FcγR(CD16) INTERACTION WITH LIGAND INDUCES Ca^{2+} MOBILIZATION AND PHOSPHOINOSITIDE TURNOVER IN HUMAN NATURAL KILLER CELLS

Role of Ca^{2+} in FcγR(CD16)-induced Transcription and Expression of Lymphokine Genes

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We reported evidence that FcγR(CD16) on human NK cells are signal-transducing molecules that, upon ligand binding, induce transcription of genes encoding cytokines (IFN-γ and TNF) relevant to NK cell biology and function (1). IL-2 also induces transcription (1) and accumulation (1, 2) of the mRNA of the two genes in NK cells, although at levels lower than those induced by FcγR(CD16) ligands. Our previous data indicated that the two stimuli act synergistically to induce mRNA accumulation and production of the cytokines, activating NK cells through mechanisms that are, at least in part, distinct.

Upon ligand binding, transmission of activating signals to the intracellular compartment occurs via mechanisms that depend on the characteristics of the ligand-receptor molecule involved. Several receptors activate protein kinase C (PKC)[1] (3), inducing phosphorylation of proteins involved in cellular functions, whereas others are autophosphorylating molecules with ligand-induced kinase activity (4). Receptor-induced hydrolysis of membrane polyphosphoinositides (PPI) has been shown to underlie activation and proliferation of lymphocytes induced by soluble stimuli (5). PPI hydrolysis results in two second messengers: inositol 1,4,5 triphosphate (IP_3), which induces a rise in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) by mobilizing this cation from intracellular stores, and diacylglycerol, which activates PKC (6, 7). It has also been suggested that receptor perturbation by the ligand may open Ca^{2+} channels within the plasma membrane (8), and there is evidence that the PPI pathway may stimulate Ca^{2+} influx through such channels (9).

Stimulation of murine macrophages with FcγR ligands (immune complexes or anti-FcγRII mAb 2.4G2) induces an increase in $[Ca^{2+}]_i$ (10), although this increase is not required for phagocytosis by these cells (11, 12). Studies on signal transduction

This work was supported in part by National Institutes of Health grants CA-10815, CA-20833, CA-32898, CA-37155, CA-40456, and CA-45284. M. A. Cassatella is recipient of a fellowship from the Associazione Italiana Ricerca sul Cancro (AIRC). B. Perussia is a Scholar of the Leukemia Society of America.

[1] *Abbreviations used in this paper:* GaMIg, goat $F(ab')_2$ anti-mouse Ig; GPI, glycosyl phosphoinositide; IP, inositol phosphate; PGK, phosphoglyceratekinase; PKC, protein kinase C; PL, phospholipase; PPI, polyphosphoinositides.

through FcγR [FcγR(CDw32)] using human granulocytes showed that $[Ca^{2+}]_i$ increases upon interaction of the cells with certain mAbs (13). It remains unclear whether one or both of the distinct FcγR present on the cells used in those studies mediated the increase in $[Ca^{2+}]_i$ and if this is related to coupling of the FcγR to IP_3 formation.

A human cDNA for FcγR(CD16) has been recently isolated (14) that encodes a protein whose mature form lacks an intracytoplasmic domain and is anchored to the cell membrane through a glycosyl-phosphoinositide (GPI) tail. Other molecules of the family of the GPI-linked cell surface glycoproteins, such as Thy-1, have been shown to participate in cell activation. Crosslinking of Thy-1 at the T cell membrane has been reported to trigger a rapid rise in $[Ca^{2+}]_i$ (15). Although other mechanisms of signal transduction through these molecules have not been investigated, such data indicate that GPI-anchored molecules, like transmembrane molecules, can play a role in intracellular signaling.

In this report, we analyzed the molecular mechanisms through which FcγR(CD16) induces NK cell activation upon ligand binding. Our data indicate that interaction of FcγR(CD16) with ligands (immune complexes or anti-CD16 antibodies) induces a rapid rise in $[Ca^{2+}]_i$ and production of both IP_3 and IP_4. Both effects are evident at significantly higher levels when anti-CD16 antibodies are crosslinked at the cell membrane. A role for Ca^{2+} in FcγR(CD16)-dependent, but not in IL-2-dependent, activation of the IFN-γ and TNF genes is demonstrated, confirming that distinct activation pathways are induced by the two stimuli in NK cells.

Materials and Methods

Cell Lines. The human B lymphoblastoid cell line RPMI 8866 and the murine mAb-producing hybrid cell clones were maintained in culture in RPMI 1640 (Flow Laboratories, Inc., Rockville, MD) supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY). All cell lines were free of mycoplasma contamination on repeated testing.

Monoclonal and Polyclonal Antibodies. The specificity, isotype, and source of mAbs B73.1 and 3G8 (anti-CD16), B36.1 (anti-CD5), B52.1 (anti-CD14), N901, and OKT3 (anti-CD3) have been reported (16). B33.1 (IgG2a, anti-HLA-DR nonpolymorphic determinant) (17, 18) was produced and characterized in our laboratory. KuFc79 (IgG1, anti-CDw32) (19) was kindly provided by Dr. M. Vaughn (Medical College of Virginia, Richmond, VA). W6.32 (IgG2a, anti-HLA class I nonpolymorphic determinant) and BBMI (IgG1, anti-β_2-microglobulin) were produced from cells obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). Anti-IFN-γ antibodies B133.1 and B133.5 and anti-TNF B154.7 and B154.9 used for the IFN-γ and TNF RIAs, respectively, were produced and characterized in our laboratory (20, 21). Purified Ig or their $F(ab')_2$ fragments were labeled with biotin or linked to CNBr-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to routine procedures. $F(ab')_2$ fragments were prepared by pepsin digestion and the purity of each preparation was confirmed by SDS-PAGE. The polyclonal FITC-labeled goat $F(ab')_2$ anti-mouse Ig (GaMIg) and the rabbit IgG anti-bovine erythrocytes (E) were purchased from Cappel Laboratories (Cochranville, PA). The $F(ab')_2$ GaMIg used to prepare the E for indirect rosetting or to crosslink antibodies at the lymphocyte surface was produced in our laboratory, absorbed on human IgG, and affinity purified on mouse IgG-CNBr Sepharose 4B columns (Pharmacia Fine Chemicals). The rabbit IgG anti-human IgG was purchased from Cappel Laboratories, absorbed on murine IgG, and affinity purified on human IgG-Sepharose columns. Human IgG1 was purified by ion-exchange chromatography from the serum of a myeloma patient as previously described (22).

NK Cell Populations. All experiments were performed on NK cells obtained from short-term cultures of PBMC with irradiated RPMI 8866 B lymphoblastoid cells as previously described (23). The NK cells were purified from the cultures, containing on average 60–80% $CD16^+/NKH\text{-}1^+/CD3^-$ NK and 20–40% $CD3^+/CD16^-/NKH\text{-}1^-$ T cells, by negative selec-

tion using indirect antiglobulin rosetting and density gradient centrifugation after sensitization of the lymphocytes with a mixture of anti-CD3, anti-CD5, and anti-CD14 mAbs (1, 23). These NK cell populations have morphologic, phenotypic, and functional properties identical to those of NK cells freshly obtained from blood (1, 23, and data not shown). However, unlike fresh NK cells, the majority of these cells express HLA-DR antigens. FcγRI or FcγR(CDw32) were never detected, nor could they be induced, as determined by binding of monomeric human IgG1 or murine IgG2a and reactivity with anti-FcγR(CDw32) antibody KuFc79 (19) or IV.3 (24) (data not shown). The purity of each NK cell preparation used was tested by indirect immunofluorescence (flow cytometry) using anti-NK (anti-CD16 and anti-NKH1) and anti-T (anti-CD3 and anti-CD5) reagents, as previously described (23). As already reported (1, 23) >98% of the NK cell populations obtained bear CD16, NKH1, CD2, and HLA-DR antigens, whereas CD3, CD5, and CD14 are not detectable on these cells.

