Journal of Cellular Biochemistry 29:289–297 (1985)
Perspectives in Inflammation, Neoplasia, and Vascular Cell Biology 77–85

Functional Ion Channel Formation by Mouse Macrophage IgG Fc Receptor Triggered by Specific Ligands

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The mouse macrophage $Fc_{\gamma 2b/\gamma 1}R$ has previously been purified with the aid of the monoclonal antibody 2.4G2. That this $Fc_\gamma R$ functions as a ligand-dependent ion channel is supported by the following evidence: (a) Employing [^{3}H]tetraphenylphosphonium ([^{3}H]Ph_4P^+) as a probe for membrane potential changes in intact cells, we found a biphasic change in membrane potential following treatment with immune complexes, monoclonal antibody 2.4G2 IgG and 2.4G2 Fab-Sephadex particles. We observed an immediate depolarization followed by prolonged hyperpolarization. (b) [^{3}H]Ph_4P^+ uptake experiments with plasma membrane vesicles prepared from J774 macrophages showed that binding of ligands to the FcR led to transmembrane monovalent cation flow. (c) Similar [^{3}H]Ph_4P^+ uptake experiments were done with phospholipid vesicles containing purified and reconstituted $Fc_{\gamma 2b/\gamma 1}R$. Following challenge with specific ligands, transmembrane monovalent cation flow was observed. (d) Purified FcR was reconstituted into planar lipid bilayers; exposure to ligands led to transient bilayer conductance increase. The conductance change was resolved into single channel events.

Quin-2 measurements showed an increase of free cytosolic calcium levels in macrophages following exposure of cells to different ligands of the FcR. An optimal range of calcium was found to be required for phagocytosis, below and above which inhibition of ingestion occurred.

Key words: Fc receptor, ion channel, ligand-dependent planar bilayer

The mechanisms by which biological signals are transduced through cell surface receptors are central problems in cell biology. For macrophages, several subclasses of receptors for the Fc domain of IgG (Fc receptors, FcR's) have been described (see [1] for review). The binding of immune complexes to the macrophage FcR's results in a number of dramatic cell responses, which include internalization of the complexes [2] and secretion of arachidonic acid metabolites [3], activated oxygen intermediates [4], and a number of hydrolytic enzymes [5]. Recently, the mouse macrophage FcR that binds to IgG2b and IgG1 aggregates ($Fc_{\gamma 2b/\gamma 1}R$) has been purified by means of affinity chromatography that uses a monoclonal anti-FcR antibody (2.4G2) as the

Received May 10, 1985; accepted August 15, 1985.

affinity reagent [6,7]. The purified antigen, which has a M_r of 47–60 kilodalton (kd) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) retains binding capacity for immune complexes [7]. Since the monoclonal antibody 2.4G2 IgG blocks rosetting of IgG-coated erythrocytes to macrophages [6] and induces macrophage responses that are normally associated only with the occupied receptor [1,3], it may be used as a functional probe for studies of the FcR-ligand interactions.

We studied the mechanism(s) by which immune complexes or 2.4G2 IgG trigger macrophage responses. We hypothesized a receptor-ionophore model in which the bound $Fc_{\gamma 2b/\gamma 1}R$ functions as a ligand-dependent ion channel and the cell signaling is attributed at least in part to a flux of ions across the cell membrane. A rigorous test of this hypothesis could be performed since we had available the purified receptor and several specific ligands. Here, we summarize our data on transmembrane ion fluxes in intact cells, plasma membrane vesicles, and FcR-containing proteoliposomes associated with binding of the $Fc_{\gamma 2b/\gamma 1}R$, and high-resolution electrical conductance events associated with individual FcR-ligand complexes.

Membrane Potential Changes Associated With Binding of the FcR

For measurements of membrane potential, [^{3}H]tetraphenylphosphonium ([^{3}H]Ph_4P^+) was used as the voltage-sensitive probe [8]. Partitioning of [^{3}H]Ph_4P^+ into cells was converted into values of membrane potential by using the Nernst equation [8]. Cells used for such measurements were either in suspension or plated on coverslips.

