γ1 FcR by dialysis against Dowex-1×8 Cl^-. When 2.4G2 Fab-Sephadex was used as the immunoabsorbent, 45% of the total trichloroacetic acid-insoluble radioactivity could be precipitated.

Reconstitution of FcR into Proteoliposomes. Purified FcR was reconstituted into vesicles by the octyl glucoside dialysis method (2). Egg phosphatidylcholine (type VI-E; Sigma) and cholesterol (CH-S; Sigma) were mixed 6:1 (wt/wt) in chloroform/methanol (2:1), dried under N_2, resuspended in diethyl ether, and dried (two cycles). Purified FcR at 35 μg/ml in buffer A/20 mM octyl glucoside was added to the lipid film at a protein/lipid ratio (wt/wt) of 1:10. This solution was sonicated for 2 min in a water-bath sonicator and dialyzed for 36 hr at 4°C against three 1-liter portions of buffer A/3 mM NaN_3. Preliminary studies using ^{125}I-labeled FcR and [*choline-methyl*-^{14}C]phosphatidylcholine (New England Nuclear) in tracer amounts showed that the FcR and lipid comigrated in the same band in flotation experiments using a linear sucrose gradient as described (8).

[^{3}H]Ph_4P^+ Uptake by Lipid Vesicles. [^{3}H]Ph_4P^+ accumulated by reconstituted proteoliposomes was separated from free [^{3}H]Ph_4P^+ by filtration through Millipore filters (0.2 μm; EGWP 02500) (9). The reaction was initiated by diluting 0.1 ml of lipid vesicles in buffer A into 0.9 ml of 0.25 M sucrose/10 mM Hepes/Tris·HCl, pH 7.4 (buffer B) containing 50 μM [^{3}H]Ph_4P^+ (0.125 Ci/mmol; 1 Ci = 37 GBq) and specific FcR ligands, including 2.4G2 IgG, 2.4G2 Fab, 2.4G2 Fab-Sephadex, the com-

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Abbreviations: $\Delta\Psi$, membrane potential; DNP, dinitrophenyl; FcR, Fc receptor(s); γ2b/γ1 FcR, IgGγ2b/γ1 FcR; HRP, horseradish peroxidase; Ph_4P^+, tetraphenylphosphonium$^+$.

plex of bovine serum albumin derivatized with dinitrophenyl (DNP) at 1:11 (DNP_{11}-albumin) and anti-DNP IgG (DNP_{11}-albumin–anti-DNP IgG), and DNP_{11}-albumin–anti-DNP IgG bound to Sephadex or to horseradish peroxidase (HRP)-anti-HRP, prepared as described (1). At given intervals, 0.1-ml aliquots of reaction mixture were filtered, the filters were washed with 5 ml of ice-cold buffer B, and the radioactivity retained on the filter was determined. The amount of nonspecific binding of $[^3H]Ph_4P^+$ associated with the filters at time 0 was subtracted from experimental values.

RESULTS

Plasma Membrane Vesicles from J774 Macrophages. We prepared plasma membrane vesicles from J774 cells by nitrogen cavitation followed by centrifugation through a dextran gradient. The yield of this membrane preparation as determined by two membrane markers is shown in Table 1. Typically, 2–4% of cell total protein was retained in the plasma membrane fraction. We obtained a 7.8-fold enrichment of alkaline phosphodiesterase activity and a 9.2-fold increase in FcR binding activity relative to the postnuclear homogenate.

