

Phospholipase D Activation in Human Natural Killer Cells through the Kp43 and CD16 Surface Antigens Takes Place by Different Mechanisms. Involvement of the Phospholipase D Pathway in Tumor Necrosis Factor α Synthesis

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Summary

We have recently described a novel glycoprotein, Kp43, expressed on the surface of human natural killer (NK) cells that appears to regulate their functional activity. In this report, signaling mechanisms through the Kp43 surface antigen have been studied. Incubation of interleukin 2 (IL-2)-treated NK cells with anti-Kp43 monoclonal antibody F(ab')₂ fragments resulted in the time- and dose-dependent stimulation of NK cell phospholipase D. Phospholipase D activation through the Kp43 surface antigen was found to take place in the absence of polyphosphoinositide turnover and appeared not to depend on the presence of Ca²⁺ in the extracellular medium. On the other hand, signaling mechanisms through the CD16 receptor (FcR-III) on NK cells were comparatively studied. Stimulation of IL-2-treated NK cells with anti-CD16 monoclonal antibody F(ab')₂ fragment also resulted in time- and dose-dependent activation of phospholipase D. However, CD16-triggered phospholipase D activation took place concomitant to phospholipase C-mediated polyphosphoinositide breakdown and showed a strong dependence on extracellular Ca²⁺. These results provide, to our knowledge, the first evidence for the presence of activatable phospholipase D in NK cells, as well as the first indication that distinct receptor-modulated pathways exist for activation of phospholipase D within the same cell type. On the other hand, phosphatidic acid, the physiologic product of phospholipase D action on phospholipids, was found to mimic the effect of anti-Kp43 monoclonal antibody regarding tumor necrosis factor α (TNF- α) biosynthesis and secretion by NK cells. Addition of phosphatidic acid vesicles to IL-2-treated NK cell cultures stimulated a TNF- α production that was abolished when the cells were previously treated with actinomycin D. Other phospholipids, including lysophosphatidic acid, were ineffective. However, phosphatidic acid-induced TNF- α production was strongly inhibited by the presence of propranolol, an inhibitor of phosphatidic acid phosphohydrolase. Moreover, in cells responding to phorbol myristate acetate, a compound that triggers activation of phospholipase D, TNF- α synthesis was also inhibited by propranolol. Thus, these data suggest a second messenger role for phosphatidic acid-derived diacylglycerol in the induction of TNF- α gene expression.

Human NK cells are phenotypically defined as CD3⁻ large granular lymphocytes expressing the CD16 (FcR-III) and the CD56 surface antigens (1). NK cells are able to mediate MHC-unrestricted cytotoxicity, secrete cytokines, and proliferate in response to IL-2. This lymphoid cell subset has been proposed to play a key effector role in nonadaptive immunity and is believed to be involved in host defense against microbial infection and tumor cell growth, as well as in the regulation of hematopoiesis (1).

Although NK cells comprise only 10–15% of total human PBL, the possibility of expanding *in vitro* NK cell popula-

tions (2) and clones (3) has enabled a considerable advance in the knowledge of NK cell biology, and of the molecular mechanisms that underlie NK cell activation. It has been reported that NK cell stimulation through the CD16 antigen triggers phospholipase C hydrolysis of polyphosphoinositides, intracellular Ca²⁺ mobilization, and Ca²⁺ influx across the plasma membrane (4). The CD16 antigen is coupled to the ζ chain of the CD3 complex, which is believed to play a key role in signal transduction (5). Phospholipase C activation via the CD16 receptor has been reported to be mediated through a cholera toxin-sensitive GTP-binding protein (6).

Nonetheless, little is known about signaling mechanisms involving activation of other intracellular phospholipases in human NK cells.

We have recently described a novel glycoprotein, termed Kp43, that is expressed on the surface of human NK cells, and appears to be involved in the regulation of IL-2-treated NK cell function (7, 8). Anti-Kp43 mAb induces cytolytic activity against normal T cell blasts (8), and enhances the synthesis of TNF- α (J. Aramburu, A. Rodríguez-Márquez, M. A. Balboa, and M. López-Botet, manuscript in preparation).

The present study was undertaken to address the issue of signal transduction through the Kp43 molecule in human NK cells. In addition, signaling mechanisms through the CD16 surface antigen were comparatively investigated. Our results demonstrate receptor-regulated phospholipase D activity through two different NK cell-associated surface molecules, namely Kp43 and CD16. This is the first report documenting phospholipase D activation in human NK cells. In addition, this study provides evidence that at least two different receptor-modulated pathways exist for activation of phospholipase D in a single cell type. Moreover, our results suggest a role for phosphatidic acid-derived diradylglycerol, a product of phospholipase D action on phospholipids, in the biosynthesis of TNF- α by NK cells.

Materials and Methods

Materials. Cell culture medium, FCS, penicillin, streptomycin, and L-glutamine were purchased from Flow Laboratories (Irvine, Scotland). [^3H]Palmitic acid (sp act, 54 Ci/mmol), *myo*[^3H]inositol (sp act, 20.8 Ci/mmol), and H_3 [^{32}P]O $_4$ (sp act, 10 mCi/mmol) were from Amersham International (Amersham, England). Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). PMA, fura-2/AM, pepsin, phospholipids, actinomycin D, and propranolol were from Sigma Chemical Co. (St. Louis, MO). G-60 thin-layer chromatography plates and the organic solvents (analytical grade) were from Scharlau (Barcelona, Spain). Phosphatidylethanol (PEt) 1 standard was prepared from egg lecithin as described (9). IL-2 was generously provided by Hoffmann-La Roche (Basel, Switzerland).

Monoclonal Antibodies. The anti-Kp43 mAb, HP3B1 (IgG2a), obtained against activated NK cells, has been previously described (7, 8). The anti-CD16 mAb, B73.1 (IgG1), was a kind gift from Dr. B. Perussia (The Wistar Institute, Philadelphia, PA). Anti-CD56 mAb, Leu19, was purchased from Becton Dickinson & Co. (Mountain View, CA). Anti-CD3 mAb, SPV-T3b (IgG2a) (10), was kindly provided by Dr. J. de Vries (Unicet Laboratories, Dardilly, France). Anti-CD2 mAb, TS2/18 (IgG2a) (11), was a kind gift from Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain). Anti- β_2 -microglobulin mAb, HP1H8 (IgG3), was obtained in our laboratory. F(ab') $_2$ anti-mouse IgG antiserum was purchased from Sigma Chemical Co.