Lymphocyte Stimulation. NK cells purified from the bulk cultures were incubated (37°C, 5×10^6 cells/ml RPMI/10% FCS) for the indicated periods of time with different inducers. FcγR(CD16) ligands were: (*a*) anti-CD16 antibodies (3G8 or B73.1, intact IgG or their $F(ab')_2$ fragments) used in either soluble form or linked to CNBr-Sepharose 4B (5 mg antibody/ml Sepharose, 5 μl beads/10^6 cells). When soluble antibodies were used, their crosslinking at the cell membrane was induced by adding a 10:1 (wt/wt) excess $F(ab')_2$ GaMIg, or a 5:1 (wt/wt) excess avidin (Sigma Chemical Co., St. Louis, MO) to biotin-labeled antibodies; (*b*) particulate immune complexes, i.e., IgG-sensitized bovine E (EA7S) prepared as previously described (25) or soluble immune complexes, i.e., human IgG1–rabbit IgG anti-human IgG at a 1:3 (wt/wt) ratio. Irrelevant, Sepharose-linked or soluble antibodies, and E were used as controls. When indicated, purified human rIL-2 was present (100 U/ml) during stimulation (rIL-2, 5×10^6 U/mg in a standard CTLL assay; Takeda Chemical Industry, Inc. Osaka, kindly provided by Dr. T. Taguchi, Osaka University, Osaka, Japan). The calcium ionophore Ionomycin (Calbiochem Behring Corp., San Diego, CA) was used at the indicated concentrations. In some cases, 1 mM EGTA and 1 mM $MgCl_2$ were added at different times after stimulation. EGTA final molar concentration in the cultures was 2.5-fold greater than that of Ca^{2+}. In these conditions, extracellular $[Ca^{2+}]$, as measured using a calcium-selective microelectrode, is reduced to ~1 μM (26). After culture, cells were collected and Sepharose beads were detached by vigorous vortexing and eliminated from the cell suspension after a 1-min centrifugation at 500 rpm. EA7S were removed by lysis with hypotonic medium. Ligand-free cells were washed and used in the different assays. Cell viability was >95% and recovery was ~80% of the original input.

$[Ca^{2+}]_i$ Measurement. $[Ca^{2+}]_i$ of NK cells was measured with the intracellular fluorescent indicator fura-2 using the procedure described by Cassatella et al. (27). Briefly, NK cells were preincubated (5×10^6/ml, 5 min at 37°C) in HBSS (Gibco Laboratories) containing 1 mM $CaCl_2$, 5.6 mM glucose, 20 mM Hepes, pH 7.4 (hereafter referred to as standard saline), and 0.025% BSA. fura-2/AM (Calbiochem Behring Corp.) was added at the optimal predetermined final concentration of 2 μM. After a 15-min incubation at 37°C with continuous shaking, the cells were diluted fivefold with standard saline and the incubation continued for 30 min. After fura-2 loading, cells were washed and resuspended in standard saline. Cells incubated under identical conditions but without fura-2/AM served as control for background autofluorescence. Fluorescence measurements were performed in a spectrofluorometer (model LS-5B; Perkin Elmer Corp., Norwalk, CT) equipped with a thermostatic cuvette holder maintained at 35°C with continuous stirring. Each sample contained 3×10^6 cells in 1.7 ml buffer. The various FcγR(CD16) ligands and controls were added after a 3- to 5-min measurement of basal fluorescence, and changes in fluorescence were recorded as a function of time, usually during 15 min. The excitation-emission wavelength pair used to monitor fura-2/AM fluorescence was 340/505 nm. $[Ca^{2+}]_i$ was calculated according to the formula: $[Ca^{2+}]_i = K_d (F\text{-}F_{min})/(F_{max}\text{-}F)$, where K_d is dissociation constant for Ca^{2+} binding to fura-2/AM (= 224 nM); F is fluorescence of the intracellular indicator; F_{min} is fluorescence detected after lysis of the cells with Triton X-100 (0.05%) in the presence of 5 mM EGTA and 40 mM Tris (final pH 8.5); and F_{max} is fluorescence of the lysed cells after addition of 5 mM $CaCl_2$ (11). Calculated $[Ca^{2+}]_i$ values were corrected for minimal changes in autofluorescence, if induced upon addition of the various reagents.

Phosphoinositide Turnover. This was measured by determining the intracellular accumula-

tion of IP, following the procedure described by Cassatella et al. (27). Briefly, purified NK cells (5–10 × 10^6/ml inositol-free RPMI 1640 supplemented with 5% FCS) were incubated for 18 h at 37°C in the presence of myo-[^{3}H]inositol, 10 μCi/ml (20 Ci/mmol sp act; Amersham Corp., Arlington Heights, IL). After incubation and washing in PBS, cells were resuspended in standard saline. Both PBS and standard saline solutions contained 10 mM LiCl. The various FcγR(CD16) ligands or controls were added at the concentrations and for the time periods indicated to duplicate aliquots of 20 × 10^6 cells/ml. The reaction was stopped with ice-cold TCA (7.5% final concentration), and samples were extracted five times using diethylether and brought to pH 8 with sodium tetraborate. Each sample was diluted to 2 ml with distilled H_2O. The different IP (IP_1–IP_4) were measured as cell-incorporated counts (cpm) after anion-exchange chromatography of each sample on a 1-ml Agl-X8 resin column (200–400-mesh formate form; Bio-Rad Laboratories, Richmond, CA). Columns were sequentially washed with H_2O to elute free [^{3}H]inositol, 5 mM tetraborate, and 60 mM Na formate to elute glycerophosphoryl [^{3}H]inositol, and a four-step discontinuous gradient of 0.1 M formic acid containing 0.2, 0.5, 0.8, and 1.0 M ammonium formate to elute IP_1, IP_2, IP_3, and IP_4, respectively (28). This method unequivocally separates IP_1, IP_2, IP_3, and IP_4 from each other, but does not enable distinction among isomers of individual IP. Radioactivity of each fraction was measured by liquid scintillation counting using a β counter (Packard Instrument Co. Inc., Downers Grove, IL). Control standard [^{3}H]IP_3 and [^{3}H]IP_4 (DuPont Co., Wilmington, DE) were run on parallel columns under identical conditions. In some cases, samples were analyzed by HPLC (Beckman Instruments, Palo Alto, CA) using a Whatman Partisil SAX10 column according to the method described by Gilligan et al. (29).

IFN-γ and TNF RIA. These were performed as previously described in detail (20, 21), using cell-free supernatants obtained from purified NK cells induced as above. Purified human natural IFN-γ (Interferon Sciences, New Brunswick, NJ) and purified human rTNF (5 × 10^7 U/mg on L929 cells; kindly provided by Dr. H. M. Shepard, Genentech Inc., South San Franscisco, CA) were used in each assay to construct the standard curve. Sensitivity of the RIAs for IFN-γ and for TNF is 0.2 U/ml and 0.1–0.2 U/ml, respectively.

