THE GLYCOSYL PHOSPHATIDYLINOSITOL-LINKED FcγRIII_PMN MEDIATES TRANSMEMBRANE SIGNALING EVENTS DISTINCT FROM FcγRII

By ROBERT P. KIMBERLY, JON W. AHLSTROM,
MICHAEL E. CLICK, AND JEFFREY C. EDBERG

*From The Hospital for Special Surgery, Cornell University Medical College,
New York, New York 10021*

The recent recognition that FcγRIII (CD16) expressed on polymorphonuclear leukocytes (FcγRIII_PMN)[1] is a glycosyl-phosphatidylinositol (GPI) anchored molecule (1–3) has heightened interest in the functional roles of human Fcγ receptors (FcγR). The potential for rapid lateral mobility within the membrane (4, 5) and the lack of an obvious mechanism to couple with guanine nucleotide–binding regulatory proteins or cytoskeletal structures have fostered the concept of FcγRIII_PMN as a binding molecule serving to present ligand to FcγRII for subsequent transmembrane signaling (1, 6, 7). In contrast, the other human FcγRs (FcγRI, FcγRII, and the isoform of FcγRIII on NK cells) are transmembrane proteins (8–14), each with the capacity for signal transduction independent of other types of FcγRs (15–21).

Two lines of evidence, however, suggest that FcγRIII_PMN is also a functionally active molecule independent of FcγRII. First, PMN can lyse chicken E opsonized with anti-FcγRIII heteroantibodies (22–24). Second, while phagocytosis of IgG-opsonized E (EA) by PMN may involve both FcγRII and FcγRIII_PMN, internalization of Con A-opsonized E requires the obligatory participation of FcγRIII_PMN and is independent of Con A-mediated engagement of FcγRII (25, 26). To explore the capacity of FcγRIII_PMN to generate intracellular signaling events that would indicate a functional role extending beyond simple ligand binding, we have analyzed cytosolic calcium and membrane potential changes elicited by FcγR-specific mAbs (27, 28). Our data indicate that multivalent, but not univalent, ligation of FcγRIII_PMN causes a rapid increase in cytosolic calcium that is derived from intracellular stores. This signal

[1] *Abbreviations used in this paper:* $[Ca^{2+}]_i$, intracellular free calcium concentration; CR3, complement receptor type 3; CT, *Vibrio cholerae* toxin; EA, IgG-opsonized E; FcγR, receptor for the Fc region of IgG; FcγRII, the 40 kD receptor for the Fc portion of IgG; FcγRIII_PMN, the 50–80 kD receptor for the Fc portion of IgG expressed on polymorphonuclear leukocytes; GAM, goat F(ab')₂ anti-mouse IgG; GPI, glycosyl-phosphatidylinositol; IP, inositol phosphates; PI-PLC, phosphatidylinositol-specific phospholipase C derived from *Bacillus thuringiensis*; PMN, polymorphonuclear leukocyte; PT, *Bordetella pertussis* toxin.

is insensitive to pertussis and cholera toxins. Ligation of FcγRII with mAb does not elicit a similar change in intracellular calcium. This inability is not merely a reflection of the lower receptor density of FcγRII, since FcγRIIIpMN reduced to a similar density level by PI-PLC, still signals effectively. These data indicate that FcγRIIIpMN, a GPI-anchored molecule, actively mediates transmembrane signaling events distinct from FcγRII. Ligand-dependent collaboration of FcγRIIIpMN with FcγRII is not required.

Materials and Methods

Preparation of PMN.	Peripheral blood, obtained from healthy volunteers, was drawn into heparinized plastic syringes. PMN were separated by two-step discontinuous density gradient centrifugation on Ficoll-Hypaque (density = 1.078 and 1.119 g/ml) (25, 26). The PMN layer was harvested and washed once in PBS at 25°C. Contaminating erythrocytes were lysed by a 10-s exposure to redistilled water (Ultrascientific, Inc., Chicago, IL) followed by 0.18% saline. Cells were resuspended in PBS at 10^7 cells/ml for loading with pertinent fluorescent probes. By microscopic examination >95% of the cells were PMN with >99% viability. Separations were completed within 90 min and all experimental procedures were completed within 4-5 h of phlebotomy.

Reagents and Buffers.	All buffers and solutions were made with redistilled water. PBS (125 mM NaCl, 10 mM PO_4) was made with 5 mM KCl and 5 mM glucose. PBS with calcium and magnesium included 1.65 mM $MgCl_2$ and 1.0 mM $CaCl_2$. Sheath fluid for flow cytometry was redistilled water with 154 mM NaCl, 1.5 mM $MgCl_2$, and 1.0 mM $CaCl_2$. Reagents were used at ambient temperature (25°C); 5 min before analysis, each sample was warmed to and maintained at 37°C (29, 30).

The chemotactic peptide, FMLP (Sigma Chemical Co., St. Louis, MO), was dissolved in ACS grade DMSO (Fisher Scientific, Pittsburgh, PA) for a stock concentration of 10^{-3} M and stored in sterile, pyrogen-free containers at -20°C. Before each experiment an aliquot of the 10^{-3} M stock was thawed and diluted with PBS to a working concentration of 10^{-5} M. For experiments with *Vibrio cholerae* toxin (CT) and *Bordetella pertussis* toxin (PT) (Sigma Chemical Co.), cells were preincubated at 37°C for 120 min with either CT (0.5 to 2.5 μg/ml with 20 mM dithiothreitol) or PT (125 ng/ml to 500 ng/ml) (19, 31, 32). Indo-1 and $DiOC_5$ (Molecular Probes, Junction City, OR), fluorochromes for the measurement of intracellular calcium and membrane potential, respectively, were used as described below. BAPTA-acetoxymethyl ester (Molecular Probes), a nonfluorogenic calcium chelator, was prepared as a 2 mM stock solution in DMSO. For chelation of intracellular calcium, cells were preincubated with 10 μM BAPTA at 37°C for 30 min.

Aggregated IgG, prepared by heat aggregation of chromatographically purified 7S human IgG from Cohn fraction II (Miles Biochemicals, Elkhart, IN), was sized by column chromatography with AcA22 (LKB, Rockville, MD) and stored in aliquots at -70°C. Goat F(ab')₂ anti-mouse IgG (GAM), free of intact IgG by silver stain analysis of SDS-PAGE analytical gels, was obtained from Tago Immunochemicals (Burlingame, CA). F(ab')₂ GAM directly conjugated to FITC was used to confirm specific binding of this reagent to mouse mAb on the cell surface.

