The Ca\(^{2+}\) Dependence of Human Fc\(\gamma\) Receptor-initiated Phagocytosis*

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Differing roles for [Ca\(^{2+}\)], transients in Fc\(\gamma\)R-mediated phagocytosis have been suggested based on the observations that antibody-opsonized erythrocyte phagocytosis by human neutrophils shows a [Ca\(^{2+}\)], dependence, while that by murine macrophages appears [Ca\(^{2+}\)],-independent. To explore whether this difference might reflect different receptor isoforms or different cell types, we studied the [Ca\(^{2+}\)], dependence of receptor-initiated phagocytosis by human Fc\(\gamma\)RIIa and a panel of Fc\(\gamma\)RIIIa cytoplasmic domain mutants expressed in murine P388D1 cells and by human Fc\(\gamma\)R endogenously expressed on human neutrophils and monocytes. Wild-type and point mutants of huFc\(\gamma\)RIIa stably transfected into murine P388D1 cells have different capacities to initiate a [Ca\(^{2+}\)], transient, which are closely correlated with quantitative phagocytosis (r = 0.94, p < 0.0001). Phagocytosis both by huFc\(\gamma\)RIIa in P388D1 cells and by huFc\(\gamma\)RIIa endogenously expressed on neutrophils and blood monocytes shows [Ca\(^{2+}\)], dependence. Phagocytosis of antibody-opsonized erythrocytes by neutrophils demonstrated greater susceptibility to [Ca\(^{2+}\)],, quenching compared with Fc\(\gamma\)RIIa-specific internalization with E-IV.3, suggesting that phagocytosis activating property of Fc\(\gamma\)RIIIb in neutrophils also engages a [Ca\(^{2+}\)],-dependent element. In contrast, phagocytosis by human Fc\(\gamma\)RIa, endogenously expressed on blood monocytes, is [Ca\(^{2+}\)],-independent. Despite the importance of a consensus tyrosine activation motif for both receptors, Fc\(\gamma\)RIa and Fc\(\gamma\)RIIa engage at least some distinct signaling elements to initiate phagocytosis. The recognition that both of the phagocytic receptors on murine macrophages and human Fc\(\gamma\)RIa associate with the Fc\(\gamma\)R \(\gamma\)-chain, which contains a tyrosine activation motif distinct from that in the Fc\(\gamma\)RIIa cytoplasmic domain, suggests that [Ca\(^{2+}\)],-independent phagocytosis is a property associated with the utilization of \(\gamma\)-chains by Fc\(\gamma\)R.

Receptors for the Fc region of IgG (Fc\(\gamma\))\(^{3}\) provide a link between antibody-antigen complexes and cellular-based effector functions and are critical in the regulation of the inflammatory response (1, 2). Significant structural diversity between the three gene families encoding Fc\(\gamma\)R is observed (1–5). Nonetheless, Fc\(\gamma\)R share certain intracellular signaling pathways. The common themes in Fc\(\gamma\)R signaling pathways involve the activation of protein tyrosine kinases followed by a transient rise in intracellular Ca\(^{2+}\) levels. The [Ca\(^{2+}\)], increase is essential for many cellular functions and is required for the phagocyte Fc\(\gamma\)R-induced oxidative burst (6, 7).

Many lines of evidence in both human and murine systems indicate that tyrosine phosphorylation events are critical for phagocyte Fc\(\gamma\)R functions, including phagocytosis (8–10). In addition, in many systems examined, tyrosine kinase activity is required for the receptor-induced rise in [Ca\(^{2+}\)], (presumably through tyrosine phosphorylation of phospholipase C\(\gamma\)1 and generation of inositol 1,4,5-trisphosphate). However, the role of [Ca\(^{2+}\)], in Fc\(\gamma\)R receptor-mediated phagocytosis has been controversial. For example, work in murine macrophage cell lines suggests that transients in [Ca\(^{2+}\)], are not essential for phagocytosis of antibody-opsonized erythrocytes (EA) (11–13). In contrast, phagocytosis of EA by human neutrophils is significantly impaired by chelation of intracellular calcium and abrogation of [Ca\(^{2+}\)], transients (14, 15). The ability of [Ca\(^{2+}\)],-depleted neutrophils to mediate phagocytosis initiated by other cell surface receptors suggests that the [Ca\(^{2+}\)],-dependent EA phagocytosis by human neutrophils may reflect a particular property of the Fc\(\gamma\) receptors on these cells (16).