Northern Blot Hybridization. Northern blots were performed as previously described in detail (1, 16, 21). The cDNA probes IL-2R2, TNF, and pSWIF detecting IL-2R, TNF and IFN-γ, respectively, were kindly provided by Drs. W. Green (National Institutes of Health, Bethesda, MD), H. M. Shepard (Genentech Co.), and S. Clark (Genetics Institute, Boston, MA). Human β-actin probe, pBR322 vector, pB_{AE} probe for 28S ribosomal RNA, and pSP64 vector were used as controls and were kindly provided by Drs. R. Weinmann and P. Curtis (Wistar Institute). Phosphoglycerate kinase (PGK) probe (30) was the pK T218 vector containing the 1.8-kb Pst I fragment of PGK, obtained from ATCC.

Nuclear Transcription Analysis (Run-On Assays). Nuclei from 20 min-stimulated and unstimulated purified NK cells, in the presence or absence of 1 mM EGTA and 1 mM $MgCl_2$, were isolated as previously described in detail (1). In vitro transcription (25 min at 26°C) was according to Groudine et al. (31) with modifications (1), and nuclear RNA was isolated according to Gariglio et al. (32). Equal amounts of ^{32}P RNA cpm per sample were hybridized (3 d) at 42°C with prehybridized nitrocellulose filters on which 10 μg of linearized plasmid cDNA had been immobilized using a slot-blot apparatus (Schleicher & Schuell, Inc., Keene, NH). After washing, filters were exposed to Kodak XAR film with intensifying screens. Extent of hybridization was quantitated by scanning densitometry with a laser densitometer (Ultrascan XL; LKB Instruments, Inc., Gaithersburg, MD) on film exposed for the appropriate length of time to produce bands with absorption within the linear response range. To calculate the rate of transcription, background hybridization to pBR322/pSP64 was subtracted from the densitometric readings and values normalized relative to those obtained for the 28S rRNA probe pB_{AE}.

Results

$[Ca^{2+}]_i$ Rise in NK Cells upon Interaction of the Cells with FcγR(CD16) Ligands. Resting $[Ca^{2+}]_i$ in cultured NK cells were similar to those reported for other mammalian cells (85–140 nM) (Table I). Addition of saturating concentrations (1 μg/ml) of anti-

TABLE I

Binding of Anti-CD16 Antibodies to NK Cells Induces $[Ca^{2+}]_i$ Mobilization

Antibody code*	Number of experiments	$[Ca^{2+}]_i$ in the presence of: No antibody (basal levels)	Antibody‡ alone	Antibody + GaMIg§
3G8, IgG1	5	140.8 ± 9.8‖	203.0 ± 11.4	327.4 ± 21.1
B73.1, IgG1	5	135.8 ± 12.3	170.2 ± 9.1	331.4 ± 18.8
B73.1, F(ab')$_2$	4	132.2 ± 7.9	184.2 ± 12.8	317.4 ± 21.3
BBM1, IgG1	3	102.0 ± 6.2	109.3 ± 7.9	111.5 ± 4.9
B33.1, IgG2a	2	126, 138	134, 145	ND, 150
KuFc79, IgG1	2	130, 84	141, 90	ND, 96

$[Ca^{2+}]_i$ was measured in fura-2/AM-loaded CD3/CD5(−) NK cells (3×10^6 cells/sample) purified by negative selection from 10-d cultures of PBMC with irradiated B-lymphoblastoid cell line as described in Materials and Methods.

* All antibody preparations were used at a dose of 1 μg/ml.

‡ $[Ca^{2+}]_i$ was measured at peak level, as described in Materials and Methods, 2 min after addition of the antibody.

§ Goat F(ab')$_2$ anti-mouse Ig (GaMIg, 50 μg/ml) was added to the cells 10 min after the first antibody, and $[Ca^{2+}]_i$ was measured at the peak level.

‖ Numbers are $[Ca^{2+}]_i$ (nM ± SE) of the number of experiments indicated.

CD16 antibodies 3G8 or B73.1 caused a significant increase in $[Ca^{2+}]_i$, with variability among donors. Both intact IgG and their F(ab')$_2$ fragments had similar effects when used at identical concentrations. A 2.4-fold average increase of basal $[Ca^{2+}]_i$ was detected when F(ab')$_2$ GaMIg was added to induce crosslinking of the primary antibody on the NK cell surface. Whereas anti-CD16 antibody B73.1 induced lower $[Ca^{2+}]_i$ levels than did 3G8, almost indentical $[Ca^{2+}]_i$ values were detected in all donors tested after crosslinking of either antibody. Irrelevant isotype-matched control antibodies to surface molecules present (β_2 microglobulin and HLA-DR antigens) or absent (FcγR(CDw32)] on the NK cell preparations did not induce a rise in $[Ca^{2+}]_i$ throughout the observation time (15 min), even after crosslinking.

Fig. 1 (experiment representative of 10 performed) shows the profile of Ca^{2+} rise induced with anti-CD16 antibodies as compared with that observed with immune complexes. The rise in $[Ca^{2+}]_i$ observed upon addition of higher concentrations of anti-CD16 antibodies occurred within 1 min and reached plateau values within 2 min, after which it remained sustained for at least 10 min. The addition of F(ab')$_2$ GaMIg (Fig. 1, *A* and *B*) or of avidin to biotin-labeled antibodies (*C*) caused a $[Ca^{2+}]_i$ rise within 10 s. Under these conditions, $[Ca^{2+}]_i$ declined to basal levels after the spike at a faster rate than that observed with anti-CD16 antibodies alone. However, plateau values after GaMIg addition were higher than those maintained after addition of the primary antibody alone. Immune complexes (human IgG anti-human IgG) caused a $[Ca^{2+}]_i$ rise in NK cells with kinetics and values similar to those observed after addition of anti-CD16 antibodies. Neither partner of the immune complex induced a $[Ca^{2+}]_i$ rise when used alone (Fig. 1, *D* and *E*).

Dose-response experiments were performed using anti-CD16 antibody 3G8 F(ab')$_2$ fragments (Fig. 2, representative of four experiments). A similar $[Ca^{2+}]_i$ rise (on average 250 nM) was observed with all saturating concentrations used (from 5 to 1.25 μg/ml), although differences in the kinetics of rise were reproducibly observed, being

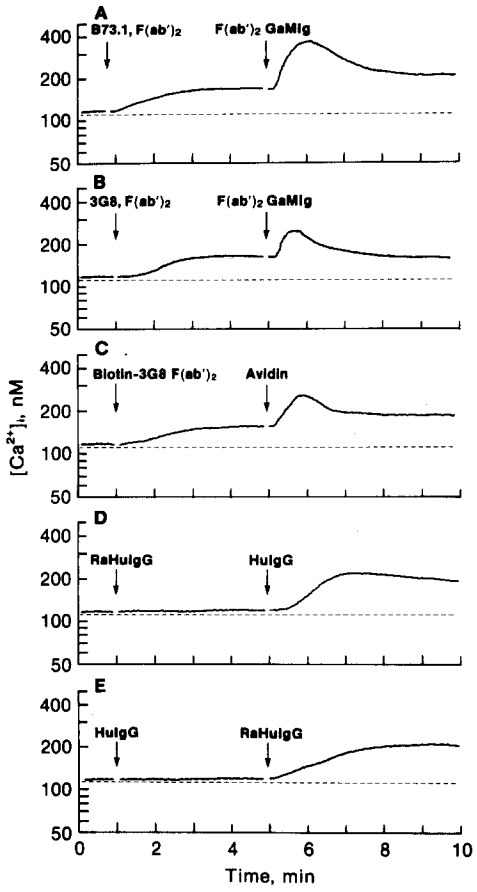

FIGURE 1. FcγR(CD16) ligands induce $[Ca^{2+}]_i$ rise in NK cells. $[Ca^{2+}]_i$ was measured in fura-2/AM-loaded cultured NK cells (3×10^6 cells/sample) as described in Table I. The doses of the inducers (added at the times indicated by the arrows) were: B73.1, 3G8, biotin-labeled 3G8 (all used as $F(ab')_2$ fragments) 2 μg/ml; $F(ab')_2$ GaMIg, 50 μg/ml; Avidin, 10 μg/ml; IgG rabbit anti-human Ig (RaHuIgG), 150 μg/ml; human IgG (HuIgG), 50 μg/ml.