Ion Flow Mediated by FcR Ligands. We tested the effects of different FcR ligands on ion flow through the membrane of the vesicles by assaying for $[^3H]Ph_4P^+$ accumulation. An ion gradient was established artificially across the membrane by preparing the vesicles in 50 mM KCl or NaCl and diluting them into equiosmotic sucrose/$[^3H]Ph_4P^+$ solutions. If the vesicles are more permeable to K^+ or Na^+ than to other ions in the reaction mixture, efflux of these ions should lead to the transient generation of a membrane potential ($\Delta\Psi$) (interior negative) that can be monitored by accumulation of the lipid-soluble $[^3H]Ph_4P^+$ (10). 2.4G2 IgG (0.1 μM) induced a 3-fold increase in total uptake of $[^3H]Ph_4P^+$, indicating rapid efflux of K^+ and Na^+ from the vesicles (Fig. 1 *A* and *B*). This increase in $[^3H]Ph_4P^+$ accumulation was transient, decaying after about 2 min. The response to 2.4G2 IgG was saturated at 2.4G2 IgG levels $\geq$ 0.1 μM and was comparable with that obtained with the K^+ ionophore valinomycin (10 μM) when KCl-loaded vesicles were diluted into equiosmotic sucrose/valinomycin/$[^3H]Ph_4P^+$ solutions (data not shown).

The Fab fragment of 2.4G2 IgG at concentrations up to 0.5 μM did not induce any net efflux of K^+ or Na^+, but a small amount of $[^3H]Ph_4P^+$ uptake was observed at 1 μM 2.4G2 Fab (Fig. 1*A*). This response was much less than that obtained with 0.1 μM 2.4G2 IgG. The decreased effect of the 2.4G2 Fab relative to the intact IgG reflects the observations made on the intact

Table 1. Distribution of membrane markers in vesicle preparations

Fraction	Protein recovery, %	2.4G2 Fab–antigen complex, cpm/μg of protein*	Alkaline phosphodiesterase activity†
Post nuclear homogenate	100	280	70
Membrane fraction			
Before dextran gradient	4.4	1,920	370
After dextran gradient	2.9	2,580	546

* Input Fab was 0.25 μg/ml radiolabeled with ^{125}I to a specific activity of 4.5×10^3 cpm/ng. Values are corrected for background binding. The protocol for the monoclonal sandwich radioimmunoassay was essentially as described (6).

† Alkaline phosphatase specific activity is expressed as (nmol/30 min)/mg of cell protein.

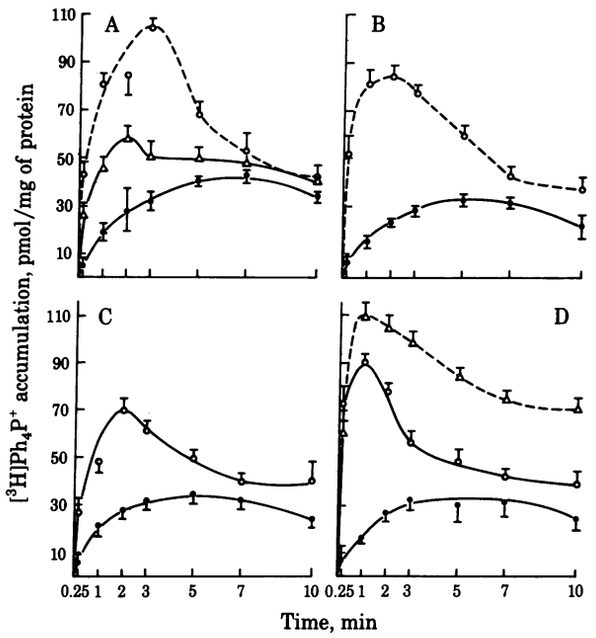

FIG. 1. Effects of various ligands on ion flow through plasma membrane vesicles of J774 cells prepared by nitrogen cavitation. Vesicles equilibrated with 0.05 M KCl (*A*, *C*, and *D*) or NaCl (*B*) were diluted (1:10) into sucrose solutions containing various ligands and 50 μM $[^3H]Ph_4P^+$. (*A*) ○, 2.4G2 IgG (0.1 μM); △, 2.4G2 Fab (1 μM). (*B*) ○, 2.4G2 IgG (0.1 μM). (*C*) ○, Soluble immunocomplex of CNBr-coupled DNP_{11}-albumin–anti-DNP–IgG, 1:6 (mol/mol) (0.1 μM). (*D*) △, 2.4G2 Fab-superfine Sephadex (1.7 mg of 2.4G2 Fab/ml of Sephadex) at 20 μl of particles/ml; ○, DNP_{11}-albumin–anti-DNP-albumin-Sephadex. In all experiments, controls (●) consisted of vesicles incubated without ligands. Data represent mean ± SD of five experiments.