Indeed, each of the studies of the [Ca\(^{2+}\)],-dependence of Fc\(\gamma\)R mediated phagocytosis has used EA probes that engage all available Fc\(\gamma\) receptor types and has not systematically distinguished between different cell types or cells derived from different species. Recent data indicate that important species differences do exist for Fc\(\gamma\) receptors. For example, human Fc\(\gamma\)RIIA and Fc\(\gamma\)RIIB, expressed on neutrophils, do not have murine homologues (1–3). Human Fc\(\gamma\)RIIA stably transfected into the murine macrophage cell line P388D1 mediates receptor-specific phagocytosis but in a [Ca\(^{2+}\)],-dependent fashion (17). In contrast, murine Fc\(\gamma\)RII (the IIb isoform) does not have a tyrosine activation motif nor does it trigger a [Ca\(^{2+}\)], transient or protein tyrosine phosphorylation (18). Murine Fc\(\gamma\)RII is also unable to mediate phagocytosis in macrophages in either a [Ca\(^{2+}\)],-dependent or independent fashion (19). These observations, coupled with the recent data of Stendahl and co-workers (20) that [Ca\(^{2+}\)], storage organelles accumulate at contact sites during phagocytosis in human neutrophils, P1, phagocytic index (number of erythrocytes phagocytosed per 100 phagocytes); E\(_{B4}\), biotinylated bovine erythrocytes; E\(_{B4A}\), avidin coated E\(_{B4}\); FMLP, formylmethionylleucylphenylalanine; E-IV.3 and E-22, erythrocytes coated with the anti-FcRII mAb IV.3 Fab fragments or the Fc(R)I mAb 22 F(ab\(^{-}\))\(_{2}\) fragments through a biotin-avidin bridge.
trophils prompted us to reexamine the question of the \(\text{Ca}^{2+}\) dependence of Fc \(\gamma\) receptor-mediated phagocytosis by human Fc \(\gamma\) receptors, endogenously expressed by human cells and stably transfected into the P388D1 murine macrophage cell line.

Our data indicate that human FcR\(\gamma\)Ia uses \(\text{Ca}^{2+}\)-dependent elements to mediate receptor-specific phagocytosis and that huFcR\(\gamma\)Ia point mutants with a varying ability to initiate a \(\text{Ca}^{2+}\)-transient show a closely corresponding variation in quantitative phagocytosis. Human FcR\(\gamma\)Ia, endogenously expressed on neutrophils and blood monocytes, also shows partial \(\text{Ca}^{2+}\)-dependence for phagocytosis. In contrast, phagocytosis by human FcR\(\gamma\)Ia, endogenously expressed on blood monocytes, is \(\text{Ca}^{2+}\)-independent. Despite the importance of tyrosine phosphorylation for phagocytosis and the use of a consen- sus tyrosine activation motif by both receptors (1–5, 21–30), they engage at least some distinct signaling elements to ini- tiate phagocytosis. The recognition that the human FcR\(\gamma\)Ia associates with the FcR\(\gamma\) \(\gamma\)-chain, which contains the tyrosine activation motif (21, 22) as do both of the phagocytic receptors on murine macrophages (23), suggests that \(\text{Ca}^{2+}\)-independent phagocytosis is a property associated with FcR utilizing \(\gamma\)-chains. Human FcR\(\gamma\)Ia, while engaging many elements in common with human FcR\(\gamma\)Ia such as p27\(\kappa\)y and phospholipase C\(\gamma\)1 (25–30), must also engage \(\text{Ca}^{2+}\)-dependent elements.