faster with higher antibody concentrations. A lower but significant $[Ca^{2+}]_i$ rise was observed after 5 min with subsaturating concentrations (0.4 μg/ml), and minimal rise was observed at 8 min with antibody concentrations as low as 0.1 μg/ml. Identical results were obtained when the intact IgG antibodies were used and addition of rIL-2, per se ineffective in inducing $[Ca^{2+}]_i$ rise, resulted in no significant variation in either the basal or the FcγR(CD16) ligand-induced $[Ca^{2+}]_i$ (data not shown).

Effect of Chelation of Extracellular Ca^{2+} on $[Ca^{2+}]_i$ Rise Induced in NK Cells by FcγR (CD16) Ligands. To determine whether the interaction of FcγR(CD16) ligands with NK cells induces the rise in $[Ca^{2+}]_i$ by releasing Ca^{2+} from intracellular stores, by increasing Ca^{2+} influx from the external medium, or by both mechanisms, we compared the ligand-induced $[Ca^{2+}]_i$ increase in the presence or absence of EGTA. When extracellular Ca^{2+} was chelated with EGTA before addition of anti-CD16 antibody 3G8, the rise of $[Ca^{2+}]_i$ induced by the antibody alone or after crosslinking by the $F(ab')_2$ GaMIg was approximately half of that observed in the absence of EGTA (Fig. 3 *B*, experiment representative of four performed with similar results). In the presence of EGTA, no significant change in the kinetics of $[Ca^{2+}]_i$ rise was observed, but the $[Ca^{2+}]_i$ returned to control resting levels within 3 min of induc-

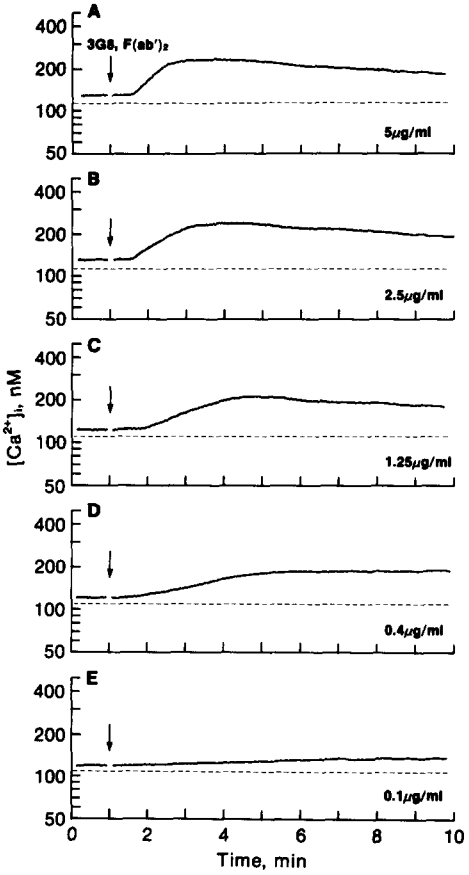

FIGURE 2. Anti-FcγR(CD16) antibodies induce $[Ca^{2+}]_i$ mobilization in NK cells in a dose-dependent fashion. $[Ca^{2+}]_i$ was measured, as described in Table I, using the indicated concentrations of 3G8 $F(ab')_2$ fragments to stimulate NK cells.

tion either with antibody alone or after crosslinking. To test whether the observed effect of EGTA on $[Ca^{2+}]_i$ increase after crosslinking of the anti-CD16 antibodies was due only to chelation of extracellular Ca^{2+} or possibly to partial depletion of intracellular stores upon prolonged (5 min) exposure of the cells to the chelating agent, EGTA was added immediately before addition of the $F(ab')_2$ GaMIg (Fig. 3 *C*). Again, the $[Ca^{2+}]_i$ rise observed in the absence of EGTA was never reduced by >50%, but it returned to the resting levels observed in untreated NK cells within 3 to 4 min.

Interaction of NK Cells with Anti-CD16 Antibodies Stimulates Polyphosphoinositide Turnover in NK Cells. Fig. 4 (representative of five experiments performed) shows the time course of accumulation of IP_1, IP_2, IP_3, and IP_4 in NK cells labeled with myo-[^{3}H]inositol and stimulated with anti-CD16 antibody B73.1 in the absence or presence of $F(ab')_2$ GaMIg. Basal cpm levels for IP_1, IP_2, IP_3, and IP_4 were 2,100, 142, 51, and 34, respectively. When NK cells were stimulated with the anti-CD16 antibody, the increase in IP_1 was low at any of the time points tested either in the absence of or after addition of the $F(ab')_2$ GaMIg. The intracellular concentrations of IP_2 and IP_3 increased to 140 and 190% of control levels, respectively, during the

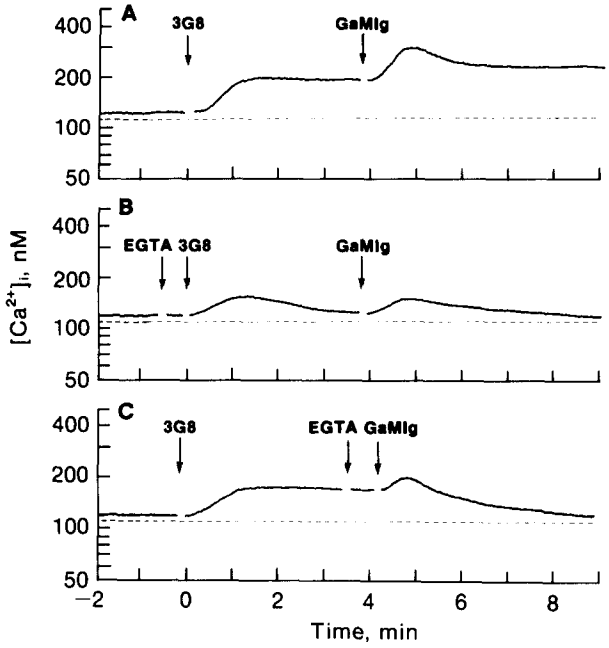

FIGURE 3. Chelation of extracellular Ca^{2+} by EGTA only partially blocks $[Ca^{2+}]_i$ mobilization induced by anti-CD16 antibodies in NK cells. $[Ca^{2+}]_i$ was measured as described in Table I in NK cells stimulated, at the times indicated by the arrows, with 3G8 [$F(ab')_2$, 2 μg/ml)] and $F(ab')_2$ GaMIg, 50 μg/ml, with or without addition of 1 mM EGTA.

first 15 s of stimulation with the anti-CD16 antibody alone. A rise in IP_4 to ~140% of control levels was detected within 30 s of stimulation. Whereas the IP_3 values remained sustained during a 3-min incubation, those of IP_2 and IP_4 decreased to control levels within 1 min. When $F(ab')_2$ GaMIg was added, a sharp further increase of IP_3 to 450% of control levels was reproducibly observed, reaching peak levels