J774 cells (1). Intact J774 cells respond to insoluble immunocomplexes with a prolonged depolarization whereas soluble complexes and 2.4G2 IgG induce only transient depolarization (1). However, the effects of the soluble immunocomplex DNP_{11}-albumin–anti-DNP (Fig. 1*C*) and of DNP_{11}-albumin-Sephadex–anti-DNP IgG and 2.4G2 Fab-Sephadex (Fig. 1*D*) were comparable with that obtained with 2.4G2 IgG. Control experiments with DNP_{11}-albumin (2 mg/ml), rabbit anti-DNP (100 μg/ml), and DNP_{11}-albumin-Sephadex did not result in any uptake of $[^3H]Ph_4P^+$ under the conditions described.

To test for selectivity between K^+ and Na^+, vesicles equilibrated with one cation were diluted into equiosmotic solutions of the other in the presence of 2.4G2 IgG. No accumulation of $[^3H]Ph_4P^+$ over the controls in which vesicles were diluted into same buffer without FcR ligands was observed (Fig. 2 *A* and *B*), indicating lack of Na^+/K^+ selectivity.

To investigate the Ca^{2+} permeability of the 2.4G2 IgG-induced ion flow, vesicles were equilibrated with 50 mM $CaCl_2$ and diluted into equiosmolar sucrose solutions. 2.4G2 IgG (0.1 μM) induced only a small increase in $[^3H]Ph_4P^+$ uptake in response to a Ca^{2+} gradient (Fig. 2*C*), suggesting low Ca^{2+} permeability relative to Na^+ or K^+. Similar experiments with other ions showed a permeability sequence of $Na^+ \approx K^+ > Li^+ > choline^+ > Ca^{2+} > Cl^-$. Thus, the permeability induced by FcR ligands is mainly monovalent cation selective.

In all experiments, after the initial rapid $[^3H]Ph_4P^+$ uptake, there was a decline to the baseline as the initial electrochemical gradient collapsed because of Cl^- permeability. To demonstrate this, we prepared vesicles equilibrated with the impermeant anion isethionate and carried out $[^3H]Ph_4P^+$ uptake experiments as described with 2.4G2 IgG as the specific FcR ligand (Fig. 2*D*). In contrast to the experiment in which vesicles were