**MATERIALS AND METHODS**

**Reagents—**NHS-LC-biotin, sulfo-NHS-biotin, and streptavidin were obtained from Pierce. BAPTA-AM and Indo-1/AM were from Molecular Probes (Eugene, OR). A 10 mM stock of BAPTA-AM in dimethyl sulfox- ide obtained from Pierce. BAPTA-AM and Indo-1/AM were from Molecular Probes (Eugene, OR). A 10 mM stock of BAPTA-AM in dimethyl sulfox- ide was prepared and stored at −20 °C. Genistein was from Life Tech- nologies, Inc. and was stored as a 1 mM stock in dimethyl sulfoxide at −20 °C. Anti-FcR\(\gamma\)Iab mAbs 32.2, 22 (FcR\(\gamma\)I, CD64) and IV.3 (FcR\(\gamma\)I, CD32) (Fab and F(ab\(^{2}\)) fragments were generated from the isotype-matched mAb by goat anti-mouse IgG (GAM) for mAb cross-linking and phyco- erythrin- and fluorescein isothiocyanate-conjugated GAM for immuno- fluorescence flow cytometry. Studies were obtained from Jackson Immuno- research (West Grove, PA) or Boehringer Mannheim. Fetal calf serum and RPMI 1640 were obtained from Life Technologies, Inc. All other reagents were from Sigma.

**Cells and Cell Lines—**Mutant FcR\(\gamma\)Ia cDNAs were made by oligonucleotide primer-directed site-specific mutagenesis of a human FcR\(\gamma\)Ia cDNA generously provided by J. Kochan, Natl. Inst. of Allergy, N.Y. (31). Mutants, confirmed by sequencing (Sequenase 2.0, U. S. Biochemical Corp.), were subcloned into pcEXV-3 (32) and transfected into EBA (17, 34). Briefly, suspensions of cells at 10\(^{6}\) cells/ml in 1.1 mM Ca\(^{2+}\) and Mg\(^{2+}\)-free phosphate-buffered saline, pH 7.4, were incubated with 5 \(\mu\)M Indo-1/AM at 37 °C for 15 min and washed in PBS. Cells preparations to be opsonized with receptor-specific mAb Fab were resuspended in Ca\(^{2+}\)- and Mg\(^{2+}\)-free RPMI PBS at 10\(^{6}\) cells/ml, incubated with saturating concen- trations of mAb IV.3 Fab (0.5 \(\mu\)g/ml) or mAb 22 F(ab\(^{2}\)) (2 \(\mu\)g/ml) at 37 °C for 5 min, and washed in PBS. All cell preparations were resus- pended in 1 mM Ca\(^{2+}\) Mg\(^{2+}\)-PBS at 37 °C for 5 min and then immediately transferred to a continuously stirred cell cuvette main- tained at 37 °C in the SLM 8000. With excitation at 355 nm, the simultaneous fluorescence emission signal at 405 and 490 nm was measured, integrated, and recorded each second. After establishing a base line for 60 s, goat anti-mouse F(ab\(^{2}\)) was added (35 \(\mu\)g/ml final concentration), and data acquisition was continued for an additional 3.5 min. Each sample was individually calibrated by lysing cells in 1% Triton X-100 to determine the maximal emission ratio and by adding EDTA (20 mM final concentration) to determine the minimal ratio. The Indo-1 fluo- rescence emission signal was converted to \(\text{Ca}^{2+}\), by the method of Gryniewicz et al. (36).

**Measurement of Phagocytosis—**Biotinylated bovine erythrocytes (E\(_{b}\)) and biotinylated anti-FcR\(\gamma\) were prepared as described previously (37). Briefly, erythrocytes (at 1 \(\times\) 10\(^{6}\) cells/ml in 0.1 M carbonate buffer (pH 8.6)) were incubated with 250 \(\mu\)g/ml of sulfo-NHS-biotin for 20 min at 4 °C with mixing. E\(_{b}\) (1 \(\times\) 10\(^{5}\) E/ml) were coated with an equal volume of streptavidin (250 \(\mu\)g/ml) for 30 min at 4 °C with mixing. The streptavi- don-coated E\(_{b}\) (E\(_{sa}\)) were then washed and resuspended to 1 \(\times\) 10\(^{5}\) erythrocytes/ml for immediate use. mAb were biotinylated with NHS- LC-biotin in 0.1 M carbonate buffer (pH 8.6). Typically, 50 \(\mu\)g/ml NHS- LC-biotin was used to biotinylate mAb (1 mg/ml for 60 min at room temperature). The free biotinylated mAb was removed by extensive dialysis against PBS (pH 7.4). Small volume dialysis (ranging from 50–100 \(\mu\)l for the mAb) was performed in a dialysis chamber (Pierce).

