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SIGNAL TRANSDUCTION IN THE LYTIC ACTIVATION OF NATURAL KILLER CELLS

BY

© ERIC A. ATKINSON

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ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
bg/bg	beige mutant
BLTE	BLT esterase
Ca^{++}	calcium ion
cAMP	cyclic AMP
cGMP	cyclic GMP
C-HS	Chediak-Higashi syndrome
CTL	cytotoxic T lymphocyte
DAG	1,2-diacylglycerol
EM	electron microscopy
E:T	effector to target ratio
IP_3	inositol-1,4,5-trisphosphate
IP_2	inositol-1,4-bisphosphate
IP	inositol-1-phosphate
LGL	large granular lymphocyte
LPA	lysophosphatidic acid
mAb	monoclonal antibody
Mg^{++}	magnesium ion
MHC	major histocompatibility complex
NK	natural killer
NKCF	natural killer cytotoxic factor
PA	phosphatidic acid
Pi	inorganic phosphate
PI	phosphatidylinositol
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol myristate acetate
RNK	rat natural killer cell line
Sr^{++}	strontium ion
SRBC	sheep red blood cells
Ti	T cell antigen receptor complex
TLC	thin layer chromatography
TNF	tumor necrosis factor

SUMMARY

We have demonstrated the production of metabolites of the inositol phospholipid pathway in the rat natural killer (NK) cell line, RNK, upon exposure to susceptible tumor targets. These metabolites include the second messengers 1,2-diacylglycerol (DAG) and inositol-trisphosphate (IP_3), as well as catabolites of these messengers, phosphatidic acid, lysophosphatidic acid, and inositol-bisphosphate. Breakdown of the phospholipid parent molecules, phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-4-phosphate, was also demonstrated to occur within a similar time span after exposure to susceptible YAC-1 tumor targets. It was also shown that the amount of DAG produced by RNK cells upon exposure to different targets appeared to coincide with the target's degree of sensitivity to lysis by RNK.

The above data suggested that second messengers generated by the inositol phospholipid pathway may have a role in NK cytotoxicity. To investigate this possibility, we employed neomycin, an agent which interacts with the inositol phospholipids and thus inhibits their hydrolysis and the subsequent formation of second messengers. Neomycin was shown to dose-dependently inhibit killing of YAC-1 by two independently-derived RNK lines, as well as by a NK cell population contained within a preparation of normal rat nylon wool-nonadherant splenocytes. Neomycin also inhibited DAG formation in RNK cells exposed to YAC-1, supporting the concept that its inhibition of cytotoxicity could be mediated by inhibition of second messenger formation.

A role for DAG in NK cytotoxicity was also suggested by experiments which demonstrated that phorbol myristate acetate (PMA), a phorbol ester which functions like DAG in that it binds and activates protein kinase C (PKC), enhanced RNK-mediated lysis of YAC-1 in a dose-dependent manner.

We next examined the hypothesis that the effects of DAG and IP_3, namely PKC activation and raised cytosolic calcium, respectively, could be employed to mediate exocytosis of cytotoxic granules. These signals were provided to RNK cells in the absence of target cells by PMA, in the place of DAG, and by the calcium ionophore A23187, in the place of IP_3. The extent of degranulation was monitored by assaying for release of a serine esterase, BLTE, which is believed to be confined to the cytoplasmic granules of NK cells and cytotoxic T lymphocytes. A23187 and PMA were shown to act synergistically to promote release of BLTE. The secretion-enhancers cytochalasin B and heavy water could both enhance this release of BLTE, suggesting that it was indeed being released via a secretory event.

Taken together, these data lead us to propose that the insositol phospholipid pathway second messengers DAG and IP_3 are formed in NK cells upon recognition of a susceptible target cell, and a central function of these messengers is to mediate exocytosis of cytotoxic granules towards the bound targets.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

TABLE OF CONTENTS (continued)

INTRODUCTION

PART I: NATURAL KILLER CELLS

A. Discovery and Identification of Cell Type

1. Theory of Immune Surveillance

The theory of immune surveillance against tumors has long been popular with cellular immunologists. First proposed by Thomas (1), and then expanded upon by Burnet (2-4), this theory suggests that malignant cells arise in the body more frequently than the clinical manifestation of recognizable tumors, and it is a host immune response that eliminates the majority of these neoplastic cells. It was assumed that cytotoxic T lymphocytes (CTL) were the primary effectors of immune surveillance against tumors, but critical experiments aimed at elucidating the role of CTL in tumor elimination failed to demonstrate a need for these cells. Noteworthy among these experiments are those that showed that athymic "nude" mice, which lack the T cell compartment, did not appear to be more susceptible to naturally occuring tumors and could still effectively eliminate tumor cell innocula (5-6).

Due to these negative results, the immune surveillance theory lost some of its popularity. However, it was not long before a newly-identified cell type came to its rescue and replaced the CTL as the major potential effector in host immunity to neoplasms. This new cell was the natural killer (NK) cell.

2. Discovery of NK Activity

For many years during the course of studying cell-mediated cytotoxicity *in vitro*, investigators noticed a certain degree of background killing by lymphocytes of normal donors and animals that had apparently not been previously sensitized to the antigen being used (7-9). This variable background level of kill was often attributed to technical error or experimental artefact. Eventually, however, a number of laboratories began studying this phenomenon and it was suggested that the cells mediating this spontaneous or "natural" cytotoxicity, NK cells, might be important *in vivo* as effectors of tumor immunity (10-13).

3. Identification of NK Cells as Large Granular Lymphocytes

Although defining NK activity to a certain cell type on the basis of unique surface markers proved elusive, NK activity eventually became associated with a population of cells known as large granular lymphocytes (LGL). LGL characteristically have a high cytoplasmic:nuclear ratio and abundant and prominent azurophilic granules in their cytoplasm. The initial observations that this cell population contained NK activity were: a) LGL were enriched in a cell preparation obtained on the basis of NK target cell adherance (14-16); b) cytotoxic activity could be correlated with the numbers of LGL binding to target cells (15); and c) NK activity and LGL peaked in the same fractions of human peripheral blood lymphocytes separated by discontinuous density gradient centrifugation (17). It has since

become largely accepted that NK cells are indeed a subpopulation of LGL.

B. In Vivo Significance

1. Studies of Naturally Occuring NK Deficiences

There are a number of lines of evidence that support the hypothesis that NK cells are important in host defence against tumors. Among the most compelling are studies of patients and animals with NK deficiencies.

1.1 The Beige Mouse

The bg/bg mutation of beige mice results in giant cytoplasmic granule formation in many cell types (18). These animals have an impairment of NK activity at a postbinding stage, but were initially reported to have normal T cell and macrophage reactivity (19-21). Since these early reports, however, it has been demonstrated that this mutation does also affect T cell (22,23) and macrophage (24) activities. Therefore, these mice do not provide an absolute model of NK defeciency as the nude mouse does for T cell deficiency, since the defect is neither total nor selective. However, it has been suggested that they do represent the best available model for assessing NK activity in vivo despite these problems (25).

Beige mice have been shown to be less effective at rejecting transplanted NK-sensitive tumors compared to their normal litter mates (+/bg) (26). In a separate report, beige mice were also shown to

allow an increased growth rate, faster induction time, and increased metastases of an innoculation of a NK-sensitive tumor line compared to control (+/bg) mice. These differences were not found with an NK-resistant cell line (27).

In other experiments, however, beige mice have been shown to be as effecient as normal mice with a similar genetic background in eliminating some NK-sensitive tumors (28). It is possible that other immune surveillance mechanisms can at times compensate for those affected by the beige mutation, thus complicating results. It has been shown that beige mice appear to greatly expand the number of CTL in response to virus infection, compared to the numbers of CTL recruited by normal mice, and it has been suggested that this increase in numbers is done to compensate for their reduced lytic capacities (23).