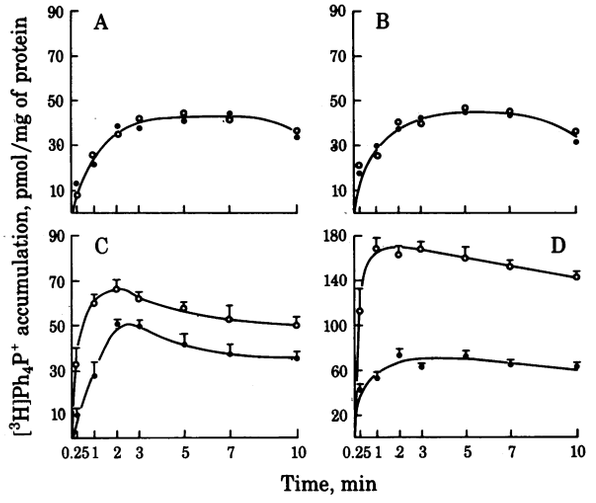

FIG. 2. Effects of various ions on vesicle accumulation of $[^3H]Ph_4P^+$. Vesicles equilibrated with 50 mM KCl (*A*) or NaCl (*B*) were diluted into buffer containing 50 mM NaCl (*A*) or KCl (*B*), 50 μM $[^3H]Ph_4P^+$, and 0.1 μM 2.4G2 IgG and, at intervals, aliquots were assayed for radioactivity. Data points (○ and ●) represent individual values. (*C* and *D*) Vesicles containing 50 mM $CaCl_2$ or Na isethionate were diluted into sucrose solutions containing 50 μM $[^3H]Ph_4P^+$ and 0.1 μM 2.4G2 IgG. Data represent mean ± SD of five experiments.

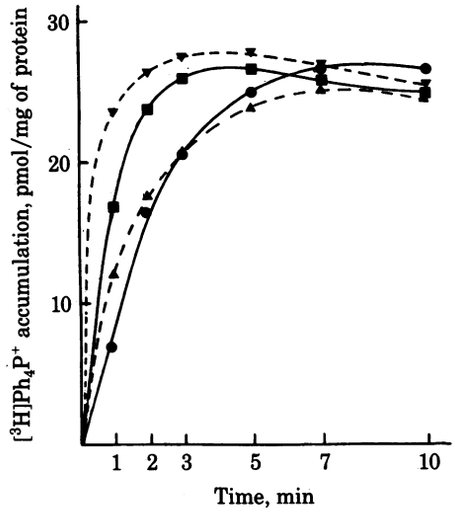

FIG. 3. Effects of three monoclonal antiplasma membrane protein IgGs on ion flow through plasma membrane vesicles. Vesicles equilibrated with 50 mM KCl were diluted into sucrose solutions containing 50 μM $[^3H]Ph_4P^+$, and 2 μM monoclonal antibody. ▼, 1.21J; ■, 2D2C; ▲, 2E2A; ●, control. Data represent means of three experiments.

equilibrated with NaCl (Fig. 1*B*), 2.4G2 IgG induced prolonged $[^3H]Ph_4P^+$ uptake in vesicles equilibrated with Na isethionate. Thus, to some degree, the transient nature of $[^3H]Ph_4P^+$ uptake by KCl- or NaCl-containing vesicles in the presence of FcR ligands was due to Cl^- permeability.

Specificity of the Response. We used three monoclonal antibodies (1.21J, 2D2C, and 2E2A) directed against major mouse macrophage plasma membrane proteins (11) to verify the specificity of the FcR-mediated response. At concentrations >2 μM, we observed a more rapid equilibration of $[^3H]Ph_4P^+$ in the vesicles but no net concentration increase over the control for 1.21J and 2D2C (Fig. 3). 2E2A had no effect on $[^3H]Ph_4P^+$ uptake (Fig. 3). Thus, the increase in cation permeability in the plasma membrane induced by 2.4G2 IgG and immunocomplexes is probably a consequence of specific ligand receptor interactions.

Ion Flow in Proteoliposomes Reconstituted with FcR. Purified FcR was reconstituted into lipid vesicles by the detergent dialysis method. We determined that 60–70% of ^{125}I-labeled γ2b/γ1 FcR-containing liposomes bound to 2.4G2 Fab-Sephadex and DNP_{11}-albumin-Sephadex coated with anti-DNP IgG (data not shown). Furthermore, labeled γ2b/γ1 FcR incorporated into reconstituted vesicles was retained on Millipore filters. $[^3H]Ph_4P^+$ uptake into K^+-loaded proteoliposomes reconstituted with purified γ2b/γ1 FcR and diluted into sucrose solution in the presence and absence of 2.4G2 IgG is shown in Fig. 4. Clearly, rapid uptake of $[^3H]Ph_4P^+$ is observed in the presence of 2.4G2 IgG. Thus, proteoliposomes reconstituted with purified receptor, mimic the basic observations with vesicles from J774 cells.