mAb-conjugated erythrocytes were prepared by incubating E\(_{ba}\) with dilutions of biotinylated anti-FcR\(\gamma\) mAb (37). mAb-coated E\(_{ba}\) were resuspended in RPMI 1640-fetal calf serum, an aliquot was removed for analysis by indirect immunofluorescence, and the remaining cells were used immediately for the phagocytosis or attachment assays. EA were prepared by incubating bovine erythrocytes with a 1:4 dilution of the maximal subagglutinating titer of rabbit anti-bovine erythrocyte IgG as described previously (38).

For quantitation of mAb-coated E\(_{ba}\) or EA phagocytosis by fresh human neutrophils (5 \(\times\) 10\(^{6}\) cells/ml) mixed with 100 \(\mu\)l of fresh neutrophils (5 \(\times\) 10\(^{6}\) cells/ml) or mAb (1 \(\times\) 10\(^{6}\) monocytes/ml), determined by myeloperoxidase staining) at a ratio of 25:1 (mAb-coated E\(_{ba}\)) or 50:1 (EA) (37). The cell mixture was pelleted for 5 min at room temperature at 44 \(\times\) g and then incubated at 37 °C for 20 min (neutrophils) or 1 h (mononuclear cells). After the nonphagocytosed erythrocytes were lysed by hypotonic saline (0.2% NaCl) for 20 s followed by the addition of an equal volume of 1.6% NaCl, phagocytosis was quan- titated by light microscopy. The data are expressed as phagocytic index (PI), the number of ingested particles/100 neutrophils or monocytes.

Phagocytosis by transfected P388D1 cells was determined in an adherent assay system. P388D1 cells (5 \(\times\) 10\(^{5}\) cells/ml) were allowed to adhere to round glass coverslips in culture dishes overnight at 37 °C. Coverslips were then transferred to clean culture dishes and EA- or mAb-coated E\(_{sa}\) were added (50 \(\mu\)l at 5 \(\times\) 10\(^{5}\) erythrocytes/ml) were added and incubated for 1 h at 37 °C. Noninternalized erythrocytes were lysed by brief immersion of the coverslip in dH\(_{2}\)O followed by immersion in buffer. Phagocytosis was quantitated by light microscopy and expressed as described above.

Heat-treated and serum-treated zymosan were prepared as de- scribed previously (38). Briefly, heat-treated zymosan were prepared by boiling 10 mg of zymosan for 10 min. Serum-treated zymosan were prepared by incubating 2 mg of zymosan with 2 ml of normal human serum for 30 min at 37 °C. Following washing, both heat-treated zymo- san and serum-treated zymosan were resuspended to 2.5 \(\times\) 10\(^{6}\) cells/ml. For phagocytosis, or serum-treated, and then resuspended with 100 \(\mu\)g/ml genistein for 30 min, and then the genis- tein was maintained at the same concentration through the phagocytic assay. Controls included loading cells with the BAPTA-AM and genis-


**Ca\(^{2+}\) Dependence of Human FcyRIIa-initiated Phagocytosis**

### A Tyrosine activation motif sequences

| Consensus sequence | Human FcyRIIa | Human γ-chain |
|--------------------|---------------|---------------|
| Rxxxxxxx | MIAIRKQKLESTNDYETADOGYMTHPRAPTDDEKNIYLTLPNDHVNSNN | RLKIGVRKAILTSYERGDOYLTRQGET----YETTEERKPP |
| Rxxxxxxx | Y252L | Y268L |
| Rxxxxxxx | Y252S | Y268S |
| Rxxxxxxx | Y252F | L255E | Y268F | L271A |
| Rxxxxxxx | Y252F | L255E | Y268F | L271A |
| Rxxxxxxx | R235E | Y245S | R258L | D263H | D263S | D263S |
| Rxxxxxxx | EE239-240QQ |

**B FcyRIIa Mutations**

Microscopy.