1.2 The Chediak-Higashi Syndrome and Other Human Disorders

The Chediak-Higashi syndrome (C-HS) (reviewed in ref. 29) is considered to be the human homologue of beige mice (18). After Roder and his co-workers had identified the NK deficiency of beige mice, they went on to study the NK activity of patients with C-HS and found these individuals to also have an immune deficiency of NK and antibody-dependent cellular cytotoxicity (ADCC) activities (30-32). Other immune deficiencies which affect NK activity include X-linked lymphoproliferative disease, severe combined immunodeficiency disease, and ataxia talangiectasia. These diseases cause numerous other immune defects, but the only defect that they share is that affecting NK cells. Patients with these diseases, as well as those with C-HS,

all exhibit an unusually high incidence of malignancies which are generally lymphoid, rather than solid, in nature (reviewed in 33, 34).

2. Studies of Experimental Modulation of NK Activity

Other evidence that NK cells have a role in tumor elimination *in vivo* has been obtained from experiments in which the level of NK activity in experimental animals was selectively diminished or enhanced.

2.1 NK Reconstitution Experiments

In one report, C57BL/6 mice which were NK-deficient due to either the beige mutation or treatment with cyclophosphamide (a drug which markedly depresses NK activity) were shown to develop pulmonary tumor colonies 2-3 weeks after injection with melanoma cells. However, when the NK activity of these mice was reconstituted by an injection of histocompatible NK clones, there was a marked reduction of lung tumor colonies. Similarly, when C57BL/6 mice were subjected to split-dose irradiation, a treatment which causes a high incidence of thymic leukemia and a concommitant severe depression of NK activity, NK reconstitution with injections of NK clones conferred a significant protection to the development of this leukemia (35). A separate report demonstrated a similar reconstitution of NK activity to cyclophosphamide-treated mice by adoptive transfer of syngeneic cells, restoring normal resistance to experimental pulmonary metastases. Evidence that the effector cells responsible for this activity were NK cells was provided by the demonstration that they were sensitive to

anti-NK antibodies plus complement (36). Similar results were reported by another group who showed that the level of anti-tumor response correlated with the levels of NK activity of the donors used for NK-reconstitution of cyclophosphamide-treated mice (37).

2.2 Asialo GM1 and NK-1.1 Depletion Experiments

A number of other studies have shown that injection of antibodies directed towards asialo GM1, a glycolipid marker of murine and rat NK cells, produced a decrease in NK activity assessed *in vitro* and a parallel increase in tumor growth in nude mice (38,39) or clearance of [^{125}IUDR]-labelled tumor cells from the lungs of rats (40). Similarly, weekly injections of a monoclonal antibody to NK-1.1, an antigen present on almost all cells with NK activity in some mice strains, were demonstrated to deplete NK cells from the spleens of adult C57BL/6 mice for up to 8 weeks. Cellular and humoral immune functions and the distribution of other lymphocyte subsets in the spleen and were unaffected by this antibody treatment. These NK-depleted mice showed significant increases in the localization and growth of tumor cells in the lung after i.v. administration, and also reduced survival time (41).

3. Correlational Studies

Another approach to elucidating the *in vivo* relevance of NK cells has been to demonstrate a correlation between the normal level of NK activity of different strains of experimental animals and their ability to reject tumor cell inocula in either long-term studies that

measured tumor growth (42) or short-term studies that measured the clearance rate of [^{125}I]dUrd-labelled tumor cells (43). Although positive results using these approaches were obtained, other experiments have given conflicting results. For instance, it has not always been possible to correlate the degree of NK-sensitivity of different tumor lines with their *in vivo* clearance rates from various organs (43). It should be remembered, however, that other immune effectors may play a role in tumor elimination, and so firm correlations with NK activity or susceptibility may not always be demonstrable. These data, therefore, do not argue against a role for NK cells in tumor elimination, but they do emphasize the need for caution when one designs and interprets experiments of this kind. One must be cognizant of the other defense mechanisms that might be recruited by the host upon tumor challenge, and attempts to control for these complications should be made.

4. Oncogene Transfection Experiments

The fact that NK cells are present in high numbers in healthy individuals and do not have a lengthy activation requirement, coupled with the observation that differentiated cells often are less susceptible to NK lysis, has led to the suggestion that the NK cell's most important role in immune surveillance is played in the early course of the disease (44). The argument has been made that continuously cultured tumor lines may not truly represent recently transformed cells, and so their use as inocula to test NK reactivity *in vivo* may not really be appropriate (45). However, gene transfection

techniques have recently provided a means of evaluating the effects of transfected oncogenes early in the transformation process.

Roder's group has shown that mouse and rat fibroblast lines transformed with the ras oncogene are more susceptible to NK lysis than the untransformed parental lines, and by using an inducible promoter, they showed that this NK susceptibility is dependent upon ras expression (46,47). They later demonstrated that this enhanced lytic susceptibility was specific to lysis by activated NK cells, and did not reflect a general increase in sensitivity to lysis mediated by other effector systems as well as NK cells, including CTL, activated macrophages, and antibody plus complement (48). This is not the case with all oncogenes, however, since it has been shown that cells transformed with the human adenovirus possessing the E1A oncogene exhibit increased susceptibility not only to NK cells, but also to lysis by activated macrophages (49,50). E1A has also been shown to induce susceptibility to tumor necrosis factor (51).

Greenberg et al. have presented data that showed that the effect of H-ras transfection on NK lytic sensitivity depended upon the recipient fibroblast cell line, since ras-transfected 10T1/2 cells became more sensitive to NK cytolysis than the parental line, and this sensitivity strongly correlated with H-ras RNA levels, while ras-transfected NIH-3T3 cells were not altered in their NK susceptibility. The authors therefore suggested that the increase in NK susceptibility seen in ras-transfected fibroblasts is not actually a direct result of transformation, but may be dependent upon certain

cellular characteristics in the recipient cell line that are induced by the unregulated activity of the oncogene (52).

Greenberg and colleagues have also provided additional evidence that NK cells are most important during the early stages of metastatic spread. They showed that lung homing, implantation, and growth within the first 48 hours of i.v. inoculation of H-ras-transformed fibroblasts were strongly regulated by NK cells, but the sensitivity to NK lysis in vitro of the individual transformed lines could not predict the metastatic ability of these lines. Escape from immune attack and the subsequent growth of metastases in the lung were shown to correlate only with the level of H-ras gene expression (53).

5. Conclusion

In light of these and other lines of evidence, it has become quite clear and widely accepted that NK cells are indeed important effectors of immune surveillance. While some of the experimental evidence supporting a role for NK cells in tumor elimination may be open to alternate interpretations, the different approaches that have been employed are highly supportive of this proposal when considered together. Obviously, if a true NK deficient animal model which is both selective and absolute can be found, this will greatly facilitate studies of the in vivo significance of NK cells. Of course, even if such an animal is obtained, it is also possible that non-NK immune effectors may be expanded and/or activated to a greater than normal degree in these animals in order to compensate for the loss of NK activity. These problems will always exist, however, and simply point

to the need to employ a variety of different but complementary approaches when studying any specific hypothesis.

Although this review has focused solely on the proposed role for NK cells in immune surveillance of neoplastic cells, there is also some evidence that they may have other functions, including:

-recognition and elimination of virally-infected cells

-maintenance of hematopoietic homeostasis

-immunoregulation

(reviewed in 33 and 54).

C. NK Reactivity

There is evidence to suggest that NK-mediated cytolysis can be divided into discrete stages, including 1) recognition and binding, 2) activation or triggering and programming for lysis, 3) delivery of the lethal hit, and 4) killer cell-independent target cell death (55-57).