The resting membrane potential of J774 cells in suspension averaged −14 mV [8]. Plated cells showed a much higher potential, reaching values of −65 to −80 mV after 6 hr of adherence [9]. This increase in membrane potential could be partially blocked by ouabain, suggesting a role for Na^+,K^+-ATPase activity which may become activated during adherence [9].

Treatment of cells with different ligands that bind to the $Fc_{\gamma 2b/\gamma 1}R$ resulted in a dramatic change in membrane potential [8]. This change was biphasic, initiating with an immediate depolarization, the magnitude and duration of which were both dose and Na^+ dependent, followed by a prolonged hyperpolarization (Fig. 1). The extent and duration of the depolarization response were also dependent on the extent of multivalent aggregation of the FcR (Fig. 1); that is, insoluble immune complexes gave larger membrane potential changes than soluble complexes, and these in turn were more effective than 2.4G2 IgG. And finally, the Fab fragment of 2.4G2 (a monovalent ligand) did not give any measurable depolarization response (Fig. 1). Whereas the depolarization was directly related to binding of FcR with specific ligands, hyperpolarization was also triggered by binding to other surface antigens (by monoclonal antibodies directed against major surface antigens of macrophages [8,10]). The hyperpolarization response could be blocked partially by ouabain and quinine, suggesting the possible involvement of Na^+,K^+-ATPase and Ca^{2+}-activated K^+-conductance in generating the hyperpolarization response.

Plasma Membrane Vesicles and FcR-Proteoliposomes

Plasma membrane vesicles from J774 macrophages were prepared by nitrogen cavitation and isolated by centrifugation through dextran or Percoll gradients [11]. Vesicles prepared this way could bind 2.4G2 IgG and immune complexes [11]. Transmembrane voltage gradients were generated by loading plasma membrane

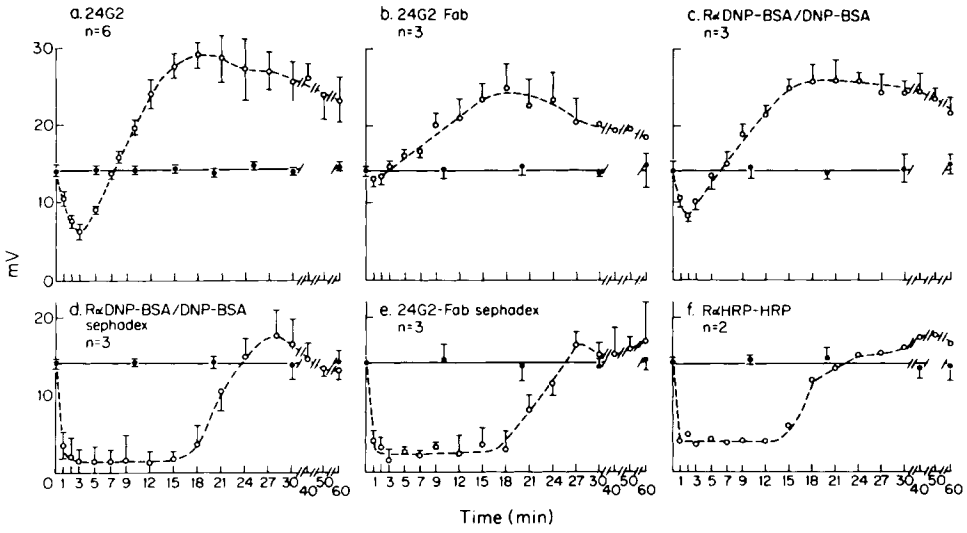

Fig. 1. Effect of specific ligands of FcR on membrane potential. Equilibration of 4×10^6 J774 cells/ml with 20 μm $[^3H]Ph_4P^+$ was done 25 min before exposure to ligands (○); control experiments represented by (●). a) 0.1 μM 2.4G2 IgG. b) 5 μM 2.4G2 Fab fragment. c) 1 μM rabbit anti-DNP IgG complexed with DNP_{11}-albumin at 1:6 molar ratio. d) 20 μl DNP_{11}-albumin-Sephadex complexed with anti-DNP IgG. e) 2.4G2 Fab-Sephadex (1.7 mg of 2.4G2 Fab/ml of Sephadex; 40 μl of particles/ml). f) 40 μg HRP complexed with rabbit anti-HRP. From [8].