Determination of Fcy Receptor Alleles---Determination of FcyRIIb alleles, NA1 and NA2, was performed by quantitative flow cytometry with mAbs CLB-FcR-gran 1, CLB-gran 11, and GRM1 (33, 38). The assignment of NA type was confirmed by leukoagglutination as described previously (38) and by immunoprecipitation of selected donors (33). Phenotyping of donors for the LR-HR alleles of FcyRIIa was performed by quantitative flow cytometry using mAbs 41H16 and IV.3 as described previously (39, 40).

Data Analysis---Phagocytosis data are displayed as the mean ± S.D. Ca\(^{2+}\) data are representative experiments. Differences in phagocytosis between phagocytic probes were compared with a Student's t-test and differences between probes over a range of BAPTA concentrations (see Fig. 4B) was determined using two-way analysis of variance.

**RESULTS**

Several recent observations including information on species differences in Fcy receptor isoforms and function (1, 2, 17, 19) have prompted a reconsideration of the studies supporting [Ca\(^{2+}\)]\(_i\)-dependent and [Ca\(^{2+}\)]\(_i\)-independent Fcy receptor-mediated phagocytosis. For example, studies by Stendahl et al. (20) have shown that there is an accumulation of [Ca\(^{2+}\)]\(_i\) storage organelles during phagocytosis in human neutrophils. These observations suggest the possibility that the Fcy receptors expressed in human neutrophils are functionally distinct from murine Fcy receptors in engaging [Ca\(^{2+}\)]\(_i\)-dependent elements for phagocytosis. Indeed, initial studies of human FcyRIIa truncation mutants stably transfected into P388D1 cells have shown that all truncations unable to initiate a [Ca\(^{2+}\)]\(_i\) transient are unable to mediate receptor-specific phagocytosis (17). The evidence for an essential role for [Ca\(^{2+}\)]\(_i\) in FcyRIIa phagocytosis is strengthened by the ability of BAPTA, a chelator of [Ca\(^{2+}\)]\(_i\), to block phagocytosis by FcyRIIa wild-type receptor in P388D1 cells (17). Accordingly, we have examined these relationships in a series of FcyRIIa transfectants with point mutations in the region of the cytoplasmic domain containing the YXXL tyrosine activation motif. Mutations in this region (Fig. 1) can lead to altered binding and activation of p72\(^{Syk}\), which in turn phosphorylates phospholipase C\(_{\gamma1}\) leading to the generation of inositol 1,4,5-trisphosphate and [Ca\(^{2+}\)]\(_i\) transients (41). Studies of transfected FcyR mutants indicate that receptor-mediated phagocytosis is also altered (42, 43).

Fifteen mutants were constructed (Fig. 1), and stable transfectants expressing between 1.1 and 2.6 × 10\(^6\) receptors/cell were selected. The [Ca\(^{2+}\)]\(_i\) transient observed after cross-linking each mutant receptor was measured and ranged from no response for several mutants of tyrosine residues within the tyrosine activation motif to a flux of approximately 500 nM (Fig. 2). The measured [Ca\(^{2+}\)]\(_i\) transients were abrogated by pre-treatment of cells with BAPTA, were unaffected by 10 mM EGTA extracellularly, and therefore were due to mobilization of [Ca\(^{2+}\)]\(_i\) from intracellular stores. Among the 15 cell lines expressing different mutant FcyRIIa, there was no significant relationship between quantitative receptor expression measured by flow cytometry and peak [Ca\(^{2+}\)]\(_i\) flux (p > 0.10; not significant).

The same mutants were probed for FcyRIIa-specific phagocytosis in human neutrophils.
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Fig. 3. Peak [Ca$^{2+}$]$_i$ flux, as demonstrated in Fig. 2, was strongly correlated with quantitative, receptor-specific phagocytosis measured with E-IV.3 (correlation coefficient = 0.94; p < 0.0001). Of note, the EE239-240Q mutant, which is located in a region outside of the YXXL dyad and has a high [Ca$^{2+}$]$_i$ flux occurs within the 7 residues defined as essential for $\Delta$[Ca$^{2+}$]$_i$, by Kolanus et al. (43). Inset, pretreatment of phagocytic cells with 50 μM BAPTA-AM-abrogated phagocytosis. A representative experiment is shown (n = 3 for each determination).