1. Recognition and Binding

1.1 Possible Dissociation of Binding from Recognition

NK binding to target cells is Mg^{++}-dependent and energy- and Ca^{++}- independent (56). One might hypothesize that NK recognition of and binding to a susceptible target cell were one and the same, with the NK receptor creating a bridge with the target structure, and this bridge holding the conjugate together. There are, however, indications that this by no means accounts for the entire binding process. While it was initially reported that murine NK cells bound to NK-sensitive targets and not NK-resistant ones (58,59), it was later

demonstrated that some NK-resistant cells also formed conjugates with NK cells (60-62). It is possible that the NK-resistant cells express a recognizable target structure and that resistance occurs at a point in the lytic cycle after the delivery of the lethal hit. Support for this proposal can be obtained from studies which showed that some NK-resistant targets become sensitive to NK lysis when exposed to metabolic inhibitors, suggesting that this treatment might interfere with protein synthesis-dependent repair processes (63). However, another possible explanation for NK cells binding targets but not lysing them is that binding and recognition and triggering are independent events, and that while some NK-resistant tumors may be able to bind NK cells, they do not have the appropriate membrane characteristics to initiate the NK lytic pathway. This proposal is supported by studies in which NK cells conjugated to normally NK-resistant targets were artificially triggered with Con A, resulting in lysis of these targets (64). Also, Targan and Newman (65) have reported that monoclonal antibody 13.1 interferes with NK cytotoxicity at a postbinding triggering stage before the Ca^{++}-dependent programming for lysis, giving further support for the proposal that binding and recognition are separate events.

Depending upon the target cell in question, either of these explanations are possible. In some cases, both may be true. That is, sub-optimal recognition and/or triggering may deliver a low dose of the lytic molecules to the target cell, which can then effectively repair any damage incurred.

It is also noteworthy to mention experiments which demonstrated that non-NK cells could bind more readily to NK-sensitive tumor cells than to NK-resistant tumor cells (66). It seems possible, therefore, that NK binding might, at least partially, employ a general adhesion system common to many different cell-cell interactions. Perhaps this general adhesion process provides the NK cell with extra time to recognize the target structures on a NK-sensitive tumor cell that will cause triggering of the lytic pathway.

1.2 Recognition Structures

The surface target structures which are recognized by the NK cell and activate the lytic pathway are presently unknown, as is the nature of the NK receptor. NK reactivity, unlike that of CTL, is MHC-unrestricted (67). In fact, there is some data that suggests that a lack of MHC molecules on the surfaces of tumor cells could make them more NK-sensitive (68), and it has been proposed that NK cells may recognize "no-self" rather than MHC "nonself" (69). Alternatively, however, a recent report has provided data that suggests the opposite might be the case in some cells: NK sensitivity of variants of the BL6 tumor line seemed to correspond directly, rather than inversely, with the level of MHC gene product expression (70). The involvement of MHC-encoded molecules in NK recognition and sensitivity is therefore somewhat controversial.

NK reactivity also differs from CTL reactivity in that NK cells do not express the T cell antigen receptor complex (Ti) (71). NK

cells also do not rearrange TiCβ (72) or Tγ genes (73) or express productive Tiβ-chain or Tiα-chain transcripts (74).

Although NK cells do not express the CD3/Ti complex necessary for normal T cell recognition and activation (75), they do express CD2, which has been shown to be used in an alternative activation pathway for T cells (76,77). It has been suggested that NK cells utilize CD2 in conjunction with other supporting and adherence receptors such as CD16 and/or CD11a/CD18 either as the major means of recognition and activation, or in association with an as yet undefined novel NK receptor (78). Support for a proposed role for CD2 in NK activation is provided in a recent paper which demonstrated that monoclonal antibodies directed towards this molecule resulted in increased conjugate formation and exocytosis of cytolytic granules (79).

Over the last decade, a number of potential target structures have been studied and suggested. Recent reviews have dealt with this controversial area, including references (80-82). Suffice it to say that to date there is no consensus as to what the NK cell recognizes on susceptible target cells.

2. Activation/Triggering and Programming for Lysis.

Once the NK cell has bound and recognized a susceptible target cell displaying the appropriate membrane characteristics, the lytic machinery must be turned on so the NK cell can deliver the lethal hit. That is, the external signal that a susceptible target cell has been bound must be internalized, or transduced across the plasma

membrane into the cell. By definition, this is the role of second messengers.

2.1 Minimal Requirements and Transmethylation of Membrane Phospholipids

It has long been known that this stage is Ca^{++}- and energy-dependent (56,83), but little data has been accumulated to shed light on the events occuring immediately before the Ca^{++} influx. Transmethylation of membrane phospholipids has been reported as an early event after NK-target cell binding (83-85). It was thought that this process could provide a mechanism for signal transduction across biological membranes (86), but this idea has since lost popularity. Although transmethylation of phospholipids is often an early event in stimulus-secretion systems, it is unlikely that it provides a direct mechanism for signal transduction. It can, however, result in altered membrane fluidity and receptor function - events which may have important consequences in cellular responsiveness (86).

2.2 Cyclic Nucleotides in Signal Transduction

The earliest described second messenger system was the cyclic AMP (cAMP) pathway. This second messenger is formed from ATP by the enzyme adenylate cyclase, which is activated via a GTP-binding protein when a stimulatory receptor on the cell is occupied by its specific ligand. cAMP exerts its effect by stimulating an A-kinase to phosphorylate and activate target enzymes (see refs. 87 and 88 for review).

Induction of cAMP in human and murine cells was found to inhibit NK cytotoxicity (89,90), suggesting that this second messenger system was not directly involved in positive signal transduction in response to binding susceptible targets. This inhibition was generally reported to occur at a post-binding stage, although at least one report suggested that increased cAMP levels decrease target cell binding (91). The finding that cyclic GMP (cGMP) modestly enhanced cytotoxicity led to the proposal that cAMP/cGMP ratios were important in NK signal transduction (89). This was further supported by the observation that raising cGMP levels could correct the depression of cytotoxicity of NK cells from Chediak-Higashi patients (92).

cGMP formation often occurs together with (i.e. immediately after) activation of another second messenger system, the inositol phospholipid pathway. This, together with the fact that cGMP is usually not connected to a cell surface receptor (see ref. 87), argues against a role for raised cGMP levels, in association with lowered cAMP levels, as the primary receptor signal in NK cells upon target cell binding. The inositol phospholipid pathway will be discussed in a later section, and reasons for suspecting that it is directly associated with receptor signalling in NK cells will be outlined.

3. The Lethal Hit

3.1 Evidence For A Stimulus-Secretion Model of NK Reactivity

Regardless of the nature of the recognition structures that mediate NK-target cell interactions and triggering, the NK cell somehow delivers a potentially fatal insult to its target. The nature

of this lethal hit is still somewhat controversial, and there are indications that the NK cell may employ a number of different mechanisms. At present, the most widely accepted theory of how the NK cell delivers the lethal hit to the target is based on a stimulus-secretion model, involving directed exocytosis of cytolytic molecules from cytoplasmic granules (see refs. 93,94 for review).

Early indications that secretion might be involved in NK-mediated cytolysis were observations that the carboxylic ionophore monensin, which inhibits secretory processes, totally inhibited NK activity at a postbinding stage (95,96). Also, massive degranulation of NK cells by Sr^{++} was shown to inhibit NK activity, and normal activity was regained only after granule regeneration (97,98). These findings, together with the observations that beige mice and Chediak-Higashi patients had defective NK cells and defective secretory processes in other cells, and also that elevated cAMP levels, known to often inhibit secretion, inhibited NK activity, strongly supported a stimulus-secretion mechanism of cytotoxicity.

3.2 Granule-Localized Cytolysin as a Major NK Cytotoxic Molecule

The facts that lysosome-type granules had been found to be a distinctive feature of NK cells (99-102), lysosomal enzyme inhibitors had been found to inhibit NK activity (103,104), and the previously mentioned data on the effects of monensin and Sr^{++}, all pointed to a secretory process being involved in NK cytotoxicity. Therefore, a crucial test of the stimulus-secretion hypothesis was to isolate the NK granules and test them for lytic potential. This was first

accomplished by Henkart's group (105). Using the rat large granular lymphocyte line RNK, they separated cell homogenates by percoll density gradient centrifugation and tested a number of different fractions for the ability to lyse SRBC. The fractions containing cytolytic activity were shown by electron microscopy (EM) to consist of pure granules. In a subsequent study, this group demonstrated that purified granules from RNK and rat peripheral blood LGL could also rapidly lyse SRBC and nucleated cells, whereas granules isolated from noncytotoxic cells could not (106). EM analysis of target cells exposed to RNK granules revealed the presence of numerous ring structures in the plasma membranes, with outside diameters of 15-28 nm and inside diameters of 5-17 nm. Similar ring structures had earlier been described in target cells attacked by NK and ADCC effectors (107, 108) and by CTL (109). It was proposed that the granule molecule responsible for these rings, termed cytolysin, acted similarly to the membrane attack complex of complement, forming transmembrane pores that caused cell death either by colloid-osmotic lysis, or by directly allowing leakage of macromolecules (106). Although granule-mediated lysis was found to be strictly Ca^{++}-dependent, in agreement with lysis caused by intact NK cells, preincubating the granules in Ca^{++}-containing medium resulted in a loss of cytolytic activity. It was suggested that nonactive cytolysin was activated by Ca^{++}, but was quickly degraded if it did not immediately insert into a membrane, similar to the complement system (106).