Monoclonal Antibodies.	3G8, a murine IgG1 mAb recognizing human FcγRIII (CD16) (33), was prepared in bulk culture and purified by ion-exchange and size-exclusion chromatography (Damon Biotechnology, Needham Heights, MA). Fab fragments were prepared by digestion with papain-Sepharose (Pierce Biochemical Co., Rockford, IL) and were purified by passage over protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at pH 8.3 and by molecular sieve chromatography with a column of TSK 3000 (LKB). Proteins were >98% pure. Fab fragment preparations contained no detectable intact IgG or heavy chains, as judged by silver stain of SDS-PAGE analytical gels.

mAb IV.3, a murine IgG2b recognizing human FcγRII (CD32) (34), was obtained as purified IgG and Fab fragments from Medarex, Inc., West Lebanon, NH. Fab fragments contained no detectable intact Ig or heavy chains as judged by silver stain of SDS-PAGE

gels. mAb preparations were sterile and contained no detectable endotoxin as determined by the limulus amoebocyte assay (Associates of Cape Cod, Woods Hole, MA). In selected experiments, mAb CIKM5, a murine IgG1 also recognizing CD32 (35), was used as an intact Ig.

Indo-1 Fluorescence. Indo-1, a calcium binding fluorescent dye whose spectral properties change with binding of free Ca^{2+} (36), was used to measure changes in intracellular calcium concentrations ($[Ca^{2+}]_i$). PMN were incubated for 15 minutes at 37°C with 5 μM indo-1 acetoxymethyl ester. After loading, the cells were washed once with PBS and maintained at room temperature in the dark. Five minutes prior to analysis the cells were warmed to 37°C in PBS with Ca^{2+} and Mg^{2+} and adjusted to a concentration of 1×10^6 cells/ml (29, 30). For selected experiments designed to test the influence of indo-1 loading conditions on assay sensitivity, PMN were incubated with varying concentrations of indo-1 acetoxymethyl ester ranging from 0.1 μM to 10 μM. Loading conditions were otherwise identical.

DiOC$_5$ Fluorescence. The lipophilic, cationic cyanine dye $DiOC_5$ was used to measure changes in membrane potential (29, 37, 38). Just prior to stimulation, cells were suspended at a final concentration of 10^6 cells/ml in PBS containing Ca^{2+} and Mg^{2+} and incubated for 5 min at 37°C with 20 nM $DiOC_5$. Cells were then analyzed directly on the flow cytometer.

Measurement of $[Ca^{2+}]_i$ and Membrane Potential. Four and five color flow cytometry was performed with a dual laser Cytofluorograf IIS with a 2151 computer (Becton Dickinson Immunocytometry Systems, Westwood, MA). Constant stirring was maintained throughout each experiment. Excitation at 488 nm for measurement of forward and right angle light scattering and for measurement of $DiOC_5$ fluorescence was provided by a Lexel 75 argon ion laser. Ultraviolet excitation at 350–360 nm for measurement of indo-1 fluorescence was provided by a Coherent 90K krypton ion laser. Peak indo-1 fluorescence emissions at 405 and 490 nm were monitored after passing through violet (395 ± 20) and blue (500 ± 20) band pass filters, respectively, as previously described (39). Physical separation of the two different incident laser beams was used to distinguish the 488-nm emission line of the argon laser from the blue emission of indo-1. $DiOC_5$ fluorescence emission was collected at >560 nm for five color analysis. Initial studies with $DiOC_5$ alone showed a linear relationship between fluorescence simultaneously collected at 510–530 nm and at >560 nm. All signals were analyzed in linear fluorescence units. The indo-1 ratio of violet to blue fluorescence was calculated in real time for each cell, multiplied by a constant to allow appropriate scaling, and displayed as a function of time. At the beginning of each experiment, the blue and violet PMTs were adjusted to provide a baseline display ratio of 10 on a scale of 1–100. The maximum ratio was determined after addition of 10 μg/ml ionomycin (Calbiochem-Behring Corp., La Jolla, CA).

For calibration of the indo-1 signal as $[Ca^{2+}]_i$ in nM, the four calibration parameters (Rmin, Rmax, Sb2, and Sf2) (36) were determined in a series of experiments on an SLM 8000 spectrofluorometer with excitation measured at 355 nm and emission measured simultaneously at 405 nm and 490 nm (SLM-Aminco, Urbana, IL). Since early time points (<10 s) were missed on the Cytofluorograf due to sample addition and mixing, the injection port on the SLM was used to facilitate analysis of these early points. All experiments were performed with constant stirring, and the mixing time was typically 2–3 s. The SLM 8000 was also used in parallel with the Cytofluorograf to analyze the influence of varying indo-1-AM loading concentrations on sensitivity for detection of changes in $[Ca^{2+}]_i$. Sensitivity was comparable for PMN loaded with indo-1 ranging from 1 to 10 μM and stimulated with both threshold and maximal stimuli. Loading with concentrations of 0.1 and 0.5 μM necessitated increased gains on the PMTs, but the sensitivity for detection of changes in $[Ca^{2+}]_i$ remained comparable for both the Cytofluorograf and the SLM 8000 measurements.

Immunofluorescent Flow Cytometry. Cells ($2.5–5 \times 10^5$), in PBS/1% FCS, were incubated with saturating doses of anti-FcγRIII mAb (3G8), anti-FcγRII mAb (IV.3), or the murine myeloma proteins MOPC21 (mIgG1) and UPC 10 (mIgG2a) as controls, for 30 min at 4°C. After washing, cells were stained with saturating amounts of FITC-conjugated rabbit anti-mouse IgG (Fab')$_2$ (Organon Technica, Malvern, PA) fragments. Additional controls included autofluorescence and staining of the cells with the FITC-conjugated rabbit anti-mouse IgG (Fab')$_2$ fragment alone. Cell-associated immunofluorescence was assayed by quantitative flow cytometry using an internal standard for each study (13, 26).

PI-PLC Digestion.   For treatment of PMN with phosphatidylinositol-specific phospholipase C (PI-PLC), a functional concentration of enzyme from *Bacillus thuringiensis* sufficient to cleave 0.39 μmol of PI/min/ml was added to the cells in PBS and incubated for 1 h at 37°C (13).