cytosis using the receptor-specific mAb IV.3 Fab conjugated to erythrocytes via a streptavidin bridge (E-IV.3). The density of mAb IV.3 conjugation to erythrocytes was monitored by flow cytometry. Nonbiotinylated erythrocytes and biotinylated but unconjugated erythrocytes neither bound nor were internalized by transfected or nontransfected P388D1 cells. Furthermore, erythrocytes coupled to an IgM anti-H2-Dd via the streptavidin bridge bound to P388D1 as expected but were not internalized (mean attachment index = 133; phagocytic index = 0; n = 3). Quantitative phagocytosis of E-IV.3 ranged from no internalization for the same tyrosine mutants that failed to elicit a [Ca$^{2+}$]$_i$ transient to a maximum phagocytic index of 126.5 ± 16 E-IV.3 ingested per 100 cells (Fig. 2B). Among the 15 different mutants, there was no significant relationship between quantitative receptor expression and E-IV.3 ingestion (p > 0.10; not significant). As with wild-type FcyRIIa (17), the protein tyrosine kinase inhibitor genistein inhibited by >95% phagocytosis of E-IV.3 by the phagocytic mutant forms of FcyRIIa and of EA by the native murine FcyR in parental P388D1.

There was, however, a striking relationship between peak [Ca$^{2+}$]$_i$ and FcyRIIa-specific phagocytosis (Fig. 3) with a correlation coefficient of 0.94 (p < 0.0001). Importantly, even the most phagocytic of the mutant receptors was sensitive to chelation of [Ca$^{2+}$]$_i$ by 50 μM BAPTA (Fig. 3, inset).

The strong correlation between [Ca$^{2+}$]$_i$ and phagocytosis for human FcyRIIa even in the environment of a murine macrophage cell line and the dependence of FcyRIIa-mediated phagocytosis on [Ca$^{2+}$]$_i$ suggested that this property might reflect the characteristics of human FcyRIIa per se. Since previous studies in human and murine cells had not probed Fcy receptor function in a receptor-specific fashion (11-15), we sought to explore the properties of FcyRIIa expressed endogenously on neutrophils. Our previous studies with neutrophils indicated that FcyRIIa alone can mediate phagocytosis (10). Therefore, using receptor-specific engagement of FcyRIIa with mAb IV.3 Fab and cross-linking with GAM F(ab)$_2$, we defined the ability of BAPTA pretreatment of neutrophils to blunt the [Ca$^{2+}$]$_i$ response (Fig. 4A). Correspondingly, we assessed quantitative receptor-specific phagocytosis (Fig. 4B). A BAPTA dose-dependent inhibition of the FcyRIIa-mediated rise in [Ca$^{2+}$]$_i$ was observed with complete inhibition achieved by 50 μM BAPTA.

BAPTA also reduced phagocytosis in a dose-dependent fashion with ~50% reduction at a loading concentration of 50 μM (p < 0.002). Neither phagocytosis of heat-treated zymosan nor of serum-treated zymosan was altered by 50 μM BAPTA pretreatment (both 99–100% of untreated control cells; p > 0.5, not significant) (Fig. 4B). Higher loading concentrations of BAPTA did not lead to further decrement of E-IV.3 internalization (PI = 50.6% of control at 100 μM). 50 μM BAPTA was also sufficient to abrogate detectable [Ca$^{2+}$]$_i$ transients initiated by 10$^{-7}$ FMLP (Δ[Ca$^{2+}$]$_i$ indistinguishable from base line, n = 7).

As an alternative technique to deplete intracellular free Ca$^{2+}$ levels, we allowed neutrophils to incubate in Ca$^{2+}$/Mg$^{2+}$-free media to exhaust intracellular Ca$^{2+}$ stores (Fig. 5) as described by Rosales and Brown (35). FcyRIIa-specific phagocytosis by neutrophils treated in this manner was markedly blunted relative to control cells in the presence of physiologic levels of Ca$^{2+}$/Mg$^{2+}$ (PI = 48.0 ± 13.1% of control, n = 3). The
extent of inhibition was comparable with that achieved by BAPTA pretreatment.