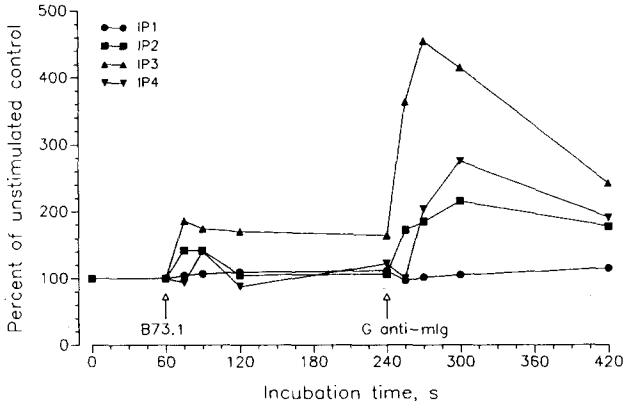

FIGURE 4 IP formation in NK cells stimulated with anti-CD16 antibodies. Purified NK cells were loaded with myo-[^{3}H]inositol as described in Materials and Methods. IP_1 (●), IP_2 (■), IP_3 (▲), and IP_4 (▼) were extracted from the cells (20 × 10^6/duplicate samples) at the indicated times after addition of B73.1 (IgG, 1 μg/ml/20 × 10^6 cells) with or without $F(ab')_2$ GaMIg (50 μg/ml). Separation of IP was by anion-exchange chromatography using a formic acid-ammonium formate discontinuous gradient buffer system as described in Materials and Methods. IP_1, IP_2, IP_3, and IP_4 basal cpm values were 2,100, 142, 51, and 34, respectively. Difference between the two duplicates was always <5%.

within 30 s. The increase in IP_2 and IP_4 levels was detected within 15–30 s and reached peak levels equal to 200 and 300% of the control, respectively, within 1 min. The levels of IP_2, IP_3, and IP_4 all slowly decreased toward control levels within 2 min. Similar results were obtained when the samples were analyzed using HPLC and, under any of the conditions tested, control anti-β_2-microglobulin antibody BBM1 did not induce IP accumulation (data not shown).

Lymphokine Production by NK Cells upon Chelation of Extracellular Ca^{2+}. To test whether NK cells require extracellular Ca^{2+} to produce lymphokines upon FcγR (CD16)-ligand interaction, 1 mM EGTA (in the presence of 1 mM $MgCl_2$) was added to the cells 30 s before addition of Sepharose-linked anti-CD16 antibodies or EA7S, in the absence or presence of rIL-2, under conditions previously shown to induce IFN-γ and TNF production (1). IFN-γ and TNF were measured in the cell-free supernatants after a 12-h culture. Cell viability after culture in the presence of EGTA was ~80%, compared with 95% of control cells. As reported (1), low but significant amounts of IFN-γ were detected in the supernatants from rIL-2-stimulated NK cells, and more so from FcγR(CD16)-stimulated NK cells (Table II). Stimulation with rIL-2 and CD16 ligands in combination synergistically induced IFN-γ production. Similar induction was observed for TNF. In the presence of EGTA, production of INF-γ induced by rIL-2 was, on average, 30 to 50% of that induced in the absence of EGTA; IFN-γ production induced by FcγR(CD16) ligand was inhibited by ~90% in the presence of EGTA. An average 95% inhibition was observed when the two stimuli were used in combination. Similar to the results obtained for IFN-γ, TNF production induced by the FcγR(CD16) ligands alone was inhibited by ~75% and the combined effect of rIL-2 and FcγR(CD16) ligands was almost completely inhibited in the presence of EGTA. Complete inhibition of lymphokine production was detected only when EGTA was either present from the beginning or added to stimulated cells within 20 min from addition of the FcγR(CD16) ligands with or without rIL-2; inhibition was significantly less (40% for IFN-γ and 50%

TABLE II

Chelation of Extracellular Ca^{2+} by EGTA Prevents IFN-γ and TNF Production by NK Cells upon FcγR(CD16)-Ligand Interaction

		IFN-γ		TNF	
Stimulus*	rIL-2	None	EGTA	None	EGTA
None	–	(4) 1.3 ± 0.9‡	1.4 ± 0.9	0.1 ± 0.1	0.1 ± 0.1
None	+	(4) 6.2 ± 2.7	2.2 ± 1.4 (74.6)§	0.1 ± 0.1	0.0 ± 0.1
Seph. B73.1	–	(4) 15.9 ± 6.2	1.5 ± 0.8 (86.7)	2.7 ± 0.9	0.6 ± 0.4 (75.2)
Seph. B73.1	+	(4) 400.4 ± 53.2	20.2 ± 11.7 (94.5)	23.7 ± 2.5	0.4 ± 0.3 (98.4)
None	–	(6) 1.0 ± 0.6	1.3 ± 0.6	0.1 ± 0.0	0.1 ± 0.0
None	+	(6) 4.6 ± 1.9	2.5 ± 0.9 (49.7)	0.1 ± 0.1	0.3 ± 0.2
EA7S	–	(6) 9.4 ± 1.7	0.9 ± 0.3 (91.0)	1.1 ± 0.2	0.2 ± 0.1 (70.6)
EA7S	+	(6) 121.4 ± 11.6	5.3 ± 1.4 (95.5)	10.7 ± 2.9	0.3 ± 0.2 (98.3)

* Purified $CD3^-/CD5^-$ NK cells were incubated (18 h, 37°C) in medium containing the indicated CD16 ligands (Sepharose-B73.1, 5 μl beads/10^6 cells; EA7S, 0.5% final concentration) in the presence or absence of 1 mM EGTA and 1 mM $MgCl_2$; rIL-2 was added at 100 U/ml.

‡ Numbers are U/ml (mean ± SE of the number of experiments indicated in parentheses) as measured by RIA.

§ Mean percent inhibition.

for TNF) when EGTA was added to NK cells 2 h after addition of any of the inducing stimuli (data not shown).

Effect of Chelation of Extracellular Ca^{2+} on IFN-γ and TNF mRNA Accumulation Induced in NK Cells upon FcγR(CD16) Ligand. To define whether the observed inhibition of IFN-γ and TNF production induced by FcγR(CD16) ligands with or without rIL-2 was due to a direct effect on mRNA accumulation, we analyzed expression of IFN-γ and TNF mRNA induced with the different stimuli in the presence of EGTA. mRNA was extracted from NK cells after a 2-h stimulation with the inducers on the basis of our previous data (1) indicating expression of IFN-γ and TNF mRNA at this time. The presence of EGTA during culture affected neither mRNA expression in control untreated cells (Fig. 5, experiment representative of four performed) nor its induction in rIL-2-treated cells. Induction of both IFN-γ and TNF mRNA accumulation by immune complexes (EA7S) was instead completely abrogated in NK cells in the presence of EGTA and the synergistic effect of rIL-2 and FcγR(CD16) ligands was abolished under the same conditions. Constitutive expression of PGK mRNA in all conditions were identical in control and EGTA-treated cells.