Data Analysis.   Flow cytometry data were collected in real time and expressed as a fluorescence ratio for indo-1 and as absolute fluorescence for DiOC$_5$ as illustrated in Fig. 1. These data were then computer-analyzed to determine time-dependent mean fluorescence ratio (R, indo-1) and mean fluorescence (F, DiOC$_5$). Changes in these values, relative to the prestimulus baseline (R$_o$ and F$_o$), were calculated for indo-1 (R/R$_o$) and for DiOC$_5$ (F/F$_o$) (Figs. 2–6). In addition, the percentage of cells responding to a stimulus was determined by selecting an arbitrary R value to give 5% responding cells in the baseline period (indo-1). The percentage of cells exceeding that value after stimulation was then calculated in a time-dependent fashion. For DiOC$_5$ fluorescence, the threshold value varied because of the broad distribu-
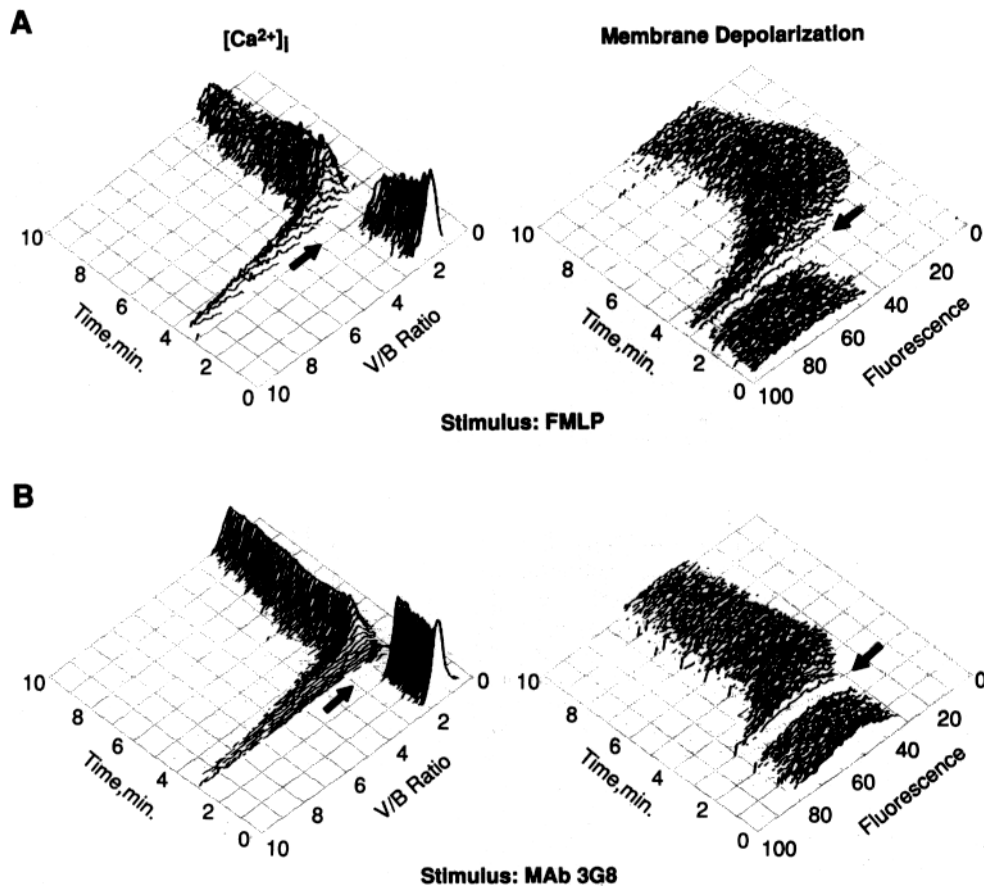

FIGURE 1.   Stimulation of PMN with FMLP and mAb 3G8. PMN were loaded with the appropriate fluorochromes and stimulated with 10^{-7} M FMLP or saturating levels of mAb 3G8. (*A*) The real-time, simultaneous measurement of time-dependent changes in [Ca^{2+}]$_i$ with indo-1 (*left*) and in membrane potential with DiOC$_5$ (*right*) elicited by 10^{-7} M FMLP. The arrows indicate the addition of FMLP. (*B*) The same parameters after stimulation by 10 μg/ml of mAb 3G8. The arrows indicate the addition of mAb 3G8. Computer analysis of these three-dimensional cytograms allowed the quantitation of mean fluorescence and of percentage of cells responding (cf., Figs. 2–6).

tion of fluorescence in the baseline state. Absolute values of $[Ca^{2+}]_i$ were determined from calibration experiments performed on the SLM 8000 spectrofluorometer as outlined above. Mean values are represented with the standard deviation as an index of dispersion.

Results

Stimulation of PMN by Chemotactic Peptide Receptors. Stimulation of freshly explanted human PMN by chemotactic peptides elicits a rapid increase in intracellular calcium levels and a change in membrane potential (29, 30). To provide a comparison for FcγRIIIPMN-induced responses, we analyzed the PMN response to FMLP by four- and five-color flow cytometry. Changes in membrane potential were monitored with DiOC5, and simultaneous changes in intracellular calcium were measured by the ratio of fluorescence emission of indo-1 (Fig. 1 *A*). Membrane potential changes showed a dose-response with hyperpolarization induced by FMLP at 10^{-11} to 10^{-9} M and increasing depolarization induced by FMLP at 10^{-8} to 10^{-6} M. The mean F/F_o for DiOC5 at maximal membrane depolarization with FMLP 10^{-7} M was 0.32 ± 0.07 (Fig. 1 *A*; $n = 12$). The magnitude of the increase in $[Ca^{2+}]_i$ also showed a dose-response relationship with the maximal change achieved by FMLP 10^{-7} M. The peak intracellular Ca^{2+} response (R/R_o) was 4.1 ± 0.9 $(n = 6)$ and was evident within 10 s after stimulation. The maximal membrane depolarization was not reached until 60–120 s after stimulation with 10^{-7} M FMLP. The initial $[Ca^{2+}]_i$ change was unaffected by chelation of extracellular Ca^{2+} with 10 mM EGTA.