To compare our results in neutrophils with those of Lew and co-workers (14), we also probed neutrophils with EA for the effects of \([\text{Ca}^{2+}]\), chelation on phagocytosis engaging both FcRIIla and FcRIIIb. Although the GPI-anchored FcRIIIb does not mediate phagocytosis itself, it does elicit a \([\text{Ca}^{2+}]\), transient and functions synergistically with FcRIIla for an enhanced phagocytic response (10). As reported by Lew (14), EA phagocytosis was profoundly reduced by \([\text{Ca}^{2+}]\), chelation (Fig. 4B) and to a significantly greater extent than E-IV.3 (EA = 23 ± 15% (n = 10) of control compared 47 ± 20% (n = 12) of control for E-IV.3 at 50 μM BAPTA, p < 0.005; over all doses of BAPTA, EA versus E-IV3, two-way analysis of variance: F = 13.2, p < 0.0002). These observations suggested that the FcRIIIib-initiated \([\text{Ca}^{2+}]\), transients might play an important quantitative role in FcRIIla internalization.

To test this hypothesis as the basis for the known difference in EA phagocytosis by individuals homozygous for the two different alleles of FcRIIla (38, 40), we examined the relative ability of FcRIIIib in NA1 and NA2 homozygotes to elicit \([\text{Ca}^{2+}]\), fluxes in neutrophils. Engagement of FcRIIIib by anti-receptor mAb 3G8 and cross-linking with either GAM or with streptavidin leads to a \([\text{Ca}^{2+}]\), flux derived from intracellular stores (34). When the anti-FcRIIIib mAb 3G8 IgG, a murine IgG1, was used to initiate the \([\text{Ca}^{2+}]\), transient, consistent differences between donors were noted that were attributable to the His-131/Arg-131 polymorphism of FcRIIla and presumably the ability of FcRIIla to engage the Fc region of 3G8 IgG and form heterotypic FcRIIla-FcRIIIib receptor clusters (Fig. 6A). However, no consistent difference in the magnitude of the \([\text{Ca}^{2+}]\), flux could be attributed to the phenotype of FcRIIIib engaged by 3G8 F(ab)2, with subsequent cross-linking in five matched pairs of NA homozygous donors (Fig. 6B). Taken together, these observations suggest that a \([\text{Ca}^{2+}]\),-sensitive signaling element is engaged by FcRIIla during EA phagocytosis but that the magnitude of the FcRIIla-induced \([\text{Ca}^{2+}]\), flux per se does not explain the difference in quantitative phagocytosis between NA1 and NA2 homozygous donors.

Demonstration of the \([\text{Ca}^{2+}]\), dependence of E-IV.3 phagocytosis in neutrophils and in murine P388D1 cells resolves the apparent controversy between DiVirgilio and others about the role of \([\text{Ca}^{2+}]\), in phagocytosis (11–15), but it does not address the issue of whether a human homologue of a "[Ca2+]-independent" murine receptor would also be \([\text{Ca}^{2+}]\), independent for phagocytosis. Accordingly, we examined the effects of BAPTA on FcRIIla-specific (E-IV.3) and FcRIIla-specific (E-22) phagocytosis by human peripheral blood monocytes. Both receptors mediate \([\text{Ca}^{2+}]\), transients after receptor cross-linking that are blocked by 50 μM BAPTA (Fig. 7A). As anticipated, E-IV.3 showed more than a 50% decrement in phagocytosis after pretreatment with 50 μM BAPTA (Fig. 7B; P = 40.5 ± 13.5% of control, p < 0.002). In contrast E-22, the specific probe for FcRIIla, showed only a minimal change that was significantly less than E-IV.3 (p < 0.002) and not significantly different from control (Fig. 7B, p > 0.5). These data for human FcRIIla are similar to those for phagocytic murine Fcγ receptors on elicited peritoneal macrophages (12, 44).