vesicles with different electrolytes and resuspending them into equiosmolar sucrose solutions containing $[^3H]Ph_4P^+$ and specific ligands of the receptor. Binding of the FcR resulted in a preferential efflux of cations from membrane vesicles, which was indicated by a transient accumulation of the voltage-sensitive probe in the vesicles (Fig. 2). The extent of $[^3H]Ph_4P^+$ uptake into vesicles was directly proportional to the extent of multivalent aggregation of the FcR [11]. The FcR-ligand bound vesicles could not distinguish significantly between Na^+ and K^+; the permeability ratios for different ions showed the sequence: $Na^+ \sim K^+ > Li^+ > choline^+ > Ca^{2+} > Cl^-$. In all experiments, $[^3H]Ph_4P^+$ uptake into vesicles was rapid and transient, suggesting a collapse of the electrochemical gradient that was due to Cl^- permeability (Fig. 2) [11]. Thus, substitution of Cl^- in the vesicles by the impermeant anion isethionate$^-$ resulted in a prolonged transmembrane potential following addition of 2.4G2 IgG [11].

Purified FcR was reconstituted into lipid vesicles (phosphatidylcholine-cholesterol) by a detergent dialysis method [11]. Reconstituted FcR was assayed directly by a monoclonal sandwich radioimmunoassay with ^{125}I-2.4G2 Fab as the labeled probe [12,13]. Incorporation of FcR into liposomes was also ascertained by isopycnic centrifugation, which showed comigration of the reconstituted protein with the lipid vesicles after flotation through a sucrose gradient [13]. In the presence of specific ligands, we observed $[^3H]Ph_4P^+$ uptake into K^+-loaded FcR-proteoliposomes diluted into equiosmolar sucrose solution [11]. Similar results were also obtained with vesicles loaded with other electrolytes [unpublished observations]. These results strongly favor the receptor-ionophore model, suggesting that the observed ionophoric activity must be a property of the occupied receptor itself.