F(ab') $_2$ Preparation. F(ab') $_2$ fragments from affinity-purified mAb were prepared by pepsin digestion and separated from undigested IgG and Fc fragments by passage through a protein A-Sepharose column as described (12).

Cells. All experiments were performed with NK cells obtained

from short-term cultures of PBMC with the irradiated Daudi tumor B cell line as previously described (2, 7, 8). NK cells were purified from 6–7-d-old cultures by negative selection using anti-CD3 mAb and rabbit complement (Behring, Marburg, Germany). These populations were later expanded with 50–100 U/ml IL-2 (7, 8), and will be referred throughout the paper to as IL-2-treated NK cells. Before performing the assays, the surface phenotype of cultured NK cells was analyzed by indirect immunofluorescence labeling with CD2-, CD7-, CD16-, CD56-, and Kp43-specific mAbs. The results were comparable to those previously described (<5% CD3 $^+$; >95% CD2 $^+$, CD7 $^+$, CD16 $^+$, CD56 $^+$, Kp43 $^+$) (7, 8).

Preparation of Labeled Cells, Incubation Conditions, and Lipid Extraction. Labeling of cells with [^3H]palmitic acid or [^3H]inositol was accomplished by adding these radioactive precursors at 10 $\mu\text{Ci/ml}$ overnight to the culture medium. [^3H]Palmitic acid in toluene was taken to dryness under a gentle stream of nitrogen, and the residue was dissolved in 0.1 M NaOH (13) and diluted with fresh medium before addition to cell cultures. For ^{32}P labeling, cells were incubated overnight with 20 $\mu\text{Ci/ml}$ H_3 [^{32}P]O $_4$. Appropriately labeled cells were resuspended in HG buffer (150 mM NaCl, 1.2 mM MgCl $_2$, 1.3 mM CaCl $_2$, 5.5 mM D-glucose, 10 mM Hepes, pH 7.5) to a concentration of 10 7 cells/ml. The reactions were initiated by adding either vehicle or the corresponding stimulus, in the presence or absence of 0.5% ethanol. Reactions were stopped by adding an equal volume of chloroform/methanol/hydrochloric acid (100:200:1) and lipids were extracted as described (14).

For preparation of Ca $^{2+}$ -depleted cells, they were incubated with 1 mM EGTA and 40 μM fura-2/AM in Ca $^{2+}$ -free HG buffer, for 60 min at 37°C (15, 16), washed twice, and stimulated as appropriate, either in presence or absence of 1 mM EGTA.

Analysis of Lipids by Thin-Layer Chromatography. After extraction, the lipids in the lower organic phase were spotted onto Silicagel G-60 plates. For PEt separation, plates were developed twice using the upper phase of a system consisting of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110:50:20:100). This system allows a good separation between phosphatidic acid (PA), PEt, and major phospholipids (17). For DG separation, plates were developed twice with *n*-hexane/diethyl ether/acetic acid (70:30:1) as a solvent system (18).

Measurement of Inositol Phosphates Production. Total inositol phosphates production was quantitated by anion-exchange chromatography as described (18, 19).

TNF- α Production Assay. NK cells were seeded in round-bottomed 96-well plates (1.5 \times 10 6 cells/ml, 200 μl /well final volume) in culture medium with 50 U/ml of rIL-2 and the different stimuli were added at the beginning of the assay. Lipids were added to the cells in the form of vesicles obtained by sonication in culture medium. After the appropriate incubation times, plates were centrifuged (200 g, 3 min), and cell-free supernatants were collected. TNF- α concentration in the supernatants was measured by ELISA (Biokine TNF Test; T Cell Sciences Inc., Cambridge, MA). When the effect of a RNA synthesis inhibitor, actinomycin D, was tested, cells were preincubated for 30 min with this compound (5 $\mu\text{g/ml}$) before addition of the different stimuli.

Results

PEt Production by IL-2-treated NK Cells. Unequivocal evidence for the involvement of phospholipase D in stimulus-response coupling is the accumulation of phosphatidylalcohols when primary alcohols, such as ethanol, are present during cell stimulation (20). These phospholipids are not normal constituents of the cell membrane, and phospholipase D-cata-

¹ Abbreviations used in this paper: DG, 1,2-diradylglycerol; PA, phosphatidic acid; PEt, phosphatidylethanol.

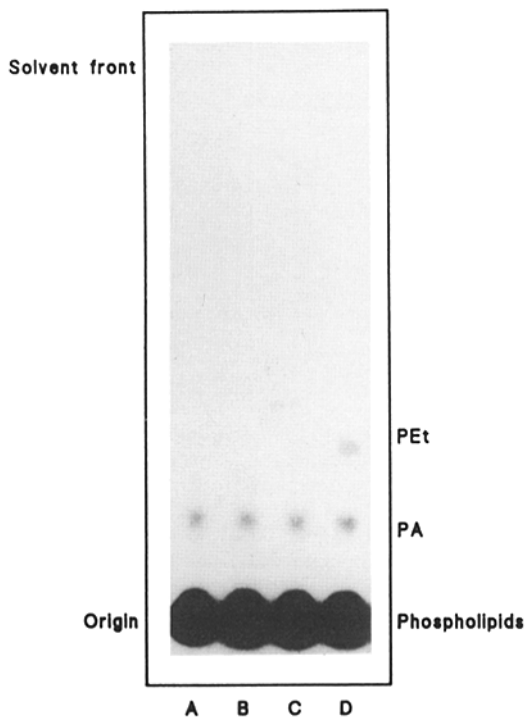


Figure 1. PEt production by IL-2-treated NK cells. ^{32}P -labeled NK cells were stimulated with PMA (100 ng/ml) for 30 min in the presence (lane D) or absence (lane B) of 0.5% ethanol. After extraction, lipids were separated by thin-layer chromatography as described in Materials and Methods, and the plate was autoradiographed. Lanes A and C denote control incubations without PMA in the absence or presence of ethanol, respectively.

lyzed transphosphatidylolation is the only known mechanism by which cells can form phosphatidylalcohols. Therefore, the formation of PEt is a sensitive and specific assay for phospholipase D activation. PEt formation was investigated in ^{32}P -labeled IL-2-treated NK cells stimulated by PMA (Fig. 1). PMA has been previously shown to optimally activate phospholipase D in human leukocytes (14, 21–23). Thin-layer chromatography analyses of lipid extracts derived from PMA-stimulated NK cells exposed to ethanol revealed the presence of a unique spot (Fig. 1, lane D) that was not found in stimulated cells in the absence of ethanol (Fig. 1, lane B) nor in unstimulated cells either in the absence or presence of ethanol (Fig. 1, lanes A and C). The spot was identified as PEt by comparison with a PEt standard (9). Thus, these results provide the first evidence for the presence of a phospholipase D activity in IL-2-activated NK cells that is regulated upon cell stimulation.