Cytolysin, also known as perforin or pore-forming protein, has recently been purified to apparent homogeneity. It appears to be a

70-75 Kd protein, and is likely identical in NK cells and CTL (110, 111). The purified protein shows the same calcium-dependent requirement for cytolytic activity as the whole granules, and is also inactivated by Ca^{++} pretreatment (111).

It is not known if membrane attack by cytolysin alone accounts for the entire lytic hit. NK and/or CTL granules contain other potentially cytotoxic molecules, including serine esterases (112-118), a cytolytic molecule antigenically related to lymphotoxin and tumor necrosis factor (TNF) (119), and possibly additional as yet unidentified toxic molecules.

One major difference between cell mediated cytotoxicity and lysis caused by complement attack is that the nucleus of target cells subjected to cytotoxic lymphocyte damage is rapidly broken down and the DNA is fragmented, whereas the complement attack has no such nuclear effects (see ref. 120 for review). It has been suggested that cytotoxic lymphocytes may somehow activate an endogenous endonuclease activity in the target cell, thereby triggering an autolytic or suicide pathway (120). Alternatively, however, it has been suggested that the pores formed by cytolysin/perforin may act as conduits, allowing entry into the cell of other granule-associated cytotoxic molecules, of which one or more may exhibit DNase activity (111). Henkart's group is presently investigating the possibility that a granule-localized serine esterase, BLT esterase, synergizes with cytolysin in causing target cell death, and recently reported experiments suggest that this may indeed occur (121). It is also interesting to speculate that the functional transmembrane pores

formed by cytolysin may act as unregulated calcium channels, activating Ca^{++}-dependent processes in the target cell, much like a Ca^{++} ionophore. Other granule molecules, or simply the effect of the bound effector cell, may direct this activation along an autolytic pathway.

3.3 NKCF As An Important NK-Derived Cytotoxic Molecule

The experiments of Wright and Bonavida have uncovered another NK-derived cytotoxic activity that is soluble and long-lived, which they have termed natural killer cytotoxic factor (NKCF). NKCF was initially generated by stimulating effector cells with lectin (PHA or Con A) in the lower chamber of a Marbrook culture vessel. Activity was assessed by trypan blue exclusion staining of NK-sensitive targets in the lower chamber, which was separated from the upper chamber by a 0.2 um nucleopore membrane. Significant lysis of the targets was achieved after incubation of the system for 30 to 40 hours at 37° C, and appeared to be specific for NK-sensitive, as opposed to NK-resistant, targets (122). Using tumor cell lines to stimulate effectors (human peripheral blood or murine spleen cells), NKCF was also released, as assessed in a similar Marbrook system (123,124). Both NK-sensitive and NK-resistant cell lines could stimulate NKCF release, but only NK-sensitive lines were killed by it (124).

A number of other experiments have supplied data in support of the hypothesis that NKCF is a major mediator of NK-mediated cytotoxicity. These include correlations of NK-mediated cytotoxicity with properties of NKCF, such as inhibition by similar sugars, species

specificity, interferon enhancement, bg/bg deficiency, etc. (reviewed in ref. 125).

A recent report suggests that NKCF activity may in part be mediated by TNF, since rTNF had similar target cell specificity to NKCF, and anti-TNF antibodies could inhibit some of the lytic activity attributed to NKCF (126). However, Ortaldo et al. (127) have raised a monoclonal antibody against NKCF which inhibits both rat and human NKCF activity, but does not affect the lytic activity of rat, mouse, or human TNF. Obviously much more information is required about NKCF at the molecular level before this intriguing activity can be fully understood.

A major problem in the hypothesis that NKCF is the major cytotoxic mediator of NK activity is its slow kinetics. NK-mediated cytotoxicity is usually detectable by 4 hours, while incubation periods in excess of 24 hours are often necessary to detect NKCF activity. In rebuttal to this criticism, Wright and Bonavida suggest that NKCF in cell-free supernatants may be greatly diluted, resulting in slow activity. Normal delivery to the target cell by the NK cell could likely achieve a much greater concentration in the target's microenvironment, leading to more efficient cytotoxicity. Also, target cell repair processes, the presence of cofactors, inhibitory or competitive molecules, and other factors, may be different in target cells conjugated with NK cells than they are in isolated targets (125). It is also conceivable that NKCF could enter the target cell via transmembrane pores produced by cytolysin.

3.4 Other Potential Mediators of NK Cytotoxicity

As mentioned earlier, there are a number of proteases in NK and CTL granules. Protease activity has long been implicated in NK reactivity, mainly on the basis of experiments showing NK inhibition by protease inhibitors (59,83,128,129). Whether or not the BLT esterase augmentation of cytolyisin-mediated kill reported by Henkart's lab (121) accounts for the entire effect of serine esterase activity in NK-mediated cytolysis is unknown. The fact that mast cell granules and granules from many other noncytolytic secretory cells also contain serine esterases suggests that these enzymes might be involved in secretory events, rather than directly involved in cytotoxicity per se.

Phospholipase A_2 activity has also been implicated in NK activity, since inhibitors of this enzyme have been shown to inhibit NK function (83,84,130,131). It has been suggested that the role of phospholipase A_2 in NK killing might lie in its ability to produce lysophosphatidylcholine, a detergent-like compound that might directly have a destructive effect on the target cell membrane (84). However, others have provided evidence that phospholipase A_2 involvement occurs in the early membrane activation events, rather than in the later lethal hit stage (83). Another product of phospholipase A_2 activity, free arachidonic acid, can be quickly metabolized along either the lipoxygenase or cyclooxygenase pathways, forming leukotrienes or prostaglandins, respectively. While prostaglandins, especially PGE_2, have been reported to inhibit NK activity (132,133), there is some evidence that products of the lipoxygenase pathway may be necessary

for maintaining NK activity (134). Therefore, another possible interpretation of the experiments showing NK inhibition with phospholipase A_2 inhibitors could be that they are inhibiting arachidonic acid formation, the appropriate metabolism of which may be important in modulating NK activity.

4. Killer Cell-Independent Target Cell Death.

By dissociating NK-target cell conjugates in dextran, Hiserodt et al. (56,57) have identified a rapid killer cell-independent cytolysis stage. This final stage in target cell lysis is also independent of divalent cations, but energy-dependent (56). It was believed that target destruction was a result of colloid-osmotic lysis, similar to that caused by complement-induced membrane lesions. However, this mechanism alone seems unlikely, since it cannot account for the DNA damage and morphological changes seen in targets lysed by cytotoxic lymphocytes, which are not seen with complement-mediated lysis (reviewed in 135). Although Ca^{++} may not be an absolute requirement for cytotoxic lymphocyte-mediated lysis, there is some evidence that raised internal Ca^{++}, caused by cytolysin/perphorin, complement or other pore-formers may contribute to target cell death (136-138).

PART II: SIGNAL TRANSDUCTION AND THE INOSITOL PHOSPHOLIPID CYCLE

A. Basic Concepts of Signal Transduction

The evolution of multicellular organisms by necessity involved the division of labour between a number of cell types. Each of these cell types has a specific task and often must respond to specific stimuli in a certain way. The orchestration of these responses is conducted by a complex and highly controlled system of messenger molecules such as hormones, growth factors, and neurotransmitters. Other stimuli may be antigens, cell-cell contacts, photons, etc. The surfaces of cells are studded with a variety of specific receptors for these external messages. When a receptor is occupied by its specific ligand, the cell is "triggered" and signalled to give an appropriate response, be it cell division, chemotaxis, pinocytosis, protein sythesis, secretion, or any one of a number of other responses.