Transmembrane Signaling Initiated by FcγRIIIPMN. To explore the capacity of FcγRIIIPMN, a GPI-anchored molecule, to elicit similar early cell activation signals, we used the anti-FcγRIII (CD16) mAb 3G8 that binds the receptor in or near the ligand binding site. At 5–10 μg/ml, a rapid increase $[Ca^{2+}]_i$ was induced by mAb 3G8 followed by modest membrane depolarization (Fig. 1 *B*). Both the cytosolic Ca^{2+} response and the membrane depolarization showed a dose-response behavior over two logs of mAb concentration (0.1–10 μg/ml; Fig. 2 *A*). Discrete, responding subpopulations were not identified (Figs. 1 *B* and 2 *A* [lower frames]), and smaller mean changes in the indo-1 signal represented smaller changes in $[Ca^{2+}]_i$ on an individual cell basis. Peak response was achieved at a final concentration of 5–10 μg/ml. Concentrations of 50 μg/ml gave responses identical to 5–10 μg/ml. As with FMLP, some variability among donors in the $[Ca^{2+}]_i$ response elicited by 3G8 was noted (Figs. 2, *A* and *B*). On average, the peak R/R_o was 3.6 ± 1.1 $(n = 25)$. In parallel studies on the spectrofluorometer this response represented a change in calculated $[Ca^{2+}]_i$ from a baseline level of 112 ± 16 nM $(n = 5)$ to 257 nM (range: 167–417 nM; $n = 5$).

Because the membrane potential changes elicited by mAb 3G8 were modest, we focused on $[Ca^{2+}]_i$ to define the properties of the cellular response induced by FcγRIIIPMN. The change in $[Ca^{2+}]_i$ was abolished by preloading cells with the nonfluorogenic Ca^{2+} chelator, BAPTA (data not shown), but was unaffected by chelation of extracellular Ca^{2+} with 10 mM EGTA (Fig. 2 *B*). These observations indicate that the indo-1 fluorescence reflected a true $[Ca^{2+}]_i$ signal and that the change in $[Ca^{2+}]_i$ was derived from intracellular stores.

Multivalent Crosslinking of FcγRIIIPMN Is Required. To examine whether univalent ligation of FcγRIIIPMN by 3G8 Fab fragments, like univalent ligation of the chemotactic peptide receptor by FMLP, could elicit a $[Ca^{2+}]_i$ response, we added 3G8
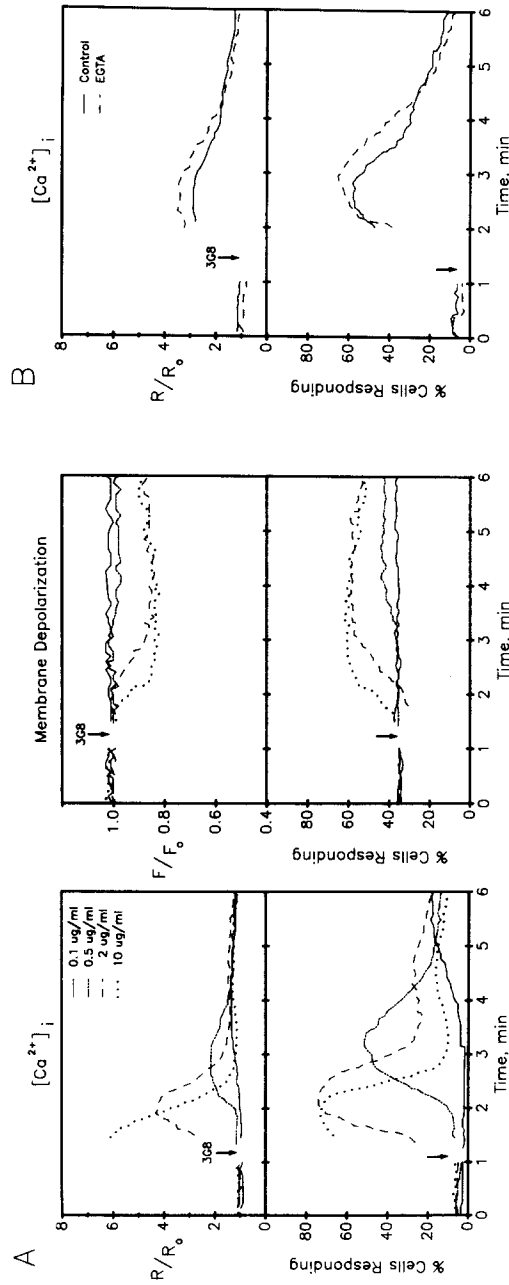
FIGURE 2.   Stimulation of PMN with anti-FcγRIII mAb 3G8. (A) The dose-response curves for mAb-induced changes in $[Ca^{2+}]_i$ (left) and membrane potential (right). Final concentrations of mAb 3G8 are given in the insert (upper left frame). The mean fluorescence ratio (R/R_o) for indo-1 and mean absolute fluorescence (F/F_o) for DiOC$_5$ are presented in the upper frames; the percent of cells responding are in the lower frames. As with FMLP-induced changes, maximal membrane depolarization followed the peak change in $[Ca^{2+}]_i$. (B) 10 mM EGTA did not alter the change in $[Ca^{2+}]_i$ elicited by mAb 3G8 ($n = 3$).

Fab to indo-1-loaded cells. Fab concentrations from 2.5 to 10 μg/ml showed no stimulation despite final concentrations of anti-receptor Fab exceeding the concentration necessary for saturation binding (Fig. 3 A; n = 1 [2.5 μg/ml], 4 [5 μg/ml], and 3 [10 μg/ml]). Addition of F(ab')$_2$ GAM, 50 μg/ml, to cells previously opsonized with 10 μg/ml 3G8 Fab, however, elicited an increase in $[Ca^{2+}]_i$ comparable to intact 3G8 (Fig. 3 B). These data indicate that univalent ligation of FcγRIII$_{PMN}$ is insufficient but that crosslinking of FcγRIII$_{PMN}$ alone is able to elicit the calcium response.