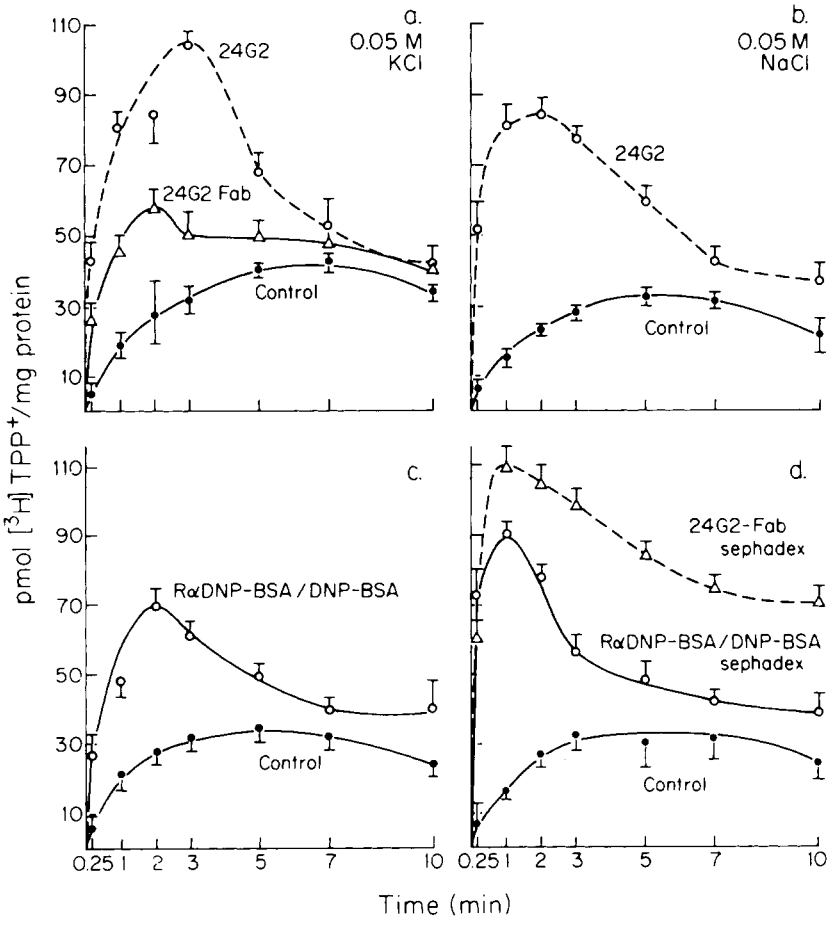

Fig. 2. Effect of ligands on transmembrane ion flow through J774 cell membrane vesicles. Vesicles equilibrated with 0.05 M KCl (a, c, and d) or NaCl (b) were diluted (1:10) into equiosmolar sucrose solutions containing ligands and 50 μM [^{3}H]Ph_4P^+. a) 0.1 μM 2.4G2 IgG (○); 1 μM 2.4G2 Fab (△). b) 0.1 μM 2.4G2 IgG (○). c) 0.1 μM soluble immunocomplex of CNBr-coupled DNP_{11}-albumin-anti-DNP-IgG, 1:6 (mol/mol) (○). 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. From [11].

Planar Lipid Bilayer Measurement of Ionic Current Associated With Bound FcR

The slow time resolution achieved with voltage-sensitive probes precludes us from drawing a more exact picture of the interactions of the receptor with the ligand at the molecular level. To overcome this problem, we reconstituted FcR-proteoliposomes into planar lipid bilayers and directly measured the transbilayer ion current associated with binding of the receptor by ligands [13]. The high impedance of the planar bilayer allows measurements of small transmembrane current changes with improved sensitivity and time resolution. In addition, the system provides built-in pharmacological control since reagents may be introduced to either side of the bilayer containing reconstituted FcR.

Monolayers of lipid containing FcR on one side (the *cis* side) could be transformed into stable bilayers [13]. Bilayers with incorporated receptor showed baseline conductance levels below 10 pS (S, siemens; 1 S = 1A/1V) and average capacitance of 0.72 μF/cm^2 [13]. The side opposite to receptor incorporation (*trans* side) was taken as virtual ground; that is, current flowed from *cis* to *trans*. Addition of ligands to the *trans* side of a bilayer containing FcR or to protein-free bilayers resulted in little or no conductance change (Fig. 3). However, when specific ligands were added to the *cis* side, the membrane conductance increased dramatically and was proportional to the concentration of both the receptor and the ligand used (Fig. 3). The sidedness suggested a vectorial orientation of the FcR in planar bilayers.

The increase in membrane conductance was not dependent on the polarity of the voltage used to clamp the bilayer. The conductance increase was activated by a variety of different specific ligands (Fig. 3). For a constant amount of receptor and ligand, the increase in membrane conductance was proportionally higher for larger multivalent ligands, in the same order observed for transmembrane fluxes in whole cells and plasma membrane vesicles. These results suggest that the state of aggregation of FcR by ligands may bear directly on the conductance levels observed. However, here, we also observed a substantial amount of ion current when monova-