The process whereby the external signal received by the cell surface receptor is internalized and a cellular response is intitiated has become known as "signal transduction" or "stimulus-response coupling". In essence, when a receptor is bound by its specific ligand, a conformational change is imparted to its transmembrane portion, often accompanied by phosphorylation of the receptor's cytoplasmic domain. This altered conformation and/or receptor phosphorylation in turn causes a conformational change in a G-protein, which acts as a signal transducer. G-proteins are so named because they become activated by binding GTP, which they quickly hydrolyze,

thereby deactivating themselves (see ref. 139 for review). In its activated state, a G protein in turn activates an internal effector molecule, usually an enzyme that exerts its functional activity at the cytoplasmic face of the membrane. This effector molecule acts as an amplifier of the transduced external signal by generating active internal messenger molecules from inactive precursors. These internal messenger molecules are called "second messengers", and their job is to travel to intracellular target sites and activate the cellular machinery necessary to produce an appropriate cellular response to the initial external signal (see Fig.1).

Amazingly few second messengers have been discovered, and it appears that a myriad of external stimuli act through the same second messenger pathways. As mentioned earlier, there are two major second messenger systems: adenylate cyclase/cAMP and the inositol phospholipid pathway. The latter generates at least two different second messenger molecules, 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). Ca^{++} has also been regarded as being an important second messenger, but as will be discussed, the discovery of IP_3 has effectively demoted Ca^{++} to "third messenger" status in many systems (140). cAMP has already been mentioned in this manuscript, and a full discussion of this second messenger is beyond the scope of this historical review, which will confine itself to a brief description of the inositol phospholipid pathway.

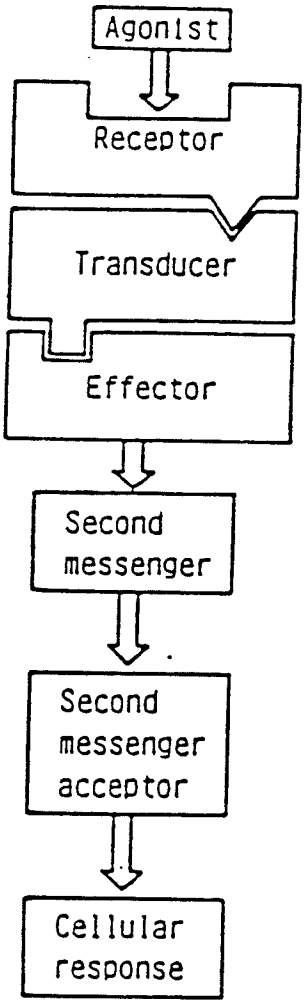

figure courtesy of Dr. A. McNicol

Figure 1. General mechanism of signal transduction.

See text for description.

B. The Inositol Phospholipid Pathway

1. Early History

1.1 The Beginnings

Although myoinositol was discovered in muscle as early as 1850, it was not until 1942 that its structure was established as 1,2,3,-4,5,6-hexahydroxycyclohexane, a fully hydroxylated 6-carbon ring which preferentially exists in a chair configuration (see 141). At approximately this time, it was recognized that inositol was a necessary component of a normal diet (see 142 for review). Later it was shown that inositol is often a requirement for *in vitro* growth of human cell lines (143).

1.2 Identification of Inositol As A Component of Membrane Phospholipids

In the 1930s and 1940s it was recognized that myoinositol was a component of the membrane lipids of prokaryotic, eukaryotic, and plant cells. In the 1950s and 1960s, phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP_2), were identified and characterized as being relatively minor membrane phospholipids, with PI normally being much more abundant than PIP or PIP_2 (144). Like other phospholipids, these 3 molecules have a sn-1,2-diacylglycerol backbone linked at the 3 position to a polar head group, in this case inositol in its phosphorylated or unphosphorylated forms. The 1 and 2 positions of the glycerol backbone have fatty acid, or acyl, sidechains that usually

consist of stearoyl and arachidonoyl, respectively (145). PI, PIP, and PIP_2 are rapidly metabolically interconverted via sequential phosphorylations and dephosphorylations, forming a "futile cycle" (see ref. 146). The kinases and phosphatases that catalyze these reactions have extremely high activity, so that these particular phospholipids label very quickly with ^{32}Pi, rapidly reaching equilibrium with the γ phosphate of ATP (144).

1.3 Identification of the "PI Response" and its Association with Secretion

Reviewers of the second messenger role of the inositol phospholipids generally point to the experiments of Hokin and Hokin in the 1950s and 1960s as being the first indications that these membrane lipids might have some role in cellular responsiveness (eg. 87,144, 147,148). These investigators demonstrated that ^{32}P incorporation into phospholipids of pancreas cells was rapidly increased upon stimulation with acetylcholine (149). They subsequently showed that this phosphate incorporation was mainly confined to PI and a phospholipid precursor molecule, phosphatidic acid (PA). Later, they showed that a variety of cell types exhibited this increase in phosphate labelling, and this "PI response" was often associated with stimuli that triggered cellular secretion (reviewed in 144). Unfortunately, the suggestion of a secretory link with the PI response was ahead of its time, and experiments of the day could not support it. Study of the phenomenon soon lost popularity, and little new biochemical work of any significance was done in this area for the next decade.

1.4 The PI Response as a Receptor-Mediated Event Not Involving cAMP

To workers remaining in this field, it eventually became clear that the PI response was a receptor-mediated event, and the receptors involved were those that did not appear to signal the cell interior via the well known cAMP second messenger pathway. These were receptors that were independently recognized as evoking an increase in either intracellular Ca^{++} concentration, cGMP levels, or both, but which caused a variety of cellular reponses in addition to secretion, including cell proliferation and phototransduction (see 144). Soon after, it became clear that the lipid labelling of the PI response was actually a secondary event which follows a receptor-mediated activation of an inositol phospholipid-specific phospholipase C (PLC) and a resultant net decrease of total cellular PI (150). This was an interesting finding, since it had earlier been found that while most PLC enzymes, which hydrolyse the glycerol-phosphate bond of intact phospholipids, generally displayed a low level of activity, those with specificity for the phosphoinositides (phosphoinositidases C) had unusually high activity (151,152). It also appeared that this extremely rapid decrease in PI was in some way associated with the increase in intracellular Ca^{++}, which was then followed by changes in cGMP levels (153,154).

1.5 The PI Breakdown/Ca^{++}-Mobilization Hypothesis

At this time it was known that phosphoinositidases C were Ca^{++}-dependent enzymes, so the initial assumption was that inositol lipid hydrolysis mediated by these enzymes, like cGMP production, was

a secondary event brought about by increases in intracellular Ca^{++}. However, it had earlier been shown that the classical PI response (lipid labelling) occured even in Ca^{++}-free conditions (155-157). This was soon confirmed to also be the case with receptor-activated depletion of PI (158,159). There were two possible explanations for these data. Either inositol phospholipid hydrolysis and intracellular Ca^{++} increases were separate but parallel events caused by activation of the same family of receptors, or inositol lipid hydrolysis occured before Ca^{++} mobilization and might in fact be essential for it (144). Michell opted for the potentially more exciting second model, thus formulating the "PI breakdown/Ca^{++} mobilization hypothesis" (154).

1.6 Requirement of Inositol For Ca^{++}-Mediated Cellular Responses

The first direct evidence that inositol was indeed important in Ca^{++} mobilization came from experiments by Fain and Berridge (160). Fluid secretion by blowfly salivary gland tissue, a Ca^{++}-dependent cellular response associated with PI breakdown, was blocked by prolonged exposure of the tissue to a physiological agonist in inositol-free medium. This cellular unresponsiveness could be reversed by adding inositol back to the medium. They also showed that Ca^{++}-independent responses were not affected by these inositol deprivation conditions. Despite this demonstration, the controversy of the temporal relationship of phospholipid breakdown and Ca^{++} mobilization remained unresolved for a number of years.