Interaction of mAb HP3B1 with the Kp43 Surface Antigen Enhances Phospholipase D Activity in IL-2-treated NK Cells. Experiments were conducted to determine the possible involvement of phospholipase D activity in NK cell stimulation through the Kp43 antigen. [^3H]Palmitic acid-labeled cells were exposed to HP3B1 mAb F(ab')₂ fragments in the presence of 0.5% ethanol, and PEt production at different times was examined (Fig. 2 A). In these experiments PMA was used as a positive control. As shown in Fig. 2 A, both

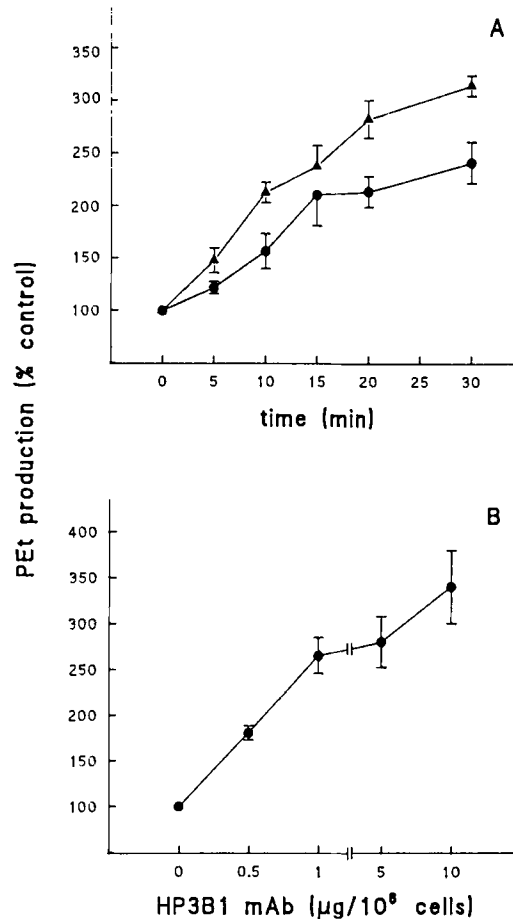


Figure 2. Formation of PEt in IL-2-treated NK cells stimulated via the Kp43 antigen. [^3H]Palmitic acid-labeled NK cells were incubated in buffer containing 0.5% ethanol and challenged by 100 ng/ml PMA (\blacktriangle) or by 3 $\mu\text{g}/10^6$ cells of HP3B1 anti-Kp43 mAb F(ab')₂ fragments (\bullet) for different times (A), or by several mAb concentrations for 30 min (B). After extraction, lipids were separated by thin-layer chromatography, and the radioactivity was quantified by liquid scintillation counting. Data correspond to the mean \pm SEM of four different experiments, and are expressed as percent production with respect to unstimulated controls taken at different times. The 100% value was 1,140 \pm 250 cpm.

PMA and HP3B1 mAb F(ab')₂ fragments induced a time-dependent accumulation of PEt in IL-2-treated NK cells. The kinetics of PEt production by both stimuli was similar, the cell response to PMA being always higher than that to HP3B1 (Fig. 2). The concentration-response relationship for HP3B1 mAb F(ab')₂ fragment-induced PEt accumulation was investigated, and the results are shown in Fig. 2 B. Increase of [^3H]PEt accumulation was observed in a dose-dependent manner, the maximal response being reached at an antibody concentration of 1 $\mu\text{g}/10^6$ cells.

PA and DG Production by IL-2-treated NK Cells Stimulated through the Kp43 Surface Antigen. Since the physiologic product of phospholipase D action on cellular phospholipids is PA, not PEt, the effect of HP3B1 mAb F(ab')₂ fragment on PA levels was investigated. PMA was used as a positive control (23). [^3H]Palmitic acid-labeled PA levels increased

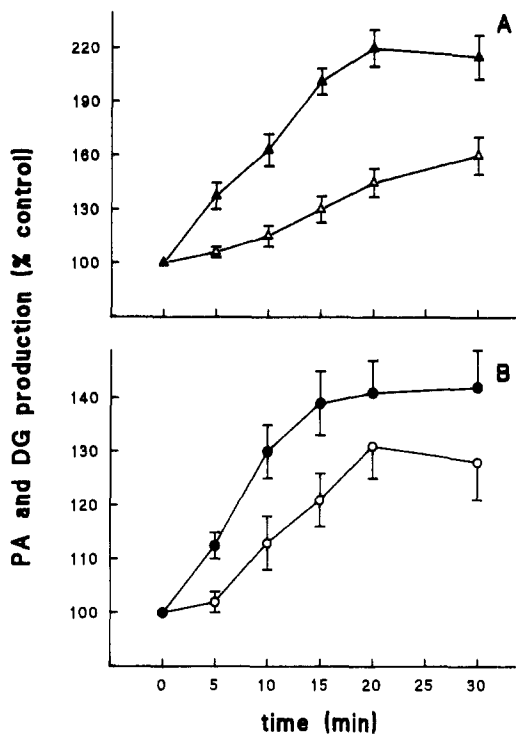


Figure 3. Accumulation of PA and DG in stimulated NK cells. NK cells, prelabeled with [³H]palmitic acid, were stimulated with 100 ng/ml PMA (A) or HP3B1 anti-Kp43 mAb F(ab')₂ fragments (3 μg/10⁶ cells) (B), for the indicated times. After extraction, lipids were separated by thin-layer chromatography, and the radiography in PA (▲, ●) and DAG (△, ○) was quantified by liquid scintillation counting. Values are shown as mean ± SEM of three different determinations in samples from different donors. The results are expressed as percent production with respect to unstimulated controls. 100% values were 5,180 ± 520 and 15,300 ± 1,860 for PA and DG, respectively.

gradually, plateauing at 15 min and remaining unchanged thereafter (Fig. 3). Stimulated PA production by both PMA and HP3B1 showed similar kinetics, although, as for PET production, the cellular response to PMA was always higher than that to HP3B1.