Interestingly, the crosslinking event did not make cells refractory to further stimulation by the same receptors. In two different experimental designs, FcγRIII$_{PMN}$, crosslinked initially with 3G8 IgG ranging in concentration from 5 to 50 μg/ml and subsequently with F(ab')$_2$ GAM, elicited a change in $[Ca^{2+}]_i$ that was not significantly different from the initial 3G8 IgG response. First, after addition of mAb 3G8 to PMN in suspension, repetitive signaling could be demonstrated with the
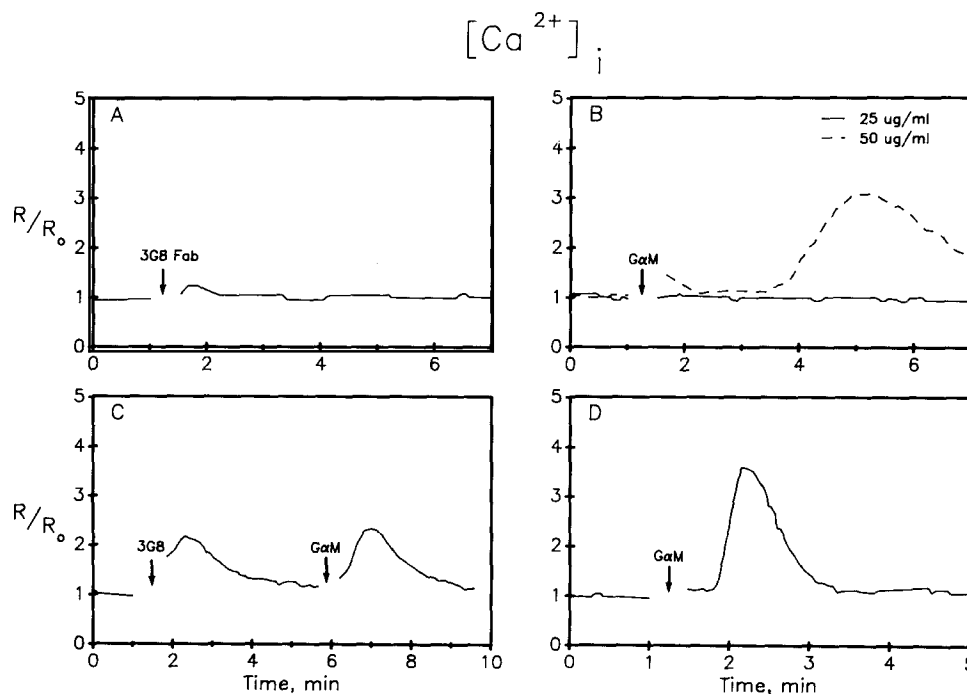

Figure 3. Crosslinking of FcγRIII$_{PMN}$. (A) Univalent ligation of FcγRIII$_{PMN}$ with 3G8 Fab, 10 μg/ml, did not elicit a $[Ca^{2+}]_i$ flux (n = 3, [10 μg/ml]). (B) When the 3G8 Fab were crosslinked by F(ab')$_2$ GAM, a calcium flux occurred. Cells were opsonized with 3G8 Fab (10 μg/ml), washed once, and then analyzed with the addition of F(ab')$_2$ GAM. F(ab')$_2$ GAM, 1–10 μg/ml, gave no response (n = 3), while F(ab')$_2$ GAM, 25 μg/ml, was variable (n = 3). F(ab')$_2$ GAM, 50 μg/ml, gave a consistent response comparable to that for 3G8 IgG (n = 3). (C) 3G8 IgG (5 μg/ml) was added to cells, the response analyzed and then F(ab')$_2$ GAM, 5 μg/ml, was added as soon as R/R$_0$ approached baseline (n = 6). (D) The $[Ca^{2+}]_i$ response to crosslinking of 3G8 IgG (10 μg/ml) with GAM (25 μg/ml) after one wash of 3G8-opsonized cells (n = 4). Low concentrations of GAM (1–5 μg/ml) gave smaller responses, as did very high concentrations (100 μg/ml). For these studies, binding of F(ab')$_2$ GAM was confirmed in selected experiments by using a FITC-conjugated preparation and monitoring cell-associated immunofluorescence in five-parameter analysis.

further addition of F(ab')₂ GAM (Fig. 3 C). Similarly, PMN, preincubated with mAb 3G8 and washed once before addition of F(ab')₂ GAM, responded to receptor crosslinking with a change in [Ca²⁺]ᵢ (Fig. 3 D). The intensity of the calcium response varied with the final concentration of GAM. For cells preopsonized with mAb 3G8, F(ab')₂ GAM 10-25 μg/ml was effective while cells preopsonized with 3G8 Fab required crosslinking with F(ab')₂ GAM 25-50 μg/ml.

The FcγRIII-induced [Ca²⁺]ᵢ Signal Is Pertussis Toxin Insensitive. In PMN, FMLP induces the breakdown of PIP₂ with the generation of diacylglycerol and IP by coupling with PT sensitive G proteins (40, 41). IP are recognized as mediators of the mobilization of calcium from intracellular stores (reviewed in reference 42). Accordingly, we looked at the toxin sensitivity of the [Ca²⁺]ᵢ signal elicited by both FMLP and FcγRIIIpmn. Preincubation of PMN with 125 ng/ml and 500 ng/ml PT significantly reduced the FMLP-induced [Ca²⁺]ᵢ response, while the FcγRIIIpmn-induced signal showed no change (Fig. 4). Cholera toxin (2.5 μg/ml) had no effect on the [Ca²⁺]ᵢ changes elicited by either stimulus (data not shown; n = 3).

Crosslinking of FcγRII Does Not Elicit a Comparable Change in [Ca²⁺]ᵢ. The experiments with 3G8 Fab and F(ab')₂ GAM demonstrated that participation of FcγRII (potentially engaged via the Fc piece of 3G8 IgG) is not necessary for generation of an intracellular calcium response. However, since mAb ligation of both human FcγRII and its murine homologue induces a change in [Ca²⁺]ᵢ in monocytes, U937 cells (15, 16), and peritoneal macrophages (28), we considered the possibility that FcγRII in PMN might function in comparable fashion. Using the anti-FcγRII mAb IV.3, we were unable to induce a calcium response with either univalent Fab fragments (Fig. 5 A) or with IV.3 Fab, crosslinked with F(ab')₂ GAM (Fig. 5 B). Intact IV.3 IgG at concentrations ranging from 0.1 μg/ml through saturation to 20 μg/ml was unable to induce a consistent change in [Ca²⁺]ᵢ (Fig. 5 C). Of note, however, in 2 of 14 separate experiments with IV.3 IgG a small change in the indo-1 ratio was observed (Fig. 5 C). Further experiments with the anti-FcγRII mAb CIKM5, known to induce a change in [Ca²⁺]ᵢ in monocytes, also showed small [Ca²⁺]ᵢ responses (R/Rₒ = 1.3 with 10 μg/ml; n = 3).