DISCUSSION

Binding of ligands to γ2b/γ1 FcR of the mouse J774 macrophage cell line results in immediate depolarization of the plasma membrane followed by a hyperpolarization (1). We have shown here that this membrane depolarization, mediated by specific ligands to the FcR, is a property of the purified receptor. In this study, we used plasma membrane vesicles prepared from J774 macrophages and proteoliposomes reconstituted with γ2b/γ1 FcR to investigate the effects of specific ligands to the FcR. For both, the evidence indicates that the ion permeability increases when the receptor is occupied with specific ligands.

We prepared membrane vesicles by nitrogen cavitation. A monoclonal antibody directed against γ2b/γ1 FcR, 2.4G2 IgG, bound to vesicles prepared by this method, indicating that right-side-out vesicles are present in the preparation, an observation that has also been made in other systems (4, 12). $[^3H]Ph_4P^+$ accumulation was used to monitor generation of a $\Delta\Psi$ secondary to an increase in membrane permeability to specific ions (9, 10). An increase in uptake of this lipophilic cation by KCl- or NaCl-containing vesicles would indicate preferential efflux of K^+ or Na^+. We observed increased $[^3H]Ph_4P^+$ uptake, relative to controls, with plasma membrane vesicles from J774 cells in the presence of immunocomplexes and 2.4G2 IgG. 2.4G2 Fab induced a response that was about half of that of 2.4G2 IgG and only at much higher concentrations. The "graded" depolarization response of intact J774 cells to ligands of various sizes (1) differs significantly from the results obtained with vesicles, which do not vary with the size of the immunocomplex. This might be

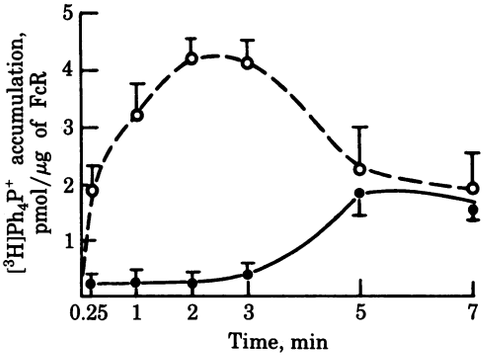

FIG. 4. Uptake of $[^3H]Ph_4P^+$ by vesicles containing reconstituted γ2b/γ1 FcR. FcR (20 μg) was reconstituted into egg phosphatidylcholine/cholesterol at a protein/lipid ratio of 1:10 by octyl glucoside dialysis. Vesicles were formed in 50 mM KCl and diluted 1:10 into an equiosmotic sucrose solution containing 100 μM $[^3H]Ph_4P^+$ (specific activity, 0.4 Ci/mmol) and 1 μM 2.4G2 IgG (○). ●, Control (reaction buffer without 2.4G2). Data represent mean ± SD of three experiments.

due in part to the high membrane-turnover rate of mouse macrophages (13), which results in rapid internalization of soluble immunocomplexes by endocytosis and thus helps to terminate the depolarization effect of membrane-bound soluble complexes. The permeability changes seen with the vesicles are specific for γ2b/γ1 FcR; monoclonal antibodies against three other membrane proteins did not result in $[^3H]Ph_4P^+$ accumulation. The ion permeability changes were induced effectively by bivalent (2.4G2 IgG) and larger ligands for FcR, which strongly suggests that bivalent crosslinking of γ2b/γ1 FcR molecules is sufficient to cause the permeability changes observed. The permeability changes observed at high 2.4G2 Fab concentration (≥ 1 μM) may be due to conformational changes induced by binding of the 2.4G2 Fab or to preferential aggregation of the γ2b/γ1 FcR–Fab complex in the plane of the membrane.