Experiments were also conducted to study 1,2-diradylglycerol (DG) production by stimulated NK cells. As shown in Fig. 3, both PMA and HP3B1 induced appreciable [³H]palmitoyl-labeled DG accumulation.

Enhancement of Phospholipase D Activity in IL-2-treated NK Cells by Anti-CD16 mAb To rule out the possibility that HP3B1-induced PET accumulation could be unspecific and not receptor mediated, we examined the effect of other mAbs on NK cell phospholipase D. The results are summarized in Table 1. It can be observed that neither HP1H8 mAb F(ab')₂ fragment (anti-β₂-microglobulin) nor SPV-T3b mAb (anti-CD3) induced PET accumulation. Therefore, these results indicate that HP3B1-induced PET formation was actually a receptor-triggered event. Remarkably, when we tested the effect of B73.1 mAb F(ab')₂ fragment (anti-CD16), an appreciable PET production of similar magnitude to that induced by anti-Kp43 mAb was observed (Table 1). These data

Table 1. PET Production by Human IL-2-treated NK Cells

Stimulus	PET production
	<i>cpm</i>
None (control)	640 ± 50 (100)
PMA	2,340 ± 240 (263)
HP3B1	1,430 ± 170 (220)
B73.1	1,320 ± 170 (210)
HP1H8	620 ± 40 (100)
SPVT3b	700 ± 100 (109)

IL-2-treated NK cells, prelabeled with [³H]palmitic acid, were incubated with stimuli listed above for 30 min, in the presence of 0.5% ethanol. PET production was determined as described in Materials and Methods. All antibody preparations were used as F(ab')₂ fragments at 3 μg/10⁶ cells. PMA was used at 100 ng/ml. Results correspond to the mean ± SEM from a representative experiment of three performed. Numbers in parentheses represent percent values referred to control.

prove that occupancy of the CD16 receptor also stimulates NK cell phospholipase D. Thus, we characterized the anti-CD16 mAb-induced PET production in IL-2-treated NK cells. As shown in Fig. 4, both the kinetics of PET accumulation and the threshold concentration giving maximal PET production in response to anti-CD16 mAb were similar to those previously observed for HP3B1-stimulated cells (compare Figs. 2 and 4). Crosslinking of CD16 receptors with F(ab')₂ mouse IgG antiserum did not appreciably modify PET production (results not shown).

Role of Ca²⁺ in Receptor Stimulation of Phospholipase D in IL-2-treated NK Cells. Ca²⁺ has been proposed to be a key regulator of phospholipase D activity in human HL60 granulocytes (21, 24), human U937 promonocytes (14), and human erythroleukemia cells and platelets (25, 26). Therefore, we examined the involvement of Ca²⁺ in IL-2-treated NK cell phospholipase D activation. The effect of both HP3B1 and B73.1 mAb F(ab')₂ fragments on phospholipase C-mediated inositol phosphates production is shown in Fig. 5. As previously reported by Cassatella et al. (4), B73.1-induced polyphosphoinositide breakdown was noticeably augmented when a crosslinker was added to the incubation mixture (Fig. 5). These data agree with the fact that occupancy of CD16 receptors on IL-2-treated NK cells triggers Ca²⁺ mobilization from internal stores (4, 8). By contrast, HP3B1 mAb F(ab')₂ fragments did not induce phospholipase C-mediated polyphosphoinositide breakdown. When a crosslinker was added, again no inositol phosphates production could be observed (Fig. 5). It is noteworthy that NK cell stimulation through the Kp43 receptor is not associated with changes in the intracellular Ca²⁺ concentration (8).

To analyze the possible involvement of extracellular Ca²⁺ in the activation of NK cell phospholipase D, experiments were carried out in the presence or absence (EGTA-containing medium) of Ca²⁺ in the incubation medium. As shown in

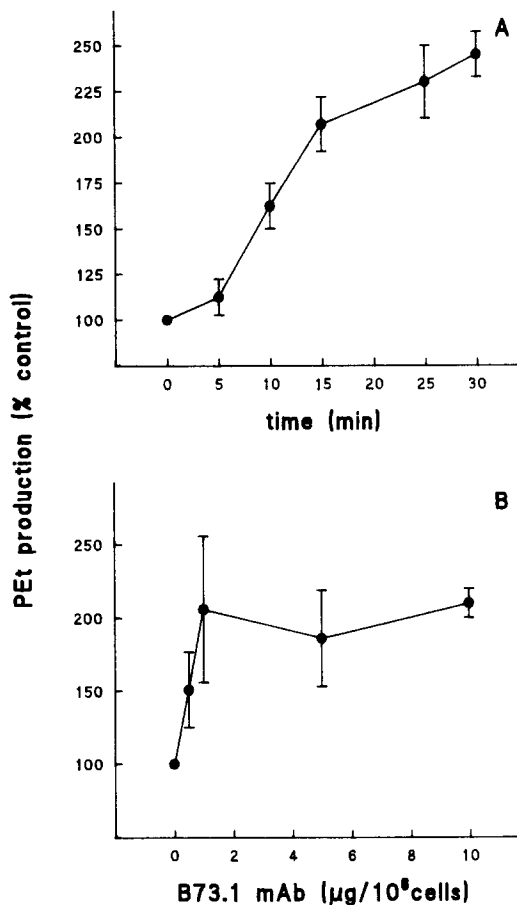


Figure 4. PEt production by NK cells stimulated via the CD16 receptor. [³H]Palmitic acid-labeled cells were stimulated by B73.1 anti-CD16 mAb F(ab')₂ fragments (3 µg/10⁶ cells) for different times (A) or by several mAb concentrations for 30 min (B), in the presence of 0.5% ethanol. Data are shown as mean ± SEM of three different experiments, and are expressed as percent production with respect to unstimulated controls. The 100% value was 1,530 ± 160 cpm.

Fig. 6, chelation of extracellular Ca²⁺ with EGTA led to a strong inhibition of B73.1-induced PEt accumulation. However, PEt production in response to HP3B1 was unaffected (Fig. 6). These data indicate, therefore, that extracellular Ca²⁺ is required for phospholipase D activation through the CD16 receptor but not through the Kp43 antigen. These findings are consistent with previous work demonstrating extracellular Ca²⁺ influx associated with CD16 receptor occupancy (4) but not with anti-Kp43 stimulation (8).