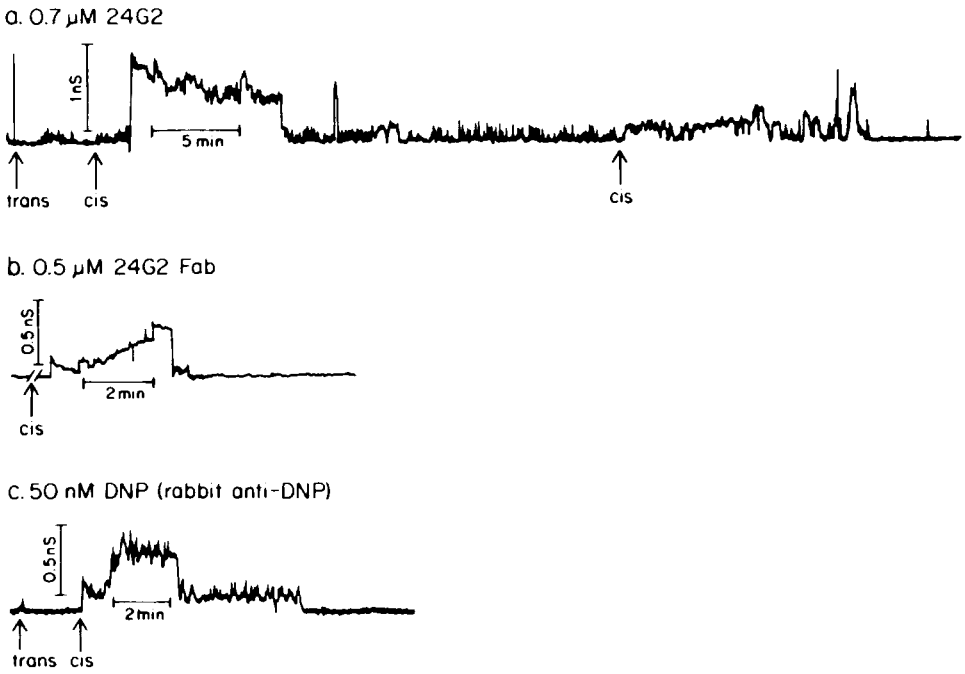

Fig. 3. Effect of specific ligands on FcR-containing planar bilayers. Each membrane contained 2 × 10^{-8} M of FcR. Ligands, 0.7 μM 2.4G2 IgG (a), 0.5 μM 2.4G2 Fab (b), and DNP-anti-DNP immunocomplexes (c), were prepared as described in Methods, and added to the side indicated by arrow. In c, rabbit anti-DNP IgG and DNP_{11}-albumin were mixed at IgG/antigen ratio of 6:1 and 50 nM of antigen complexed with IgG added to the membrane. In b, the noise owing to stirring was deleted (parallel bars). Membranes were clamped at a constant +30 mV. Temperature, 22°C. Time resolution, 0.6 msec. From [13].

lent ligand 2.4G2 Fab was used as a triggering ligand (Fig. 3c). This result may have been due to artificially imposed receptor aggregation during purification and/or reconstitution.

The increase in bilayer conductance levels, which occurred rapidly within the first few minutes following addition of ligands, thereafter decayed spontaneously to baseline levels. Subsequent addition of excess ligand resulted in only small conductance increases (Fig. 3a). The self-inactivation of the receptor observed here suggests that following binding and channel activation, the receptor relaxes to a new, nonconductive state.

To resolve single channel fluctuations associated with the bound receptor, the amount of receptor used for bilayer incorporation was reduced to picomolar amounts, and the ionic strength of the bathing buffer increased in order to enhance the current driven through each individual channel. Figure 4 shows single channels resolved in 1 M KCl with 2.4G2 IgG as the specific ligand. The mean conductance per channel was 60 pS, and the average lifetime was 250 mS, within the first 5 min after exposure to ligand. The single channel current-voltage relationships showed that the receptor-channel behaved as an ohmic resistor. Thus, the time-variant conductance (that is, the phenomenon of conductance relaxation) could not be explained by a variable conductance of the channel itself but rather by a decreased frequency of opening of the bound receptor as a function of the length of time during which the receptor was exposed to the ligand.