While IV.3 Fab crosslinked by F(ab')₂ GAM were unable to generate a change

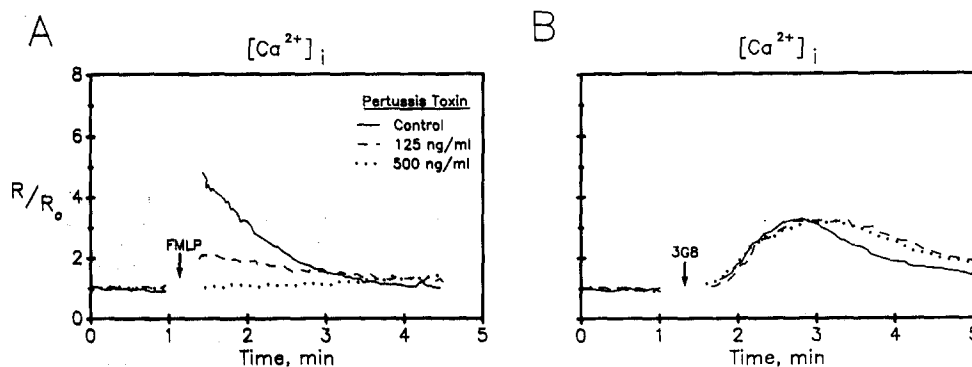

FIGURE 4. Comparative sensitivity to pertussis toxin. (A) The effect of PT on the [Ca²⁺]ᵢ response to FMLP. For FMLP, PT (125 ng/ml) reduced the [Ca²⁺]ᵢ response with an even greater effect at 500 ng/ml. (B) PT had no effect on the [Ca²⁺]ᵢ response elicited by mAb 3G8.
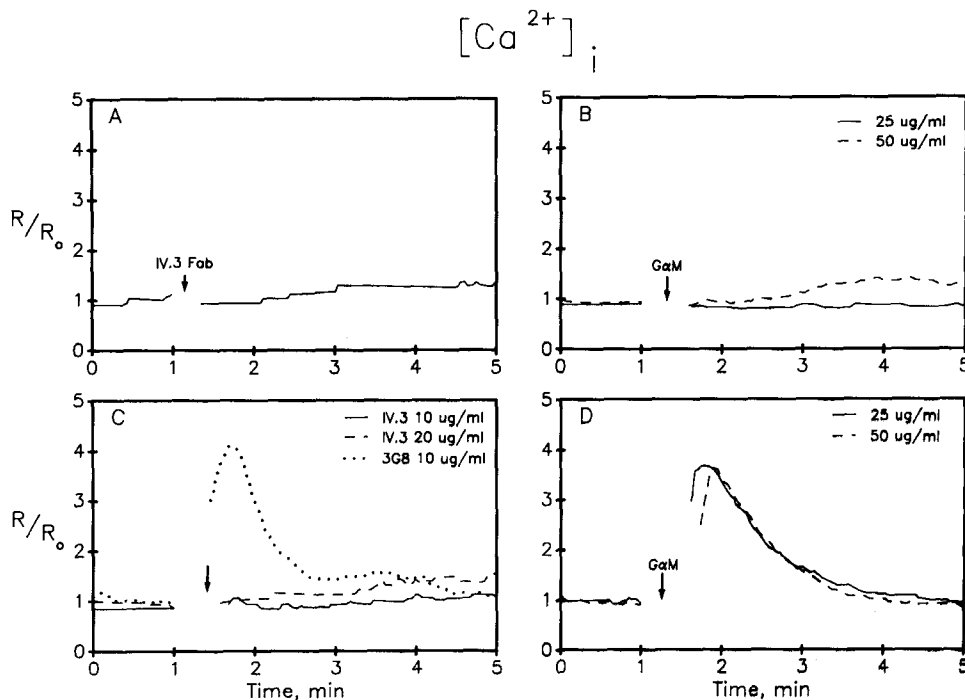
$$[Ca^{2+}]_i$$

A
IV.3 Fab

B
— 25 ug/ml
- - 50 ug/ml
GaM

C
— IV.3 10 ug/ml
- - IV.3 20 ug/ml
··· 3G8 10 ug/ml

D
— 25 ug/ml
- - 50 ug/ml
GaM

R/R_0

Time, min

FIGURE 5. Changes in $[Ca^{2+}]_i$ with mAb IV.3. (A) Univalent ligation of FcγRII with IV.3 Fab, 10 μg/ml, did not elicit a $[Ca^{2+}]_i$ flux (n = 3). Nor did a calcium flux occur when the IV.3 Fab (10 μg/ml) were crosslinked by F(ab')$_2$ GAM (25 and 50 μg/ml). (B) Cells were opsonized with IV.3 Fab, washed once, and then analyzed with the addition of F(ab')$_2$ GAM. F(ab')$_2$ GAM, 25-50 μg/ml gave little or no response (n = 6). (C) IV.3 IgG (5-20 μg/ml) was added to cells but no consistent response was evident (see text). The 20 μg/ml IV.3 represents the maximal $[Ca^{2+}]_i$ response seen with IV.3. The mAb 3G8 response on the same day is included for comparison. (D) The $[Ca^{2+}]_i$ response to crosslinking of IV.3 IgG (10 μg/ml) with F(ab')$_2$ GAM (25 μg/ml) after one wash of IV.3-opsonized cells (n = 3). The response is comparable to the 3G8 response.

in $[Ca^{2+}]_i$, IV.3 IgG crosslinked by the same reagent gave a rapid calcium response (Fig. 5 D) comparable to that elicited by 3G8 IgG. Both IV.3 Fab and IV.3 IgG bound to the PMN equally, as judged by quantitative flow cytometry. Thus, the effectiveness of GAM with IV.3 IgG may reflect the formation of heterotypic clusters of both FcγRII engaged by the Fab end of IV.3 and FcγRIII$_{PMN}$ engaged by the Fc region. Interestingly, aggregated human IgG generated a change in $[Ca^{2+}]_i$ comparable to crosslinked 3G8 Fab, 3G8 IgG and crosslinked IV.3 IgG (data not shown). Thus, both ligand and mAb induced similar $[Ca^{2+}]_i$ fluxes, but ligand was unable to distinguish between FcγRIII$_{PMN}$ signaling and synergistic signaling of FcγRIII$_{PMN}$ and FcγRII through heterotypic clusters.