Receptor activation of PEt synthesis was also investigated in IL-2-treated NK cells pretreated with 40 µM fura-2/AM in the presence of 1 mM EGTA, a procedure that depletes the intracellular Ca²⁺ stores and buffers and clamps the intracellular Ca²⁺ concentration at very low levels (~10⁻⁸ M) (15, 16). Under these conditions, PEt production by HP3B1-stimulated IL-2-treated NK cells was completely abolished (data not shown). Thus, the results suggest that a threshold concentration of Ca²⁺ inside the cell is minimally required

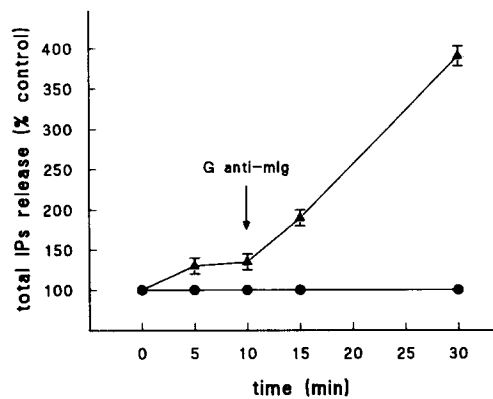


Figure 5. Phospholipase C-mediated hydrolysis of inositol phospholipids in NK cells stimulated either by anti-Kp43 or anti-CD16 mAbs. NK cells, prelabeled with myo[³H]inositol, were treated with HP3B1 anti-Kp43 (●) or B73.1 (▲) mAb F(ab')₂ fragments (3 µg/10⁶ cells) for the indicated times. Total inositol phosphates production was determined as described in Materials and Methods. The arrow indicates addition of a F(ab')₂ goat anti-mouse Ig antiserum (G anti-mIg) (50 µg/ml) as a crosslinker. Results are given as mean ± SEM from a representative experiment of two similar ones, and are expressed as percent production with respect to unstimulated controls taken at different times. The 100% value was 860 ± 100 cpm.

to sustain receptor-mediated activation of phospholipase D in NK cells.

Stimulation of TNF-α Biosynthesis in IL-2-treated NK Cells by PA-derived DG. From the results shown in Fig. 3, it is apparent that stimulated PA accumulation in NK cells precedes that of DG. This suggests that PA may be a source of DG generated upon cell activation, as has been described in other cellular systems (27–31). A common feature of IL-2-treated NK cells exposed either to PMA, HP3B1 mAb, or B73.1 mAb is the induction of TNF-α biosynthesis (32) (J. Aramburu, A. Rodríguez-Márquez, M. A. Balboa, and M. López-Botet, manuscript in preparation). As all of these stimuli

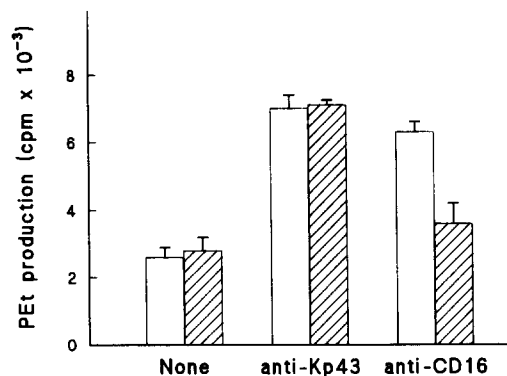


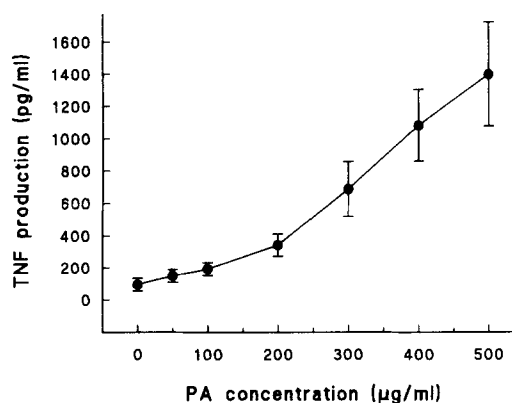
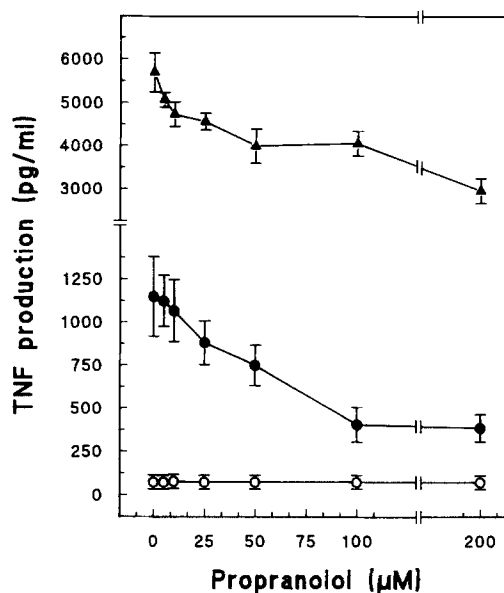
Figure 6. Effect of Ca²⁺ on receptor-stimulated PEt production in NK cells. [³H]Palmitic acid-labeled NK cells were placed in buffer containing 0.5% ethanol and stimulated by HP3B1 (3 µg/10⁶ cells) or by B73.1 (3 µg/10⁶ cells) for 30 min, in the presence (open bars) or absence (hatched bars) of 1.3 mM CaCl₂ in the extracellular medium. PEt production was determined as described in Materials and Methods. Results are given as mean ± SEM of three different experiments.

Table 2. TNF- α Production by Human IL-2-treated NK Cells

Stimulus	TNF- α	
	- Act D	+ Act D
	<i>pg/ml</i>	
None (control)	105 \pm 12	110 \pm 12
PMA	5387 \pm 1,004	420 \pm 110
HP3B1	1,228 \pm 480	95 \pm 12
B73.1	208 \pm 75	106 \pm 9
PA	1,200 \pm 148	394 \pm 28

NK cells were treated for 3 h with the stimuli listed above in a IL-2-containing medium (50 U/ml), either in the presence or absence of 5 μ g/ml actinomycin D (Act D). The mAbs were used as F(ab')₂ fragments at a concentration of 5 μ g/ml. PMA was used at 20 ng/ml, and PA at 500 μ g/ml, in the form of sonicated vesicles. At the end of the 3-h period, cell-free supernatants were collected and assayed for TNF- α content as described in Materials and Methods. Values correspond to the mean \pm SEM of four determinations in samples from different donors.