2. The Last Decade: A New Era in Understanding

Two discoveries occured in the late 1970s-early 1980s that yielded the missing links necessary to form a convincing model of cellular responsiveness mediated by inositol phospholipid breakdown.

2.1 Identification of DAG as a Second Messenger

One of these key discoveries was that 1,2-diacylglycerol (DAG), a product of phosphoinositide hydrolysis by phosphoinositidase C, was a second messenger, necessary for activating an ubiquitous enzyme, protein kinase C (PKC) (161). This enzyme had been characterized as being Ca^{++}- and phospholipid-dependent. DAG was shown to act by increasing its affinity for Ca^{++} approximately 1000 fold, allowing it to function at resting cytosolic Ca^{++} concentrations (161). It was further realized that the stimulated cells that seemed to utilize DAG-activated PKC also employed increased cytoplasmic Ca^{++} as another second messenger. Using suboptimal amounts of a calcium ionophore (to raise intracellular Ca^{++} levels), and a synthetic diacylglycerol (to activate PKC), it was further shown in a model system involving platelet secretion that these agents separately were not very effective, but together they could synergize to cause significant secretion (162).

2.2 Identification of IP_3 as a Second Messenger

The other important discovery was that it was PIP_2, not PI, that was the inositol phospholipid initially hydrolysed upon receptor activation (163,164). This meant that phosphoinositidase activity

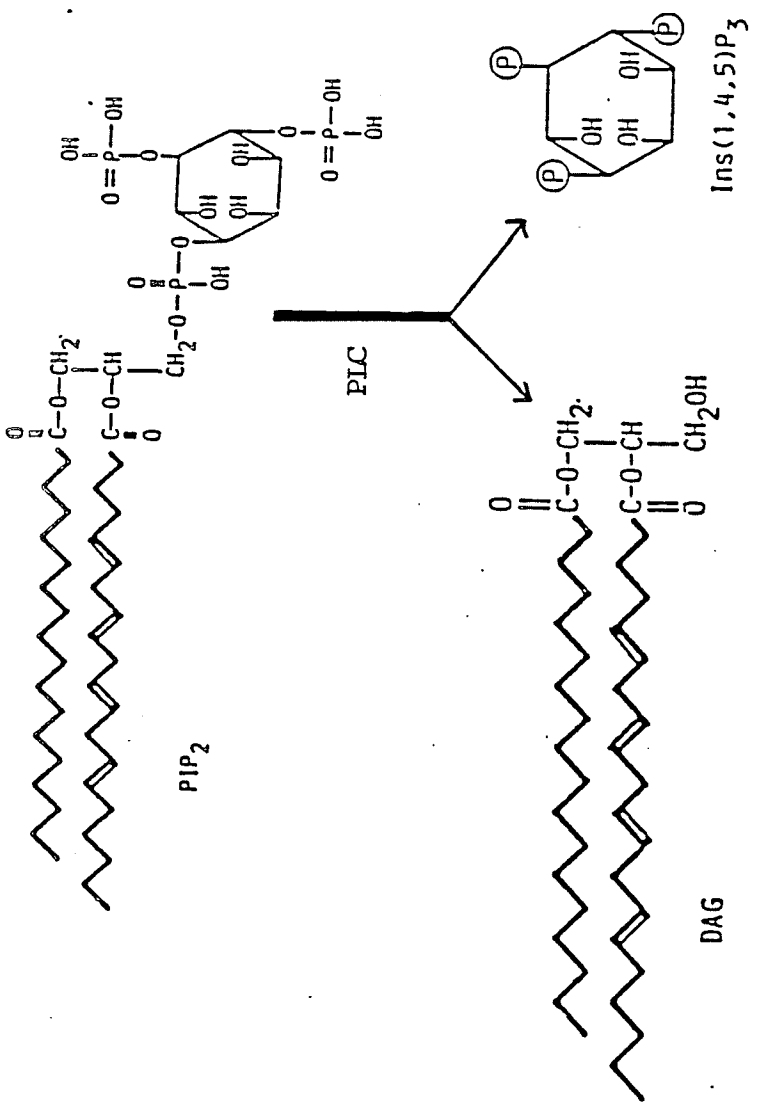

Figure 2. Hydrolysis of PIP_2 by phospholipase C yields DAG and IP_3.

would produce DAG and inositol-1,4,5-trisphosphate (IP_3) (see Fig. 2). IP_3 was soon found in several stimulated tissues that PI breakdown had been demonstrated in (165,166). Since IP_3 was shown to be rapidly produced and rapidly degraded in stimulated cells, it was proposed that this molecule might have some important biological function (167). In 1983, Berridge and colleagues proposed that IP_3 might act to mobilize Ca^{++} (165), and soon extensive data was accumulated that suggested that this might be the case (reviewed in ref. 147). IP_3 was demonstrated to release Ca^{++} from permeabilized pancreatic acinar cells from a nonmitochondrial intracellular store, giving credence to Berridge's proposal (168). This observation was soon extended to include a wide variety of other cell types (see ref. 147 for review).

2.3 Ca^{++} and PKC in Granule Movement and Fusion

Clues to how Ca^{++}-mediated events synergize with PKC-mediated events to promote secretion have come from studies in the platelet model system. By experimentally activating the two different arms of this pathway separately - using Ca^{++} ionophores and PKC-activating phorbol esters or synthetic diglycerides - the relative contributions of each have been studied. It appears that raised internal Ca^{++} may be responsible for granule redistribution, while PKC activity may be important in granule membrane fusion (reviewed in refs. 169,170).

3. Conclusion

The action of a receptor-linked PLC enzyme upon a membrane phospholipid, PIP_2, simultaneously forms two molecules, each with important second messenger functions. IP_3 causes an increase in intracellular Ca^{++} levels, and DAG activates PKC. Moreover, as Nishizuka and his colleagues have shown, the effects of these two signals are synergistic in producing a cellular response (162). In the 5 years since this model was proposed, it has gained wide acceptance and has been verified in countless numbers of cell types. A number of recent reviews in this area (eg. 87,147,148), and especially an excellent new book that details the involvement of this second messenger pathway in a variety of systems (171), have been published. Today, this second messenger pathway continues to be an active area of research, and new significant findings are constantly occuring. One recent discovery is that IP_4, a metabolite of IP_3, may be a signal for external Ca^{++} to enter the cell (see 172), sustaining and amplifying the internal Ca^{++} elevation elicited by IP_3 mobilzation of internal stores of Ca^{++}. As Figure 3 shows, a number of additional metabolites of this cycle have been identified, including cyclic forms of phosphoinositides. Further work will no doubt increase our understanding of the intricacies of this pathway.