Effect of Chelation of Extracellular Ca^{2+} on Transcription of Lymphokine Genes Induced by FcγR(CD16) Ligands in NK Cells. To evaluate whether the inhibition of IFN-γ and TNF mRNA accumulation observed in the presence of EGTA is due to inhibition of transcription of the genes encoding the two lymphokines, nuclear run-on experiments were performed on nuclear nascent RNA obtained from NK cells stimulated for 20 min with rIL-2 or Sepharose-linked anti-FcγR(CD16) antibody 3G8 (Fig. 6, experiment representative of three performed). The presence of EGTA did not modify either endogenous transcription rates of IFN-γ and TNF genes or the increased rate of transcription observed upon stimulation with rIL-2 (Fig. 6, *top and middle*). As reported (1), Sepharose-linked anti-CD16 antibody induced higher transcription rates for both lymphokines than did rIL-2 (23- and 3.5-fold increase in the experiment reported, as compared with 3.5- and 2.5-fold increase induced by rIL-2, for TNF and IFN-γ, respectively). Unlike the observations using rIL-2, FcγR(CD16)-induced transcription of TNF and IFN-γ genes were decreased by 78.3 and 60.1%, respectively, in the presence of EGTA.

Dependence of Lymphokine Production by NK Cells on Ca^{2+} Mobilization. To test whether Ca^{2+} mobilization from intracellular stores is sufficient to induce lymphokine production by NK cells, IFN-γ production was measured in the supernatants from cells treated, in the absence and presence of rIL-2, with doses of ionomycin (1.2 and 30 nM) that induce $[Ca^{2+}]_i$ rises similar to those detected upon stimulation of the same cells with anti-CD16 antibodies alone or in the presence of cross-linking $F(ab')_2$ GaMIg. Supernatants from NK cells stimulated with soluble or Sepharose-linked anti-CD16 antibody B73.1 with or without rIL-2 added were used as controls. As shown in Fig. 7, only concentrations of ionomycin as high as 250 nM and able to induce $[Ca^{2+}]_i$ rises significantly higher than those ever observed with anti-CD16 antibodies were able to induce IFN-γ production at levels similar to those induced by Sepharose-linked anti-CD16 antibodies. Ionomycin used at lower doses and soluble antibodies were ineffective, both in the absence and presence of rIL-2, though they induced a significant $[Ca^{2+}]_i$ rise.

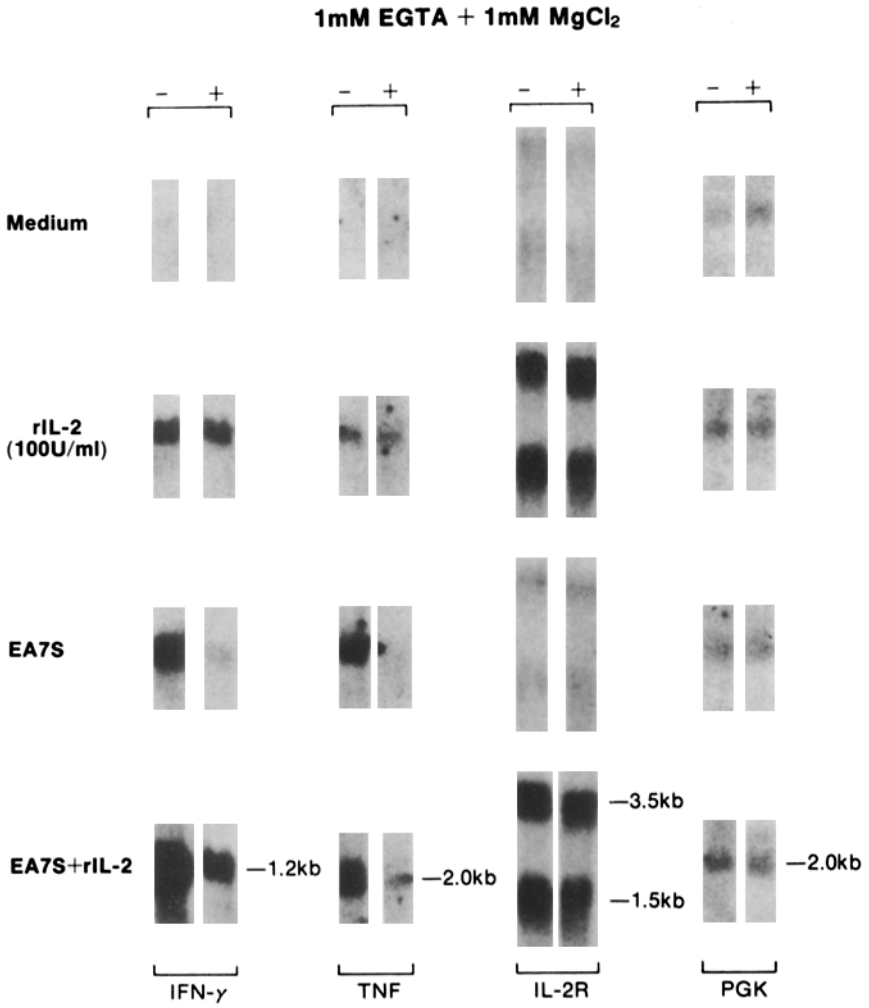

FIGURE 5. EGTA inhibits accumulation of IFN-γ, TNF, and IL-2-R mRNA in NK cells stimulated with FcγR(CD16) ligands. Cytoplasmic RNA, extracted from purified NK cells incubated (2 h, 37°C) in the presence of the indicated inducers in the absence (−) or presence (+) of 1 mM EGTA and 1 mM $MgCl_2$ were electrophoresed on 1% agarose/6% formaldehyde gel (20 μg mRNA per lane). After transfer to nylon, the RNA was hybridized to the appropriate cDNA probes. Amount and integrity of RNA was checked by ethidium bromide staining.

Discussion

In this study we demonstrate that FcγR(CD16) on NK cells transduce intracellular signals, upon interaction with ligands, resulting in a rapid $[Ca^{2+}]_i$ rise and IP accumulation, and we provide evidence that FcγR(CD16)-dependent NK cell activation occurs through molecular mechanisms in which Ca^{2+} plays an essential role.