The ion-selectivity was determined from the reversal potentials required to null current flow from a ten-fold higher KCl or NaCl concentration across the bilayer. The ion-selectivity between NA^+ and K^+ was poor. We obtained a ratio of 3.5–4:1 for K^+ and Na^+ over Cl^- and 12:1 for Na^+/Ca^{2+}. Thus, the receptor behaves as a monovalent nonspecific cation-selective ion channel.

The kinetics of the channel opening and closing was related to the lipid composition of the lipid bilayer used. Lipids extracted from J774 macrophages with chloro-

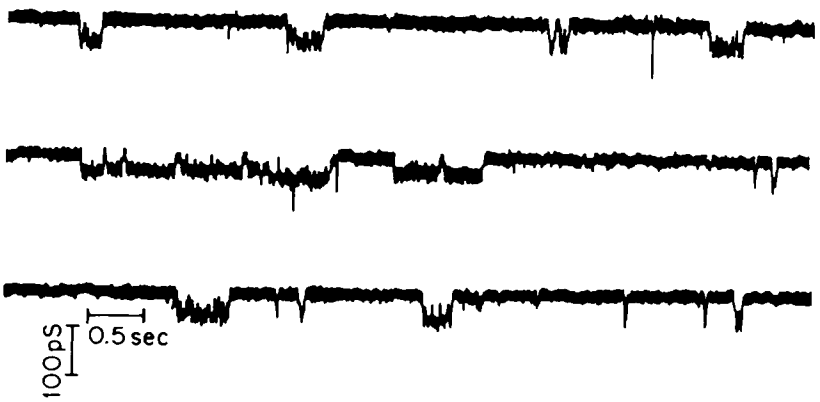

Fig. 4. Single FcR-channel fluctuations activated by 2.4G2 IgG. The bilayer contained 10 pM FcR and was treated with 0.1 μM 2.4G2 IgG. The bilayer was formed from egg lecithin:cholesterol in 1 M KCl, 3 mM $CaCl_2$, 10 mM Hepes, pH 7.4, and clamped at +100 mV. Opening of channels is indicated by downward deflections. Temperature 22°C. Time resolution, 1 msec. From [13].

form and methanol and used for bilayer formation gave much faster single channel events, with shorter open times.

Cytosolic Free Calcium Changes Associated With FcR-Ligation

Changes in cytosolic free calcium levels ($[Ca^{2+}]_i$) have been implicated in a number of cell surface-activated responses (see [14] for review). We investigated for a rise in macrophage $[Ca^{2+}]_i$ following binding by specific ligands of the $Fc_{\gamma 2b/\gamma 1}R$. The concentration of $[Ca^{2+}]_i$ was measured in J774 macrophages employing the fluorescent indicator quin-2 [15]. Quin-2 has a K_d of 0.1 μm for Ca^{2+}, appears to have no affinity for membranes or mitochondria, and provides therefore a sensitive, noninvasive assay for calcium levels found normally in the cell [16,17]. Resting $[Ca^{2+}]_i$ in macrophages averaged around 90 nm. Exposure to different specific ligands of the FcR increased $[Ca^{2+}]_i$ several-fold, the magnitude of the increase depending again on the extent of receptor aggregation. Exposure of the cells to the monovalent ligand 2.4G2 Fab gave only a small Ca^{2+} signal but blocked cell response to subsequent addition of multivalent ligands. Incubation of cells with antibody-coated erythrocytes increased macrophage $[Ca^{2+}]_i$ to micromolar levels. The cytosolic calcium changes were only partially blocked by depletion of extracellular calcium, indicating that Ca^{2+} was also being released from internal stores in response to receptor ligation, in addition to an influx of Ca^{2+} from the medium. The internal stores were not restricted to mitochondria, since mitochondrial inhibitors such as valinomycin, FCCP, and oligomycin did not inhibit the increase in $[Ca^{2+}]_i$ induced by ligands of the FcR in the absence of external calcium. The calcium signal appears to be associated with binding of the receptor rather than due to membrane depolarization per se, since depolarization of cells with high K^+-containing medium had little effect on $[Ca^{2+}]_i$ (employing 50 mM K^+ in the medium, which caused 70% membrane depolarization, increased $[Ca^{2+}]_i$ only to 180 nM).