activate phospholipase D, we examined the effect of adding PA directly to IL-2-treated NK cell cultures on TNF- α production. As well as HP3B1, B73.1, and PMA, PA by itself was able to induce appreciable TNF- α production (Table 2). In as much as cell viability remained constant along the experiments, as measured by ⁵¹Cr release from ⁵¹Cr-labeled cells (data not shown), increase in supernatant levels of TNF- α was not due to PA-induced cell damage. Cell-associated TNF- α levels before incubation with the different stimuli were 75 \pm 25 pg/ml, as measured in NK cell homogenates obtained by freezing/thawing. To assess that PA-stimulated TNF- α production was actually the consequence of a biosynthetic process, experiments were performed in the presence of actinomycin D, an inhibitor of mRNA transcription. As for

**Figure 7.** Effect of PA on TNF- α production by NK cells. Different concentrations of PA, in vesicle form, were added directly to cell cultures. After 3 h, the concentration of TNF- α in the supernatants was measured as described in Materials and Methods. Results are shown as mean \pm SEM of four different experiments.**Figure 8.** Effect of propranolol on PMA- and PA-induced TNF- α production by NK cells. Cells were treated with PMA (20 ng/ml) (▲), 500 μ g/ml PA (●), or neither (○) in the presence of the indicated amounts of propranolol. After 3 h, the concentration of TNF- α in the supernatants was measured as described in Materials and Methods. Results are shown as mean \pm SEM of three different experiments.

HP3B1 mAb-, B73.1 mAb-, or PMA-mediated effects, actinomycin D blunted PA-induced TNF- α production (Table 2). The PA effect on TNF- α biosynthesis was readily observable at concentrations between 200 and 500 μ g/ml (Fig. 7). It is interesting to note that optimal PA concentrations necessary to yield maximal TNF- α production are in the range of intracellular PA levels attained in response to agonist stimulation, as reported by Bocckino et al. (33), who calculated that PA levels rise to 360–720 μ g/ml in hormone-treated hepatocytes. To assess PA specificity, other phospholipids were assayed for their ability to stimulate TNF- α production: phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, lysophosphatidylethanolamine, lysophosphatidylserine, and lysophosphatidic acid. None of these compounds were able to mimic the effect of PA in stimulating TNF- α biosynthesis in IL-2-treated NK cells (data not shown). Remarkably, when PA-stimulated TNF- α production was examined in the presence of propranolol, the response was strongly reduced (Fig. 8). Propranolol, an inhibitor of phosphatidic acid phosphohydrolase (34), has been previously shown to prevent phospholipase D-derived DG production in human leukocytes (23, 27, 28, 31, 35, 36). Thus, these data suggest a role for PA-derived DG in TNF- α synthesis by NK cells. Very interestingly, PMA-induced TNF- α biosynthesis was partially inhibited by propranolol (Fig. 8), suggesting that a portion of the PMA effect was secondary to enhanced DG production via PA dephosphorylation.

Discussion

The present work has provided, to our knowledge, the first evidence for receptor-mediated stimulation of phospholi-

pase D in human IL-2-treated NK cells. In addition, this study has demonstrated that at least two different mechanisms exist for receptor activation of NK cell phospholipase D. Thus, it has been shown that phospholipase D activation through the CD16 receptor takes place concomitant to phospholipase C-catalyzed polyphosphoinositide breakdown and depends on extracellular Ca^{2+} . On the other hand, Kp43-induced phospholipase D activation occurs in the absence of polyphosphoinositide turnover and does not depend on external Ca^{2+} . Altogether, these results give further support to the notion that different pathways exist for activation of phospholipase D (14, 20, 22, 37). Moreover, this study has documented for the first time the existence of two distinct receptor-modulated pathways for phospholipase D activation within the same cell type. Both routes require, however, a threshold Ca^{2+} concentration inside the cell, thus indicating that, in common with several other phospholipase Ds (37), NK cell phospholipase D requires Ca^{2+} for activity. This finding also suggests that occupancy of both Kp43 and CD16 may lead to activation of the same enzyme. We cannot exclude, however, the possibility that two different phospholipase D isoenzymes may exist; one activated through the Kp43 antigen, and the other one activated through the CD16 receptor. Phospholipase D activity appears to be present in cells under multiple isoforms (38, 39). Indeed, involvement of different phospholipase D isoenzymes during cell activation has recently been suggested in human neutrophils (40). The fact that activation of the Ca^{2+} -requiring NK cell phospholipase D appears to be sustained by Ca^{2+} in one case (CD16) but not in the other one (Kp43) is somewhat reminiscent of that occurring with phosphoinositide-specific phospholipase C from different tissues. Thus, phosphatidylinositol hydrolysis by phospholipase C is dependent on and stimulated by Ca^{2+} ; whereas, phospholipase C attack on phosphatidylinositol 4,5-bisphosphate, although Ca^{2+} -dependent, is not stimulated by Ca^{2+} (41).

An important aspect of the present work is the finding that PA, the physiologic product of phospholipase D action on phospholipids, is able to induce TNF- α biosynthesis and secretion, a process triggered by mAbs specific for both

Kp43 and CD16 (32) surface antigens. PA has previously been implicated in the regulation of nuclear events, such as the induction of *c-myc*, *c-fos*, and platelet-derived growth factor genes, and the stimulation of DNA synthesis (42–45). In addition, PA has also been suggested to act as a second messenger in receptor-triggered secretory processes in human neutrophils (31, 46). Our data using propranolol suggest, however, that in NK cells, the stimulatory effect of PA on TNF- α synthesis is accounted for by PA-derived DG rather than by PA itself. In addition, our results have revealed that a part of the TNF- α produced by PMA-stimulated cells is propranolol inhibitable, thus suggesting that PMA effects on TNF- α synthesis involve DG generation via PA dephosphorylation. Four distinct pathways have been described for DG generation in stimulated cells. These are phospholipase C-mediated breakdown of polyphosphoinositides, phospholipase C attack on phospholipids other than phosphoinositides, ceramide/phosphatidylcholine exchange, and phospholipase D/PA phosphohydrolase (47). In as much as the only route for DG production that is sensitive to propranolol is one involving phospholipase D (47), the data of this study suggest a second messenger role for phospholipase D-derived lipid messengers in the induction of TNF- α gene transcription in human NK cells. The putative role of phospholipase D-derived lipid messengers obviously does not exclude the involvement of other messenger molecules, particularly for the CD16-triggered TNF- α gene expression, where polyphosphoinositide-specific phospholipase C is certainly activated (see reference 4).