Binding of immune complexes, but not of monomeric IgG, to NK cells, and stim-

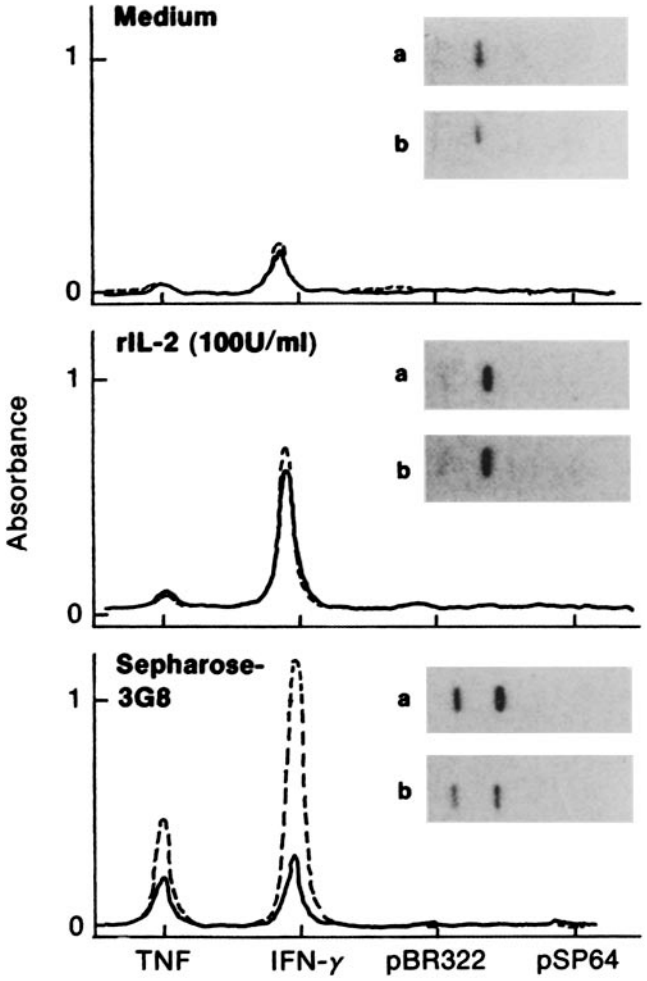

FIGURE 6. Chelation of extracellular Ca^{2+} by EGTA inhibits the FcγR(CD16) ligand-induced transcription of TNF and IFN-γ genes in NK cells. Purified NK cells were incubated (20 min, 37°C) in medium in the absence (*top*) or presence of rIL-2, 100 U/ml (*middle*) or Sepharose-B73.1 (*bottom*). 1 mM EGTA and 1 mM $MgCl_2$ were either absent (- - - -) or present (——) during incubation. Transcription run-on assays were performed as described in Materials and Methods. Labeled transcripts from each reaction were hybridized to excess denatured cDNA immobilized on nitrocellulose filter. The histograms are densitometric readings of the bands in the respective films (inset in the right corner of each panel; *a*, in the absence of EGTA; *b*, in the presence of EGTA).

ulation of these cells with saturating concentrations of anti-CD16 antibodies, result in a $[Ca^{2+}]_i$ increase. The rapid $[Ca^{2+}]_i$ rise induced by the antibodies is due to direct interaction of their antigen-binding portion with the CD16 molecule: $F(ab')_2$ fragments of both B73.1 and 3G8 are as active as the intact IgG. By contrast, antibodies directed to distinct surface molecules expressed on the NK cells from the preparations used are ineffective. Anti-FcγR(CDw32) antibody KuFc79, which reportedly induces a $[Ca^{2+}]_i$ rise in granulocytes after crosslinking at the cell surface (13), does not exert this effect on NK cells. Unlike polymorphonuclear cells and monocytes, NK cells bear only one FcγR type (CD16), through which antibody-dependent cytotoxicity is induced (17, 33, 34). The present results confirm our previous observations (1) that anti-CD16 antibodies can mimic the effect of natural ligands to this FcγR and that interaction of immune complexes with FcγR(CD16) alone is sufficient to transduce signals intracellularly.

The $[Ca^{2+}]_i$ rise induced by the CD16 ligands is sustained at plateau levels for

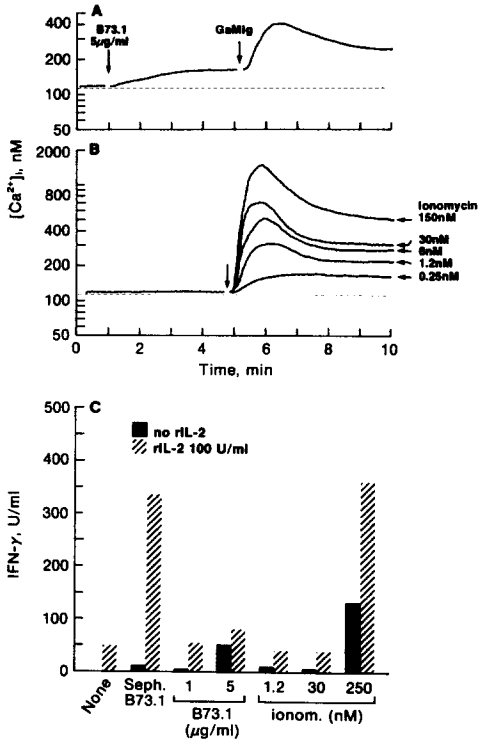

FIGURE 7. Concentrations of the Ca^{2+} ionophore ionomycin able to induce the same $[Ca^{2+}]_i$ increases as FcγR(CD16) ligands are unable to induce IFN-γ production by NK cells. $[Ca^{2+}]_i$ (*A* and *B*) was measured in purified NK cells as described in Table I. IFN-γ (*C*) was measured by RIA in the cell-free supernatants collected after culture (18 h at 37°C) of purified NK cells in the absence (none) or presence of the inducers (indicated in abscissa) with (▨) or without (■) 100 U/ml rIL-2.

a considerable time period. The methodology we used does not allow detection of $[Ca^{2+}]_i$ at the single cell level, and it is possible that the overall rise reflects accumulation of signals from NK cells that are stimulated at different times, possibly depending on variable degree of expression of the CD16 molecule at the membrane. This hypothesis could also explain why similar plateau levels are reached, independent of the antibody dose used, although with different kinetics, at any of the saturating antibody concentrations. Alternatively or in addition, FcγR(CD16) might behave as an ion channel upon ligand interaction or induce opening of ion channels at the cell membrane. A role for moFcγRII as an ion channel upon ligand binding, though not Ca^{2+} specific, has been demonstrated (10).

Occupancy of two receptor molecules by $F(ab')_2$ fragments is sufficient to induce a $[Ca^{2+}]_i$ rise, but crosslinking of the antibody at the cell membrane induces a rapid rise of $[Ca^{2+}]_i$ to levels over those induced by the antibody alone with identical kinetics, independent of the concentration of the primary antibody (data not shown), suggesting that, as in other receptor-ligand systems (35), the ligand requires engagement of more than two recepter molecules at the cell membrane for optimal stimulation of the cells. The transient increase in $[Ca^{2+}]_i$ observed upon crosslinking has faster kinetics than that induced in the absence of crosslinking and is followed by a decline to values higher than those maintained after addition of the primary antibody alone. The spike observed after crosslinking probably reflects simultaneous stimulation of most cells. However, it is also possible that two distinct pools of intra-

cellular Ca^{2+} stores are mobilized, under the two different conditions of stimulation, as it has been suggested to occur in T cells upon perturbation of the T cell receptor (36).

Chelation of extracellular Ca^{2+} significantly reduces, but never abolishes, the initial induction of the $[Ca^{2+}]_i$ rise induced by the FcγR(CD16) ligand both in the absence and presence of its crosslinking. The decreased Ca^{2+} levels observed after crosslinking induced in the presence of EGTA do not depend on depletion of intracellular Ca^{2+} stores induced by stimulation with the first antibody, because similar effects are evident independent of the time at which EGTA is added to the cells. Thus, our data indicate that the initial induction of the $[Ca^{2+}]_i$ rise by anti-CD16 antibodies, either alone or after crosslinking, is due to mobilization of Ca^{2+} from intracellular stores but that the sustained $[Ca^{2+}]_i$ levels are maintained through extracellular Ca^{2+} influx.

Crosslinking of FcγR(CD16) at the NK cell membrane results in significantly higher levels of IP_3 and IP_4 than those induced in the absence of crosslinking. This effect, like the rise in $[Ca^{2+}]_i$, is specifically mediated by anti-CD16 antibody $F(ab')_2$ fragments, thus excluding the participation of other molecules not linked to FcγR in the phenomenon. $[Ca^{2+}]_i$ mobilization from intracellular stores induced upon FcγR(CD16)-ligand interaction is likely to be mediated, at least in part, through receptor-mediated hydrolysis of phosphoinositides, as reported for other signal-transducing transmembrane molecules. We reported (37) that upon crosslinking, CD16 is internalized in NK cells, as indicated by the detection of anti-CD16 antibodies in digested form in the supernatants of fresh NK cells incubated at 37°C after reaction with ^{125}I-anti-CD16 antibody and GaMIg. The observation that significant IP accumulation is observed in NK cells only upon CD16 crosslinking suggests that internalization and IP accumulation occur under the same conditions of stimulation. As discussed before, crosslinking of two receptor molecules is likely even when soluble antibody alone is used. The detection, under these conditions, of a significant $[Ca^{2+}]_i$ rise in the absence of significant IP formation suggests that either the receptor is not internalized under these conditions or the extent of internalization is insufficient to determine IP production. It is also possible that the mechanisms mediating $[Ca^{2+}]_i$ rise and internalization of the receptor differ.