FcγRIII$_{PMN}$ Signals Effectively at Reduced Receptor Density. Since PMN express approximately 150-200,000 copies of FcγRIII$_{PMN}$ per cell but only 40-60,000 copies of FcγRII per cell, we explored the possibility that the higher receptor number of FcγRIII$_{PMN}$ might explain the differential ability of the two FcγR on PMN to initiate a change in $[Ca^{2+}]_i$. We incubated PMN with PI-PLC to decrease the number

of FcγRIIIpmn on the cell surface. As measured by flow cytometry, FcγRIIIpmn density was reduced by ~70% to a level similar to that of FcγRII (Fig. 6 A). PI-PLC treatment did not alter PMN responsiveness to FMLP (Fig. 6 B). Similarly, cross-linking of FcγRIIIpmn with 3G8 IgG still elicited a rapid change in $[Ca^{2+}]_i$ that was indistinguishable from control samples (Fig. 6 B, *bottom*). Since PI-PLC treated and control cells showed identical response characteristics over a three log dose range of mAb 3G8 (0.05–50 μg/ml; n = 11), differences in receptor density cannot explain the different signaling properties of FcγRIIIpmn and FcγRII.

Discussion

The potential for rapid lateral mobility in the cell membrane and lack of an obvious signal transducing structure for the GPI-anchored form of FcγRIII expressed
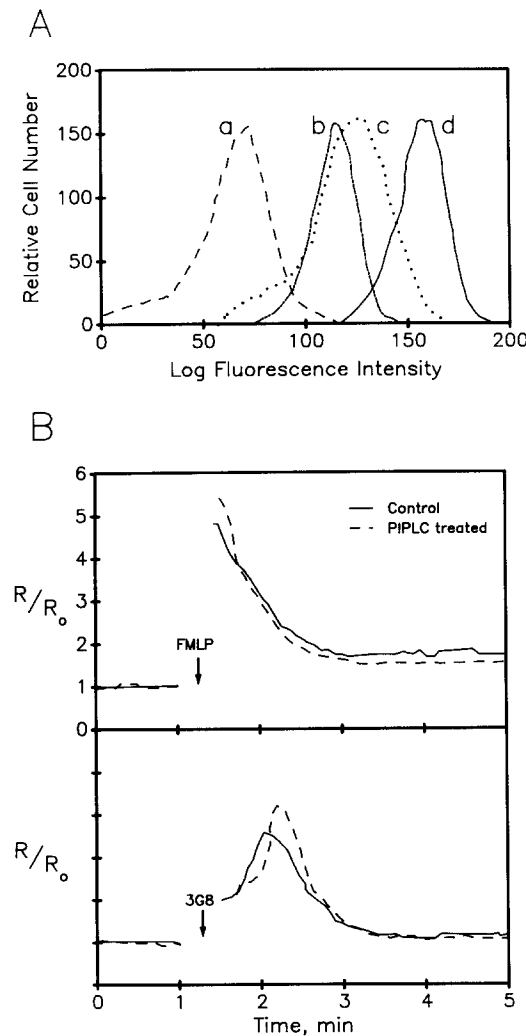

Figure 6.   Reduced FcγRIIIpmn density does not alter the change in $[Ca^{2+}]_i$. (A) After incubation of cells with PI-PLC, FcγRIIIpmn density is reduced to a level similar to FcγRII. Histogram *d* is mAb 3G8 fluorescence before PI-PLC; histogram *c* is 3G8 following PI-PLC. Histogram *b* shows that FcγRII (mAb IV.3) was unchanged by PI-PLC. Histogram *a* is the isotype control. (B) PI-PLC digestion did not alter the $[Ca^{2+}]_i$ response to FMLP (*top*). Similarly, the bottom panel shows that the response to 3G8, 1.0 μg/ml, was unchanged. A three log dose range of mAb 3G8 (0.05–50 μg/ml) was used for stimulation. No differences were detectable at any dose.

in PMN has raised the possibility that FcγRIIIpMN may serve only as a focusing molecule for ligand. The ability of various GPI-anchored molecules to mediate transmembrane signaling in T cells (43–48), however, prompted us to investigate the signaling properties of this receptor as a functionally active molecule in a non-T cell system. Using resting PMN, we have shown that oligovalent, but not univalent, ligation leads to a rapid increase in $[Ca^{2+}]_i$ derived from intracellular stores through a PT and CT insensitive process. This capacity to initiate changes in $[Ca^{2+}]_i$ is distinct from that of FcγRII and is not simply a reflection of the relative difference in cell surface density of the two FcγRs. The transmembrane signaling event initiated by FcγRIIIpMN requires the engagement of only FcγRIIIpMN by crosslinked Fab fragments. These data indicate that FcγRIIIpMN is not merely a focusing molecule for FcγRII but rather an active participant in integrated cell processes.

The mechanism of signal generation after ligation of FcγRIIIpMN differs from that used by the FMLP receptor in several significant ways. Univalent ligation of the FMLP receptor leads to both the generation of IP and a rapid initial increase in $[Ca^{2+}]_i$ derived from intracellular stores. Membrane depolarization is maximal at 2–3 min after stimulation and is followed by a transmembrane calcium flux and a secondary increase in cytosolic calcium (29, 30, 49, 50). These FMLP-induced changes are inhibitable by preincubation with PT (40, 41, 51–53). In contrast, univalent ligation of the FcγRIIIpMN ligand binding site by mAb 3G8 does not elicit any change in $[Ca^{2+}]_i$. Multivalent crosslinking of the receptor leads to a change in $[Ca^{2+}]_i$ derived from intracellular stores that is not sensitive to PT. While PT and CT insensitivity does not exclude the involvement of G proteins, it does suggest a receptor coupling pathway distinct from the FMLP receptor as well as from FcγRII, both of which are PT sensitive (19, 40, 41, 51–53). If G proteins are activated by FcγRIIIpMN, perhaps through another surface molecule, the rapidity of the response would suggest that FcγRIIIpMN and this other molecule co-exist in close physical association.