Plasma membrane vesicles incubated with FcR ligands show only a small increase in permeability to Ca^{2+}. This supports our observation that depletion of Ca^{2+} from the medium has only minimal effects (10%) on the $\Delta\Psi$ (1). This relatively small increase in permeability to Ca^{2+} may reflect a significant flow of Ca^{2+} into the cell because of the enormous Ca^{2+} gradient that exists across the cell membrane (14, 15). However, since macrophages and monocytes can ingest IgG-coated particles in the absence of extracellular Ca^{2+} (16, 17), a change in the Ca^{2+} permeability of the plasma membrane may not be physiologically important.

Increased uptake of $[^3H]Ph_4P^+$ by plasma membrane vesicles treated with specific ligands to the FcR was transient and decreases substantially after 7 min of incubation. Substitution of Cl^- inside the vesicles with an impermeant anion, isethionate$^-$, results in a high level of uptake of the lipophilic cation for at least 15 min. The transient nature of $[^3H]Ph_4P^+$ uptake triggered by FcR ligands in buffers with the permeant anion Cl^- is due to the collapse of a $\Delta\Psi$ established by the efflux of Na^+ or K^+ by the subsequent flow of permeant anions down the electrochemical gradient. Thus, accumulation of $[^3H]Ph_4P^+$ by the vesicles can be used to monitor ion fluxes.

We have reconstituted γ2b/γ1 FcR, purified by using the monoclonal antibody 2.4G2, into proteoliposomes. The reconstituted receptor retains both antigenic activity and the ability to bind to immobilized immunocomplexes, showing that at least a portion of the reconstituted FcR molecules are undenatured and have a right-side-out orientation. Binding of specific ligands to receptor-containing proteoliposomes induces cation efflux, as measured by $[^3H]Ph_4P^+$ accumulation when a K^+ diffusion gradient is imposed ($K^+_{in} \rightarrow K^+_{out}$), indicating that the purified receptor functions as an ionophore when occupied. Although our protocol for the purification of γ2b/γ1 FcR uses an elution scheme at high pH in the presence of deoxycholate, which may denature part of the receptor population, we believe our observations with the purified receptor represent at least a qualitative picture of FcR–ligand interactions.

An increase in ion permeability associated with the occupied FcR can be due to the opening of an ion channel or to an ion carrier mechanism (18). The use of $[^3H]Ph_4P^+$ as a probe cannot distinguish between these two mechanisms. We have also incorporated purified FcR into planar bilayer membranes and monitored the conductance across the membranes in the presence of specific ligands. The results are consistent with a receptor-ion channel model. This may be related to the physiologically important responses mediated by FcR. Besides triggering the cell to internalize membranes, binding of FcR in macrophages results in secretion of a number of oxygen metabolism intermediates (19), lysosomal enzymes (19, 20), and arachidonate metabolites (21, 22). Thus, the occupied FcR may be coupled to the secretion of physiologically important metabolites via an influx of ions into the cell, in a way similar to the stimulus–secretion coupling observed in gland cells (23, 24) or to the activation of lymphocytes via mitogenic stimulation (25). In addition, mouse macrophages have recently been shown to exhibit complex membrane conductances with rectifying properties (26–28) and the changes in ion permeability induced by the occupied FcR further emphasize the role played by ion fluxes in the normal physiology of these cells.

Ligand-dependent ion flow has been described for the acetylcholine receptor (29, 30) but this receptor, unlike the γ2b/γ1 FcR, consists of four polypeptide chains (31). Thus, our observations suggesting that a single polypeptide chain that may function as an ionophore by crosslinking with specific ligands offers an unparalleled opportunity for studying ion gating by membrane proteins.

Crosslinking and clustering of receptors in the presence of ligands have been shown to be involved in the functional activation of a number of different receptors (32–34). In particular, crosslinking of the IgE FcR in mast cells and basophils has been shown to be the signal for histamine secretion (34, 35). In this system, Ca^{2+} influx has been associated with the crosslinked receptor (36, 37). It may be possible that the receptor-ionophore model suggested for the γ2b/γ1 FcR is applicable to other receptors, particularly in those involving stimulus–secretion coupling.

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