An optimal range of cytosolic calcium appeared to be required for phagocytosis of opsonized particles by J774 and peritoneal macrophages [15]. Both lowering and increasing $[Ca^{2+}]_i$ produced inhibitory effects on particle ingestion [15]. $[Ca^{2+}]_i$ of macrophages was effectively buffered with quin-2 in the absence of external calcium, under which conditions phagocytosis was inhibited. This inhibitory effect was reversed with addition of external calcium. Alternatively, raising $[Ca^{2+}]_i$ to micromolar levels with the calcium ionophore A23187 (0.5 μM) also resulted in similar inhibitory effects. Again, the effect was reversible, indicating that A23187 was not toxic to cells at the levels used. High external $[Ca^{2+}]$ was only partially effective in inhibiting phagocytosis at levels over 10 mM. Moreover, such high levels of external calcium brought only little changes in the resting $[Ca^{2+}]_i$ of macrophages, reinforcing the well known fact that cells have powerful regulatory mechanisms for maintaining transmembrane calcium gradients.

The rise in cytosolic $[Ca^{2+}]$ observed here could occur in part as a result of direct permeation of calcium through the occupied receptor-channel. Although the reconstituted FcR-channel shows low permeability to Ca^{2+}, calcium influx through the receptor could be significant in view of the normally large transmembrane calcium gradient (at least over 10,000 fold).

DISCUSSION

It is known that the surface phenomena of phagocytosis and secretion of inflammatory metabolites by macrophages require extensive membrane movement. It

is possible that local rearrangement of cytoskeleton brought forth by an initial influx of ions (Na^+, Ca^{2+}) may lead to directed membrane movement. It is conceivable that a change in local ionic environment could provide the regulatory control or signal for such proteins as gelsolin, which is known to confer Ca^{2+} dependence to contractile proteins in macrophages (see [18] for review). Macrophages also show a number of complex membrane electrophysiological properties compatible with excitable cells [19,20], so that our observations are consistent with a role for ion fluxes in the normal physiology of these cells. A depolarizing current mediated by the FcR could initiate a series of membrane-activated conductances, including the generation of action potentials in human macrophages [20], all of which may be required for the movement of membranes.

The response of macrophages to specific ligands of the FcR appears to be graded in accord to the extent of receptor aggregation. These results mirror the response of intact macrophages to immunocomplexes in the triggering of release of mediators of inflammation [21]. Intriguing also is the observation that the receptor-channel decays spontaneously in channel activity. This time-variant phenomenon suggests a built-in control that inactivates the receptor, avoiding electrolyte leakage from the cell, which might otherwise have lytic effects on the macrophages. Other intracellular components may also play a role in regulating the receptor-channel activity. For instance, we have found that intracellular calcium levels modulate the kinetics of the receptor-channel, the channel remaining open for longer durations in the presence of at least micromolar levels of calcium.

Our results do not rule out that the bound receptor may have some kind of enzymatic activity or that membrane-associated enzymes may be activated in conjunction to the ion permeability increase. The receptor-channel model for FcR described here may be applicable to a number of other receptors that mediate important surface responses.

ACKNOWLEDGMENTS

We thank S.S. Ko for excellent technical assistance during part of this work and Drs. S.C. Silverstein and H.R. Kaback for helpful discussions. This work was performed with the aid of grants AI-14603 and CA-30198 from the Public Health Service and a Fellowship from Jane Coffin Childs Memorial Research Fund for Medical Research to J.D.-E Young.

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