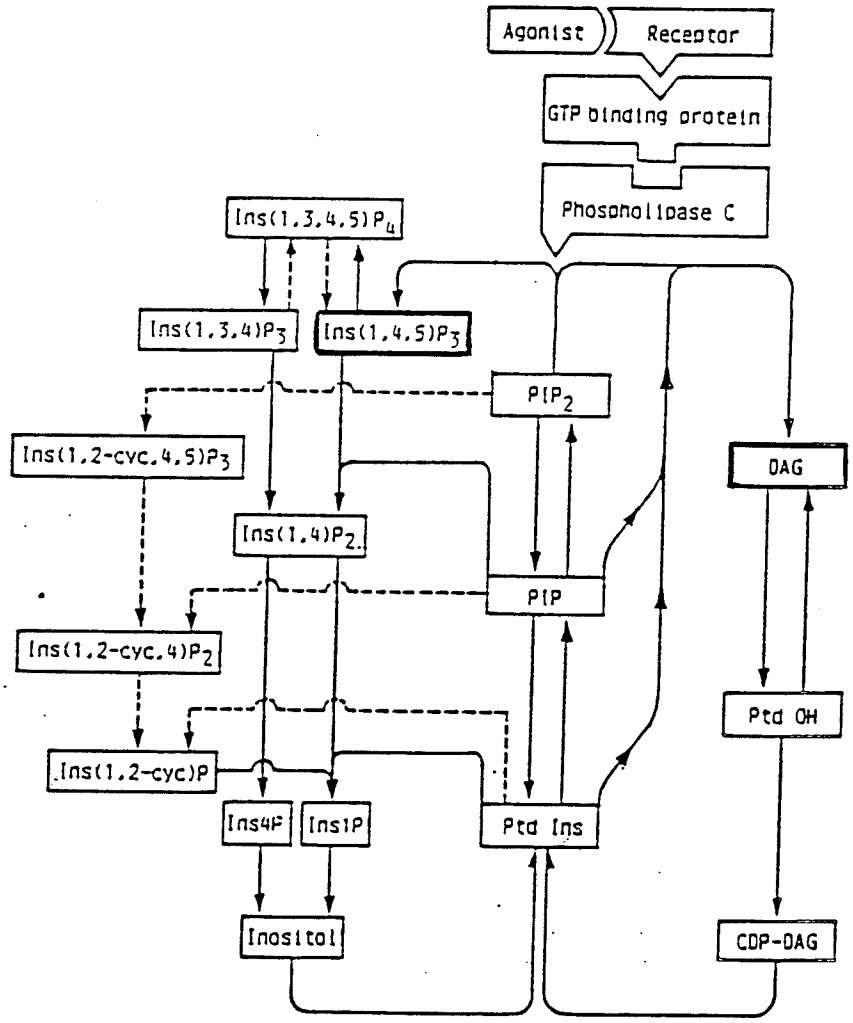

figure courtesy of Dr. A. McNicol

Figure 3. The inositol phospholipid pathway as it is known today. As yet only DAG and Ins(1,4,5)P_3 (IP_3) are accepted as having second messenger functions, although Ins(1,3,4,5)P_4 is strongly suspected to have a role in mediating external calcium influx.
As can be seen in this figure, a number of cyclic inositol phosphates with unknown function are also formed.

RATIONALE OF PROJECT

This project was undertaken to examine the hypothesis that NK lytic activation might involve phosphoinositide breakdown, and the resultant second messengers, IP_3 and DAG, might somehow be involved in the lytic mechanism. The basis for this hypothesis are simple and clear. NK lysis of tumor cells best fits a stimulus-secretion model (93,94) and is Ca^{++}-dependent (56). Increases in cAMP were shown not to be directly involved in a positive manner in signalling the NK cell to lyse a bound target cell, though cGMP levels were so implicated (89). As discussed earlier, many cellular responses, including secretion, that are cAMP-independent, but Ca^{++}-dependent and associated with increases in cGMP have recently been shown to involve PIP_2 breakdown and the actions of IP_3 and DAG. It was therefore reasonable to suspect that this newly-identified second messenger system might be involved with NK lytic activation, degranulation, and lytic activity.

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CHAPTER I.

FORMATION OF INOSITOL PHOSPHOLIPID METABOLITES IN A RAT NK LINE EXPOSED TO SUSCEPTIBLE TARGETS

ABSTRACT

It is currently believed that natural killer (NK) cells kill bound target cells by exocytosis of cytotoxic granules via a calcium-dependent process. After confirming that NK-mediated killing was indeed dependent upon extracellular calcium, we investigated the production of inositol phospholipid-derived second messengers in a rat NK cell line, RNK, upon exposure to susceptible target cells. These messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG), are associated with calciumdependent secretory processes in a number of cell types. When RNK cells were exposed to susceptible YAC-1 tumor targets, significant amounts of both IP_3 and DAG were produced. Levels of the membrane phospholipid parent molecules of these second messengers declined in similarly stimulated RNK cells over a comparable time period. Using three different target cell lines, it was found that the levels of DAG that RNK produced in response to the different targets followed the same rank order as their susceptibility to RNK-mediated lysis. These data suggest that IP_3 and DAG are produced in NK cells in response to tumor target cells, and these second messengers may have a functional role in NK-mediated killing.

INTRODUCTION

Natural killer (NK) cells are a heterogeneous population of large granular lymphocytes (LGL) that are capable of lysing a variety of tumor cells and some normal cells without prior sensitization (1). The granules of NK cells contain a number of potentially cytotoxic molecules, including one termed cytolysin which is similar to C9 of the complement membrane attack complex (2-5). According to the stimulus-secretion model of NK cytotoxicity, when an NK cell binds a susceptible target cell, the cytotoxic granules move towards the point of contact and exocytose their contents into the space between the two cells (6,7). Molecules of cytolysin then polymerize within the target cell membrane, forming pores which can disrupt the ionic and osmotic balance of the target cell, contributing to cell lysis. Though NK-mediated killing is believed to be energy- and calcium-dependent (8), very little is known about the exact biochemical requirements for activation of the lytic process.

Many other cell types with calcium-dependent secretory processes initially respond to external stimuli by rapidly hydrolysing the membrane phospholipid phosphatidylinositol-4,5-bisphosphate (PIP_2), which is formed by phosphorylation of phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol (PI). Hydrolysis of PIP_2 by phospholipase C yields inositol-1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), products which are potent synergistic second messengers. IP_3 acts to mobilize intracellular calcium and DAG activates the membrane-associated enzyme protein kinase C (PKC) (reviewed in 9). IP_3 is rapidly converted to inositol bisphosphate (IP_2) and inositol

monophosphate (IP), and DAG may be further metabolized to phosphatidic acid (PA) and lysophosphatidic acid (LPA).

The purpose of the present study was twofold: First, to verify that NK killing is strictly dependent upon external calcium, since a number of recent reports have cast doubt upon this requirement in some cytotoxic T lymphocyte systems (10-12). Second, to examine whether NK killing is associated with the formation of second messengers formed from the inositol phospholipids.

MATERIALS AND METHODS

Effector Cells

1. RNK Cells

RNK-O and RNK-16, two independently-derived rat large granular lymphocyte cell lines with functional NK characteristics (13,14), were obtained from Dr. C. Reynolds (NCI, Frederick, MD) and were maintained by serial passage in the peritoneal cavity of young Fischer rats. These rats were injected intraperitonealy with 1 ml 2,6,10,14 tetramethylpentadecane (Aldrich Chemical Co., Milwaukee, Wis.) 4-10 days prior to injecting $2x10^7$ RNK cells, which were then allowed to grow for approximately 14 days. They were harvested in RPMI-1640 (Gibco, Grand Island, NY) containing 100 units heparin (Sigma Chemical Co., St. Louis, MO) per ml, washed twice by centrifugation and then resuspended in RPMI containing 10% heat-inactivated fetal calf serum (Gibco) and 15 mM Hepes (Sigma) (RPMI-FCS-Hepes). Cells were not used unless >90% viable.

2. Nylon Wool-Nonadherant Splenocytes

Normal rat spleen cells obtained from 250g male Fischer rats

injected intraperitoneally 18 hours previously with 1 mg polyinosinic-polycytidylic acid (poly I:C) (Sigma) or saline, were passaged through a nylon wool column as described (15). Essentially, the spleens were removed and disaggragated by gently pushing them through a fine-mesh nylon screen stretched above a Petri dish containing RPMI or Hanks solution. The collected cells were then washed once by centrifugation, and the RBCs were lysed by adding approximately 2 ml of a 0.83% NH_4Cl solution to the pellet and incubating at 37° C for 4 minutes. The cells were then washed two more times, resuspended to approximately 1 ml in RPMI-FCS, and applied to a nylon wool column that had been previously prepared as follows:

Approximately 1 gram of nylon wool per 10 ml column volume was used. The nylon wool was gently teased under water to remove air bubbles and achieve a uniform consistency throughout. Still under water, it was then stuffed into a column (3 grams into a 30 ml syringe) such that it was a uniform density from top to bottom. After removing the column from the water, the water in the nylon wool was displaced with Hanks. The column was then kept in a 37°C incubator until the splenocytes were ready, at which time the column was removed from the incubator and the Hanks was displaced with 37°C RPMI-FCS.