In conclusion, our results suggest that phospholipase D activation in IL-2-treated NK cells, and the subsequent generation of PA and DG, may provide a route for signal transduction of great importance for the understanding of NK cell biology. The fact that NK cell stimulation via the Kp43 surface antigen triggers cytotoxicity (8) and leads to phospholipase D activation in the absence of a detectable rise in the intracellular Ca^{2+} concentration suggests a role for phospholipase D-derived products in the induction of Ca^{2+} -independent NK cell cytotoxicity (48, 49). Studies are currently in progress to elucidate these questions.

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References

1. Trinchieri, G. 1989. Biology of natural killer cells. *Adv. Immunol.* 47:187.
2. Perussia, B., C. Ramoni, I. Anegón, M.C. Cuturi, J. Faust, and G. Trinchieri. 1987. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. *Nat. Immun. Cell Growth*

- Regul.* 6:171.
3. Ciccone, E., O. Viale, D. Pende, M. Malnati, R. Biassoni, G. Melioni, A. Moretta, E.O. Long, and L. Moretta. 1988. Specific lysis of allogeneic cells after activation of CD3⁻ lymphocytes in mixed lymphocyte culture. *J. Exp. Med.* 168:2403.
 4. Cassatella, M.A., I. Anegón, M.C. Cuturi, P. Griskey, G. Trinchieri, and B. Perussia. 1989. FcγR(CD16) interaction with ligand induces Ca²⁺ mobilization and phosphoinositide turnover in human natural killer cells. Role Ca²⁺ in FcγR (CD16)-induced transcription and expression of lymphokine genes. *J. Exp. Med.* 169:549.
 5. Lanier, L.L., G. Yu, and J.H. Phillips. 1989. Co-association of CD3 with a receptor (CD16) for IgG Fc on human natural killer cells. *Nature (Lond.)*. 342:803.
 6. Procopio, A.D.G., R. Paolini, I. Vecchio, L. Frati, and A. Santoni. 1991. GTP-binding proteins transduce signals generated via human Fc receptor IIIA (CD16). *J. Immunol.* 146:3550.
 7. Aramburu, J., M.A. Balboa, A. Ramírez, A. Silva, A. Acevedo, F. Sánchez-Madrid, M.O. de Landázuri, and M. López-Botet. 1990. A novel functional surface dimer (Kp43) expressed by natural killer cells and T cell receptor-gamma/delta T lymphocytes. I. Inhibition of the IL-2 dependent proliferation by anti-Kp43 monoclonal antibody. *J. Immunol.* 144:3238.
 8. Aramburu, J., M.A. Balboa, M. Izquierdo, and M. López-Botet. 1991. A novel functional surface dimer (Kp43) expressed by natural killer cells and gamma/delta TcR⁺ T lymphocytes. II. Modulation of natural killer cell cytotoxicity by anti-Kp43 monoclonal antibody. *J. Immunol.* 147:714.
 9. Kobayashi, M., and J.N. Kanfer. 1987. Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J. Neurochem.* 48:1597.
 10. Spits, H., H. Yssel, J. Leeuwenberg, and J.E. de Vries. 1985. Antigen specific cytotoxic T cell and antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. *Eur. J. Immunol.* 15:88.
 11. Sánchez-Madrid, F., A.M. Krensky, C.F. Ware, E. Robbins, J.L. Strominger, and T.A. Springer. 1982. Three distinct antigens associated with T lymphocyte-mediated cytotoxicity: LFA-1, LFA-2, and LFA-3. *Proc. Natl. Acad. Sci. USA.* 79:7489.
 12. Parham, P. 1983. On the fragmentation of monoclonal IgG1, IgG2a, and IgG2b from BALB/c mice. *J. Immunol.* 131:2895.
 13. Balsinde, J., A. Schüller, and E. Diez. 1988. The interaction of ethanol and exogenous arachidonic acid in the generation of extracellular messengers by mouse peritoneal macrophages. *Biochim. Biophys. Acta.* 970:83.
 14. Balsinde, J., and F. Mollinedo. 1991. Platelet-activating factor synergizes with phorbol myristate acetate in activating phospholipase D in the human promonocytic cell line U937. Evidence for different mechanisms of activation. *J. Biol. Chem.* 266:18726.
 15. Di Virgilio, F., D.P. Lew, and T. Pozzan. 1984. Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic Ca²⁺ levels. *Nature (Lond.)*. 310:619.
 16. Rossi, F., V. Della Bianca, M. Grzeskowiak, and F. Bazzoni. 1989. Studies on molecular regulation of phagocytosis in neutrophils. Con A-mediated ingestion and associated respiratory burst independent of phosphoinositide turnover, rise in [Ca²⁺]_i and arachidonic acid release. *J. Immunol.* 142:1652.
 17. Pai, J.K., M.I. Siegel, R.W. Egan, and M.M. Billah. 1988. Activation of phospholipase D by chemotactic peptide in HL60 granulocytes. *Biochem. Biophys. Res. Commun.* 150:355.
 18. Balsinde, J., and F. Mollinedo. 1990. Phosphatidylinositol hydrolysis by human plasma phospholipase D. *FEBS (Fed. Eur. Biochem. Sci.) Lett.* 259:237.
 19. Balsinde, J., B. Fernández, and E. Diez. 1990. Regulation of arachidonic acid release in mouse peritoneal macrophages. The role of extracellular calcium and protein kinase C. *J. Immunol.* 144:4298.
 20. Billah, M.M., J.C. Anthes, and T.J. Mullmann. 1991. Receptor-coupled phospholipase D: regulation and functional significance. *Biochem. Soc. Trans.* 19:324.
 21. Billah, M.M., J.K. Pai, T.J. Mullmann, R.W. Egan, and M.I. Siegel. 1989. Regulation of phospholipase D in HL60 granulocytes. Activation by phorbol esters, diglyceride, and calcium ionophore via protein kinase C-independent mechanisms. *J. Biol. Chem.* 264:9069.
 22. Reinhold, S.L., S.M. Prescott, G.A. Zimmermann, and T.M. McIntyre. 1990. Activation of human neutrophil phospholipase D by three separable mechanisms. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:208.
 23. Mullmann, T.J., M.I. Siegel, R.W. Egan, and M.M. Billah. 1990. Phorbol 12-myristate, 13-acetate activation of phospholipase D in human neutrophils leads to the production of phosphatides and diglycerides. *Biochem. Biophys. Res. Commun.* 170:1197.
 24. Pai, J.K., M.I. Siegel, R.W. Egan, and M.M. Billah. 1988. Phospholipase D catalyzes phospholipid metabolism in chemotactic peptide-stimulated HL60 granulocytes. *J. Biol. Chem.* 263:12472.
 25. Halenda, S.P., and A.G. Rehm. 1990. Evidence for the calcium-dependent activation of phospholipase D in thrombin stimulated human erythroleukaemia cells. *Biochem. J.* 267:479.
 26. Huang, R., G.L. Kucera, and S.E. Rittenhouse. 1991. Elevated cytosolic Ca²⁺ activates phospholipase D in human platelets. *J. Biol. Chem.* 266:1652.21.
 27. Billah, M.M., S. Eckel, T.J. Mullmann, R.W. Egan, and M.I. Siegel. 1989. Phosphatidylcholine hydrolysis by phospholipase D determines phosphatidate and diglyceride levels in chemotactic peptide-stimulated human neutrophils. *J. Biol. Chem.* 264:17069.
 28. Mullmann, T.J., M.I. Siegel, R.W. Egan, and M.M. Billah. 1990. Complement C5a activation of phospholipase D in human neutrophils. A major route for the production of phosphatidates and diglycerides. *J. Immunol.* 144:1901.
 29. Gruchalla, R.S., T.T. Dinh, and D.A. Kennerly. 1990. An indirect pathway of receptor-mediated 1,2-diacylglycerol formation in mast cells. I. IgE receptor-mediated activation of phospholipase D. *J. Immunol.* 144:2334.
 30. Martinson, E.A., I. Trilivas, and J.H. Brown. 1990. Rapid protein kinase C-dependent activation of phospholipase D leads to delayed 1,2-diglyceride accumulation. *J. Biol. Chem.* 265:22282.
 31. Kanaho, Y., H. Kanoh, K. Saitoh, and Y. Nozawa. 1991. Phospholipase D activation by platelet-activating factor, leukotriene B₄ and formyl-methionyl-leucyl-phenylalanine in rabbit neutrophils. Phospholipase D activation is involved in enzyme release. *J. Immunol.* 146:3536.
 32. Anegón, I., M.C. Cuturi, G. Trinchieri, and B. Perussia. 1988. Interaction of Fc gamma receptor (CD16) ligands induces transcription of interleukin-2 receptor (CD25) and lymphokine genes and expression of their products in human natural killer cells. *J. Exp. Med.* 169:549.
 33. Bocckino, S.B., P.B. Wilson, and J.H. Exton. 1991. Phosphatidate-dependent protein phosphorylation. *Proc. Natl. Acad. Sci. USA.* 88:6210.