**DISCUSSION**

Recent observations have prompted a re-evaluation of the role of \([\text{Ca}^{2+}]\), in Fcγ receptor-mediated phagocytosis. In previous work, both murine macrophage cell lines and human neutrophils were used, which may have confused the issues since the cells express structurally distinct Fcγ receptors and may have intrinsically different signaling capacities and cell...
The mechanism underlying the variation in \([Ca^{2+}]\) transients and phagocytosis for different FcRIIA mutants may relate to variable efficiency in engaging the SH2 domain of the protein tyrosine kinase p72\(^{\text{\beta}}\). FcRIIa does bind p72\(^{\text{\beta}}\) (25, 26, 28, 29), and the binding of the ZAP70 homologue to a similar YXXL tyrosine activation motif shows a high degree of sensitivity to mutations in the flanking sequences (41, 46–48). In the FcRRI model system, which incorporates the YXXL tyrosine activation motif in \(\gamma\)-chain, p72\(^{\text{\beta}}\)-binding and phosphorylation leads to tyrosine phosphorylation of phospholipase C-\(\gamma\) (either directly by p72\(^{\text{\beta}}\) or through an intermediary kinase(s)), inositol lipid breakdown with generation of inositol 1,4,5-trisphosphate, and a \([Ca^{2+}]\) (49–53). Disruption of this sequence by inhibition of p72\(^{\text{\beta}}\) with picetannol or specific inhibitory peptide abrogates the \([Ca^{2+}]\) flux (53, 54). The essential role for \([Ca^{2+}]\), in FcRRIIa-mediated phagocytosis is strongly supported by the correlation of \([Ca^{2+}]\) with phagocytosis between the 15 different FcRRIIa mutants, by the ability of BAPTA to abrogate phagocytosis by both wild-type and mutant receptors (Fig. 3) and by the ability of BAPTA to abrogate phagocytosis by FcRRIIa in neutrophils without affecting ingestion of zymosan and in monocytes without affecting internalization by FcRRIa. However, the critical \([Ca^{2+}]\)-dependent element(s) for FcRRIa signaling remain unidentified at present. Preliminary experiments with cyclosporin A, an inhibitor of calcineurin that is a \([Ca^{2+}]\)-dependent phosphatase, show little or no effect on human FcRRIa phagocytosis in transfected P388D1 cells, although calcineurin activity is essential for neutrophil motility in some circumstances (55). Recent data suggest that l-plastin, a \([Ca^{2+}]\)-regulated actin-bundling protein, may be a candidate for a \([Ca^{2+}]\)-dependent element essential for Fc receptor-mediated phagocytosis in neutrophils (56).

The greater susceptibility of EA phagocytosis to \([Ca^{2+}]\) buffering compared with FcRRIIa-specific phagocytosis with E-IV.3 suggests that the activation of phagocytosis by FcRRIIib in human neutrophils has \([Ca^{2+}]\)-dependent elements. Since FcRRIIib elicits a \([Ca^{2+}]\) flux and functions synergistically with FcRRIIa for phagocytosis, an allele-specific variation in FcRRIIib-initiated \([Ca^{2+}]\), would provide a straightforward mechanism for the quantitative difference in phagocytosis shown by donors homozygous for different FcRRIIib alleles. Although we could define consistent differences in \([Ca^{2+}]\) transients between individuals, with those elicited by intact anti-receptor IgG relating to the His-131/Arg-131 polymorphism of FcRRIa (57–59), donors homozygous for the NA1/NA2 FcRRIIib alleles were not different in their ability to generate FcRRIIib-specific fluxes in \([Ca^{2+}]\). Thus, some other mechanism, perhaps relating to the differences in glycosylation of the NA1 and NA2 alleles and potential carbohydrate-mediated interactions with other cell surface molecules such as CD11b/CD18 (60, 61) must be involved. While we cannot explain the NA1/NA2 difference in phagocytosis on the basis of quantitative differences in \([Ca^{2+}]\) interacting with the partially \([Ca^{2+}]\)-dependent phagocytosis of FcRRIa, the difference in \([Ca^{2+}]\) dependence between FcRRIIa and FcRRIa underscores the fact that different Fc receptors can engage distinct signaling elements. Whether these distinct elements reflect primary sequence differences in the tyrosine activation motifs used by each of the receptors or some modifying contribution of the cytoplasmic domain of the ligand binding FcRRIa α chain (compare Ref. 52) remains to be determined. These distinct elements may converge on some cell programs, but they also provide the foundation for differences in elicited cell programs and for selective therapeutic intervention.