A cDNA for human FcγR(CD16) isolated from placenta has been shown to encode a protein that, in its mature form, lacks an intracytoplasmic domain and is anchored to the cell membrane through a GPI tail (14). A transmembrane form of FcγR(CD16) has been reported to exist in in vitro-cultured macrophages from patients affected with paroxysmal nocturnal hemoglobinuria (PNH) (38). CD16 is not completely released from PBMC upon GPI-specific phospholipase C (PLC) treatment (14) and our preliminary data indicate that the molecules on both fresh and cultured NK cells are insensitive to doses of PI-PLC that cleave 90% of FcγR(CD16) on neutrophils, CD14 on monocytes, or Thy-1 on murine T cells. These data are difficult to reconcile, at present, with the report of a single FcγR(CD16) mRNA species in human NK cells (14). Although they could reflect resistance of a proportion of the molecules to PLC digestion, as described for other GPI-anchored proteins (39, 40), they also suggest the possibility that CD16 on NK cells are, at least in part, transmembrane molecules. Conventionally anchored CD16 in NK cells could mediate signal transduction through the short hydrophilic terminus encoded in the cDNA,

as reported for other transmembrane receptor molecules such as IL-2R(CD25) (41, 42) or sIg (43). Alternatively, signal transduction through the GPI-anchored form of CD16 could be mediated through other transmembrane molecules linked to CD16, similar to what has been suggested for the Thy-1 molecule (44, 45), or through a direct action of the IP anchor, as hypothesized by Low et al. (46).

We confirm here that, under the conditions in which FcγR(CD16) is crosslinked by ligands at the NK cell membrane, intracellular signals are transduced that activate expression of lymphokine genes. Chelation of extracellular Ca^{2+}, while not abolishing intracellular Ca^{2+} mobilization, results in complete inhibition of lymphokine secretion, and of accumulation and transcription of the mRNA for both IFN-γ and TNF upon stimulation with either immune complexes or anti-CD16 antibodies. Neither antigen-antibody interaction nor Ig binding ability of FcγR are abolished upon chelation of extracellular Ca^{2+} (47, and data not shown) so that the lack of activation does not reflect inability of NK cells to bind the ligand. In the presence of EGTA, NK cells still respond with IFN-γ and TNF production to IL-2 or to a B-lymphoblastoid cell-produced factor inducing IFN-γ production, though at levels lower than control (data not shown). Under the same conditions, the ability of rIL-2 to induce transcription and accumulation of mRNA for both lymphokines and IL-2R is unaltered and similar levels of PGK transcripts are present in the same amounts of RNA. These data show that EGTA treatment does not affect NK cell viability or their ability, under appropriate stimulation, to transcribe lymphokine genes and to accumulate their transcripts in the cytoplasm, and only partially prevents translation and/or secretion of lymphokines. Rather, they indicate that Ca^{2+} represents a stringent requirement for the FcγR(CD16)-induced activation of NK cells, consistent with the known requirement for Ca^{2+} in NK cytotoxicity (48). It seems unlikely that Ca^{2+} plays an indirect role by allowing synthesis of other proteins needed for cytokine gene expression because cytokine gene transcription induced in NK cells by either FcγR(CD16)-ligand interaction or by rIL-2 does not depend on de novo protein synthesis (1).

Intracellular Ca^{2+} may play a role in the induction of cytokine gene expression in NK cells and it is possible that the threshold of activation for them is reached at $[Ca^{2+}]_i$ higher than those reached when CD16 ligands interact with FcγR in the presence of EGTA. This possibility is supported by the observation that neither IFN-γ production (these data) nor accumulation of its mRNA (our unpublished data) can be detected in NK cells stimulated with anti-CD16 antibody alone, under conditions in which $[Ca^{2+}]_i$ is always lower than that observed upon crosslinking of anti-CD16 antibodies to the NK cell membrane. However, because EGTA completely prevents the sustained $[Ca^{2+}]_i$ plateau levels in either case, our data suggest a primary role for extracellular Ca^{2+} and, possibly, for the duration of the increased Ca^{2+} levels under the different conditions. The observation that EGTA inhibits cytokine production even when added to cells 20 min after stimulation with ligand (data not shown) is compatible with the hypothesis that signals and second messengers critical for induction of FcγR-dependent gene expression are generated during the period in which $[Ca^{2+}]_i$ remains elevated over basal levels. The lack of correlation between IFN-γ production and $[Ca^{2+}]_i$ rise induced in NK cells stimulated with either anti-CD16 antibodies or doses of ionomycin to levels similar to those observed upon FcγR(CD16) triggering suggests that the $[Ca^{2+}]_i$ levels obtained after

FcγR (CD16) crosslinking are not sufficient to induce cytokine production. Ionomycin at high doses is likely to induce activation of additional second messengers of signal transduction and our data are compatible with the hypothesis that FcγR(CD16) crosslinking generates additional transducing mechanisms that synergize with IL-2 to induce maximal NK cell activation.

Summary

In this study, we present evidence that interaction of FcγR(CD16) with ligands (immune complexes or anti-CD16 antibodies) induces a rapid rise in $[Ca^{2+}]_i$ and fast production of both inositol 1,4,5 triphosphate (IP_3) and IP_4 in homogeneous NK cell preparations. Part of the initial $[Ca^{2+}]_i$ rise observed upon stimulation of NK cells with either anti-CD16 antibodies alone or after their crosslinking at the cell membrane depends on Ca^{2+} mobilization from intracellular stores, but sustained $[Ca^{2+}]_i$ levels are maintained, after the initial spike, through influx of extracellular Ca^{2+}. The $[Ca^{2+}]_i$ rise is mediated, at least in part, by increases in IP_3 after receptor-induced hydrolysis of membrane polyphosphoinositides (PPI). The role of extracellular Ca^{2+} in FcγR(CD16)-dependent induction of lymphokine gene expression has been tested by evaluating production, mRNA accumulation and transcription of IFN-γ and TNF in NK cells stimulated with FcγR(CD16) ligands and/or rIL-2 in the presence of EGTA. Under these conditions, accumulation and transcription of both IFN-γ and TNF mRNA induced by CD16 ligands, but not that induced by rIL-2, is completely abolished and neither cytokine can be detected at significant levels in the supernatant fluids of cells so treated. These data confirm that NK cell activation by specific ligands occurs through mechanisms distinct from those induced by IL-2, and indicate that extracellular Ca^{2+} represents a stringent requirement for cytokine production induced in NK cells through specific (FcγR) stimulation. Our data also indicate that the $[Ca^{2+}]_i$ rise induced upon FcγR(CD16) crosslinking, though necessary, is not sufficient per se to induce activation of lymphokine genes, compatible with the hypothesis that FcγR(CD16) crosslinking generates additional transducing signals that synergize with IL-2 to maximally activate NK cells.

We thank Marina Hoffman for editing and Marion Kaplan for secretarial assistance.

Received for publication 1 August 1988 and in revised form 17 October 1988.

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