34. Koul, O., and G. Hauser. 1987. Modulation of rat brain cytosolic phosphatidate phosphohydrolase: effect of cationic amphiphilic drugs and divalent cations. *Arch. Biochem. Biophys.* 253:453.
35. Bourgoin, S., E. Plante, M. Gaudry, P.H. Naccache, P. Borgeat, and P.E. Poubelle. 1990. Involvement of a phospholipase D in the mechanism of action of granulocyte-macrophage colony-stimulating factor (GM-CSF): priming of human neutrophils in vitro with GM-CSF is associated with accumulation of phosphatidic acid and diradylglycerol. *J. Exp. Med.* 172:767.
36. English, D., G. Taylor, and J.G.N. García. 1991. Diacylglycerol generation in fluoride-treated neutrophils: involvement of phospholipase D. *Blood.* 77:2746.
37. Billah, M.M., and J.C. Anthes. 1990. The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* 269:281.
38. Balsinde, J., E. Diez, B. Fernández, and F. Mollinedo. 1989. Biochemical characterization of phospholipase D activity from human neutrophils. *Eur. J. Biochem.* 186:717.
39. Wang, P., J.C. Anthes, M.I. Siegel, R.W. Egan, and M.M. Billah. 1991. Existence of cytosolic phospholipase D. Identification and comparison with membrane-bound enzyme. *J. Biol. Chem.* 266:14877.
40. Agwu, D.E., C.E. McCall, and L.C. McPhail. 1991. Regulation of phospholipase D-induced hydrolysis of choline-containing phosphoglycerides by cyclic AMP in human neutrophils. *J. Immunol.* 146:3895.
41. Cockcroft, S. 1987. Polyphosphoinositide phosphodiesterase: regulation by a novel guanine nucleotide binding protein, G_p. *Trends Biochem. Sci.* 12:75.
42. Moolenaar, W.H., W. Kruijer, B.C. Tilly, I. Verlaan, A.J. Bierman, and S.W. de Laat. 1986. Growth factor-like action of phosphatidic acid. *Nature (Lond.)* 323:171.
43. Siegmann, D.B. 1987. Stimulation of quiescent 3T3 cells by phosphatidic acid-containing liposomes. *Biochem. Biophys. Res. Commun.* 145:228.
44. Yu, C.L., M.H. Tsai, and D.W. Stacey. 1988. Cellular *ras* activity and phospholipid metabolism. *Cell.* 52:63.
45. Knauss, T.C., F.E. Jaffer, and H.E. Abboud. 1990. Phosphatidic acid modulates DNA synthesis, phospholipase C, and platelet-derived growth factor mRNAs in cultured mesangial cells. Role of protein kinase C. *J. Biol. Chem.* 265:14457.
46. Cockcroft, S. 1984. Ca²⁺-dependent conversion of phosphatidylinositol to phosphatidic acid in neutrophils stimulated with fMetLeuPhe or ionophore A23187. *Biochim. Biophys. Acta.* 795:37.
47. Dennis, E.A., S.G. Rhee, M.M. Billah, and Y.A. Hannun. 1991. Role of phospholipases in generating lipid second messengers in signal transduction. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:2068.
48. Zanovello, P., A. Rosato, V. Bronte, V. Cerundolo, S. Treves, F. Di Virgilio, T. Pozzan, G. Biasi, and D. Collavo. 1989. Interactions of lymphokine-activated killer cells with susceptible targets does not induce second messenger generation and cytolytic granule exocytosis. *J. Exp. Med.* 170:665.
49. Richards, A.L., and J.Y. Djeu. 1990. Calcium-dependent natural killer and calcium-independent natural cytotoxic activities in an IL-2-dependent killer cell line. *J. Immunol.* 145:3144.