Transmembrane signaling and stimulation of cell proliferation have been demonstrated for other GPI-linked proteins, most notably in T cells (43–48). With both Thy-1 and TAP there is a requirement for coexpression of the T cell antigen receptor complex (CD3-TCR), implying the need for an additional molecular species for signal transduction (54–57). Since shared binding of antigen with CD3-TCR is not required for activation (43–47), a non–ligand-dependent interaction between Thy-1 (TAP) and some shared component(s) of the CD3-TCR may be important. For example, the ζ-chain of the TCR has been proposed as a more general signal-transducing molecule (58). Interestingly, the ζ-chain has homology to the FcεRI γ-chain which associates with the murine homologue of the transmembrane form of human FcγRIII (59). Given the incomplete removal of FcγRIIIpMN with PI-PLC treatment (1–3, 13, 14) and the lack of a decrement in the $[Ca^{2+}]_i$ signal, it is tempting to speculate that there may be a signal-transducing subpopulation of FcγRIIIpMN that is associated with another integral membrane protein, such as the γ-chain of FcεRI, and that is thereby relatively PI-PLC resistant. However, incomplete removal of many different GPI-anchored molecules by PI-PLC has been observed by many investigators (reviewed in references 60, 61), and recent data in the Qa-2 system, a GPI-anchored class I molecule on murine T cells, suggests that it is the GPI anchor per se that is essential for signal transduction (48).

No matter what molecular mechanism mediates signal transduction by FcγRIIIpMN, the absence of a $[Ca^{2+}]_i$ signal elicited by crosslinking of FcγRII alone with IV.3 Fab fragments and $F(ab')_2$ GAM highlights the potential for different roles served by FcγRII and FcγRIIIpMN. For example, PMN have the capacity to use several different pathways for FcγR-mediated phagocytosis. With resting PMN, EA phagocytosis is $[Ca^{2+}]_i$-dependent and PT-insensitive like the anti-FcγRIII mAb-initiated $[Ca^{2+}]_i$ signal (31, 62). In contrast, activated PMN have both PT-sensitive and insensitive components of EA phagocytosis, and many FcγRII-initiated processes, such as the generation of reactive oxygen intermediates and enzyme release by PMN and EA phagocytosis by monocytes, are also PT sensitive (19, 32). Thus, it may be possible that resting PMN primarily use a single signal transduction pathway while activated PMN use several receptor and/or signal transduction systems for phagocytosis. Of note, the occurrence of rapid $[Ca^{2+}]_i$ responses with crosslinking of IV.3 IgG and the presumed formation of heterotypic clusters of both FcγRII (engaged by Fab) and FcγRIIIpMN (engaged by the Fc region) suggest that synergism between the two receptors may be important in PMN function.

The role of the $[Ca^{2+}]_i$ signal per se, generated by FcγRIIIpMN, may be as a "priming" or "triggering" event that serves in conjunction with other events generated through FcγRIIIpMN or other surface molecules. The general model of a rapid change in $[Ca^{2+}]_i$ as a "priming" event is applicable to a number of receptor systems. For example, in PMN, superoxide anion generation seems to require both an initial rapid change in $[Ca^{2+}]_i$ and a more sustained signal (63). By analogy, the intracellular calcium signal elicited by FcγRIIIpMN might serve as a triggering event for interaction with another membrane molecule such as CR3 of the β2 integrin family, that can influence phagocytosis by FcγRs in PMN (64). A physical interaction between the FcγR and certain domains of CR3 may occur after engagement of the FcγR by ligand, and CR3 may couple with the cytoskeleton to assist in FcγR phagocytosis. Since FcγR and CR3 probably do not coexist in a multimolecular receptor complex, it is possible that, as a consequence of engagement of FcγR by ligand, a transmembrane signal and/or conformational change in the receptor initiates the collaboration between FcγR and CR3. If applied preferentially to FcγRIIIpMN, this model might explain the different roles for both $[Ca^{2+}]_i$ transients (62, 65) and PT-sensitive G proteins in EA phagocytosis by resting PMN and monocytes (31).

The full range of cell functions initiated by ligation of FcγRIIIpMN is not clearly established. Evidence that both FcγRII and FcγRIIIpMN contribute to the generation of reactive oxygen metabolites has been presented (19, 34, 66). Given the PMN's capacity to use different pathways for the same function (31) and to alter its biology depending on the state of activation and/or adherence (67-69), interaction between receptor systems can be anticipated. However, it is clear that FcγRIIIpMN, a GPI-anchored molecule, can mediate transmembrane signaling independent of ligand-mediated engagement of FcγRII. Analysis of the different transmembrane signals initiated by FcγRIIIpMN and FcγRII in PMN and initiated by FcγRIII on PMN and on NK cells may provide important insights into structure-function relationships in human FcγR.

Summary

To investigate the ability of FcγRIII$_{PMN}$, the GPI-anchored isoform of FcγRIII (CD16) in polymorphonuclear leukocytes (PMN), to mediate transmembrane signaling events, we measured changes in membrane potential with DiOC$_5$ and in intracellular calcium with indo-1. FcγR were ligated by anti-FcγRIII mAb 3G8 (IgG and Fab), anti-FcγRII mAb IV.3 (IgG and Fab), and human IgG aggregates. Cell bound mAbs were also crosslinked by goat F(ab')$_2$ anti–mouse IgG. 3G8 IgG elicited a rapid change in [Ca^{2+}]$_i$, which was unaffected by EGTA, *Vibrio cholerae* toxin (CT), or *Bordetella pertussis* toxin (PT), and was abolished by BAPTA. Univalent receptor binding with 3G8 Fab gave no response but crosslinking with F(ab')$_2$ GAM gave a rapid [Ca^{2+}]$_i$ response. Neither IV.3 Fab, IV.3 IgG, nor crosslinking of IV.3 Fab elicited a calcium signal. PI-PLC–treated PMN with the density of FcγRIII$_{PMN}$ reduced to that of FcγRII showed an unattenuated change in [Ca^{2+}]$_i$ with a 3G8 stimulus. The effects of IgG aggregates paralleled those of 3G8 mAb. These data indicate that multivalent ligation of FcγRIII$_{PMN}$ initiates an increase in [Ca^{2+}]$_i$, derived from intracellular stores, that is distinct from both the FMLP- and FcγRII–induced responses. Ligand-dependent interaction with FcγRII is not required. Since FcγRIII$_{PMN}$ can internalize the FcγRIII-specific probe Con A–opsonized E and lyse anti-FcγRIII heteroantibody-opsonized chick E, this GPI-anchored molecule mediates both signal transduction and integrated cell responses.

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