The cells were then added to the top of the column and allowed to migrate approximately half the way down by opening up the bottom stop cock. RPMI-FCS was added to the top to replace the medium lost during this process. The column with the cells in it was then incubated at 37°C for 45 minutes to 1 hour. After this incubation, the nylon wool-nonadherant cells were collected by slowly flushing the column with more than two volumes worth of 37°C RPMI-FCS and collecting the

eluant. After centrifugation, the collected cells were resuspended in RPMI-FCS and counted.

Target Cells

YAC-1 lymphoma cells, P815 mastocytoma cells, and SL2-5 lymphoma cells were grown in RPMI-1640 supplemented with 10% FCS and were used in RPMI-FCS-Hepes for lytic assays. For studies of RNK lipid metabolism, the target cells were first lightly fixed in 0.52% glutaraldehyde (Fisher Scientific Co., Fairlawn, NJ) at 37°C for 30 minutes to ensure that they were not metabolically active and could not be implicated in the lipid changes seen. The fixed tumor cells were washed, incubated for 30 minutes in RPMI-FCS, then washed twice in RPMI-FCS and resuspended in RPMI-FCSHepes before use.

Labelling of RNK Cells

The RNK cells were labelled at 37°C for 24 hours with 0.025 uCi/ml ^{3}H-arachidonic acid for studies of DAG production. Other cells were labelled for 2 hours with 80 uCi/ml ^{3}H-inositol for evaluation of inositol phosphates or phosphoinositides (New England Nuclear, Dupont Canada, Lachine, Quebec). The labelled cells to be used for studies of production of inositol phosphates were washed and incubated another hour in the presence of 10mM LiCl (Fisher), while the labelled cells to be used for evaluation of phosphoinositides were washed and incubated a further 30 minutes in the presence of 5 mM unlabelled inositol. All labelled cells were then washed twice in buffered medium and resuspended in RPMI-FCS-Hepes before use.

Measurement of Diacylglycerol, Inositol Phosphates, and Phosphoinositides

For assays of stimulated RNK cells, 10^7 labelled cells in 0.25 ml were mixed with $3x10^7$ fixed YAC-1 cells in an equal volume (effector to target ratio, 1:3) and centrifuged at 60 xg for 30 seconds to promote cell-cell contact. Timing started the instant the centrifuge reached its maximum RPMs. At the indicated time points, the reaction was stopped by adding and vigorously mixing ethyl acetate (for the DAG assay) or chloroform:methanol:HCl 100:200:2 (for the phosphoinositides and the inositol phosphates) (Fisher). For assay of unstimulated RNK cell samples, target cells were not added, but an equal volume of medium was used. Alternatively, immediately after the addition of the appropriate stopping reagent and mixing, fixed target cells were added to each unstimulated sample tube to equilibrate them with the stimulated samples.

Extraction of DAG (16), inositol phosphates (17), and phosphoinositides (18) was then carried out as follows.

DAG:

Briefly, DAG was extracted by 4 additions of 3 ml EtAc each. After each addition, the tubes were violently vortexed and often exposed to prolonged agitation on a shaker or nutator in order to promote phase mixing. The tubes were then centrifuged at 1000-3000 RPM for 10 minutes and the organic phase was removed, combined, and saved. After the first two extractions, the aqueous phase containing the cell pellet was acidified by adding 20 ul of 1N HCl and lightly mixed before the next two extractions were performed. The combined

organic phases (total of 12 ml each sample) were then evaporated to dryness under N_2 and resuspended in 35 ul EtAc. Often, samples were left overnight at this point. DAG production was assessed by carefully spotting the samples (35 ul each) onto Baker-H Si250 thin layer chromatography (TLC) plates that had been heat activated in a 110°C oven for 1 hour (12 samples per plate). Spotting was done with a 50 ul Hamilton syringe in a spotting chamber under N_2. The solvent system used to separate DAG from the other extracted lipids was benzene:diethylether:ethanol:acetic acid 50:40:2:0.2 (19). This solvent system effectively separates the 1,3-diacylglycerol from the 1,2-diacylglycerol isomers, as verified by high quality standards (Serdary Research Labs., London, Ontario). The appropriate bands were visualized by comparison with standards either using iodine vapor, UV light, or autoradiography. Samples were quantitated on a Bioscan System 200 imaging scanner and/or by scraping the appropriate bands, extracting them in methanol and counting in PCS/xylene using a Beckman scintillation counter.

Phosphoinositides:

For the phosphoinositides, 1.8 ml chloroform:methanol:HCl (100:200:2) was mixed with each 0.5 ml sample and left overnight. Then an additional 0.6 ml chloroform and 0.6 ml 2M KCl were added to separate the phases. The organic layer was then removed, evaporated under N_2, resuspended in 40 ul chloroform:methanol (1:2) and the phosphoinositides separated by TLC using heat-activated Baker-H Si250 plates impregnated with 1% potassium oxalate and 2 mM EDTA, and run with chloroform:ethanol:4N NH_4OH (35:45:10).

Inositol Phosphates:

Inositol phosphates were extracted as for phosphoinositides, with the exception of 0.6 ml H_2O replacing the 0.6 ml 2M KCl. The inositol phosphates were separated from the aqueous phase by ion exchange chromatography using Dowex-formate columns. These columns were made by stuffing the bottoms of the bodies of 9 inch Pasteur pipettes with glass wool and filling them with Dowex-formate. 5 ml syringes were then attached to the tops of each column in order to be able to add the samples and solvents to them. The aqueous phases of each sample were added to different column/syringe assemblies, immediately followed by 6 ml H_2O. The eluate was collected and saved. ^{3}H-inositol was eluted with 3 additions of 5 ml each of 0.06 M ammonium formate / 5mM disodium tetraborate. ^{3}H-IP was eluted with 4 additions of 5 ml each of a solution of 0.2 M ammonium formate / 0.1 M formic acid. ^{3}H-IP_2 was eluted with 4 additions of a solution of 0.4 M ammonium formate / 0.1 M formic acid. ^{3}H-IP_3 was eluted with 4 additions of 3 ml each of a solution of 1.0 M ammonium formate / 0.1 M. formic acid. All fractions were collected and counted in PCS scintillation fluid (Amersham Corp., Arlington Heights, Il.) on a Beckman scintillation counter.

NK Lytic Assays

This ^{51}Cr-release assay has been described in detail elsewhere (20). Briefly, a cell pellet containing approximately 10^7 target cells was labelled with about 145 uCi ^{51}Cr for 45 minutes at 37° C, washed once by adding about 10 ml HBSS and centrifuging for 8-10

minutes at 1000 RPM, resuspending in RPMI-FCS and incubating a further 30 minutes at 37°C. After this, the cells were centrifuged and washed 2 or 3 times with ice-cold HBSS, resuspended in RPMI-HIFCS-HEPES, counted, and the concentration of cells adjusted appropriately. 10^4 target cells were usually added to V-bottom microplate wells in 80 ul after doubling dilutions of effector cells had been added in a similar volume. E:T ratios usually started at 100:1 or 80:1.

When EGTA was used, it was added to the wells after both effectors and targets had been added, to achieve the final concentration reported. Each lytic assay was run in triplicate. Assay time was 4-5 hours when nylon wool-nonadherant splenocytes were used, and 18 hours when RNK cells were used.

The per cent specific lysis (% SL) was calculated as follows:

$$\% \text{ SL} = \frac{\text{(experimental cpm - spontaneous cpm)}}{\text{(total cmp - spontaneous cpm)}} \times 100.$$

One lytic unit is the number of effector cells necessary to achieve 30% SL.

RESULTS

Normal rat nylon wool-nonadherant splenocytes, a population of cells containing NK activity, effectively lyse ^{51}Cr-labelled YAC-1 cells when the lytic assay is performed in buffered RPMI containing 10% heat-inactivated FCS. However, sequestering external calcium with EGTA abolished killing of YAC-1 cells by the splenocytes, indicating that calcium is strictly required in this system (Fig. 1). EGTA did not appear to be toxic to the splenocytes in the conditions used in the assay, nor did it significantly increase the background ^{51}Cr

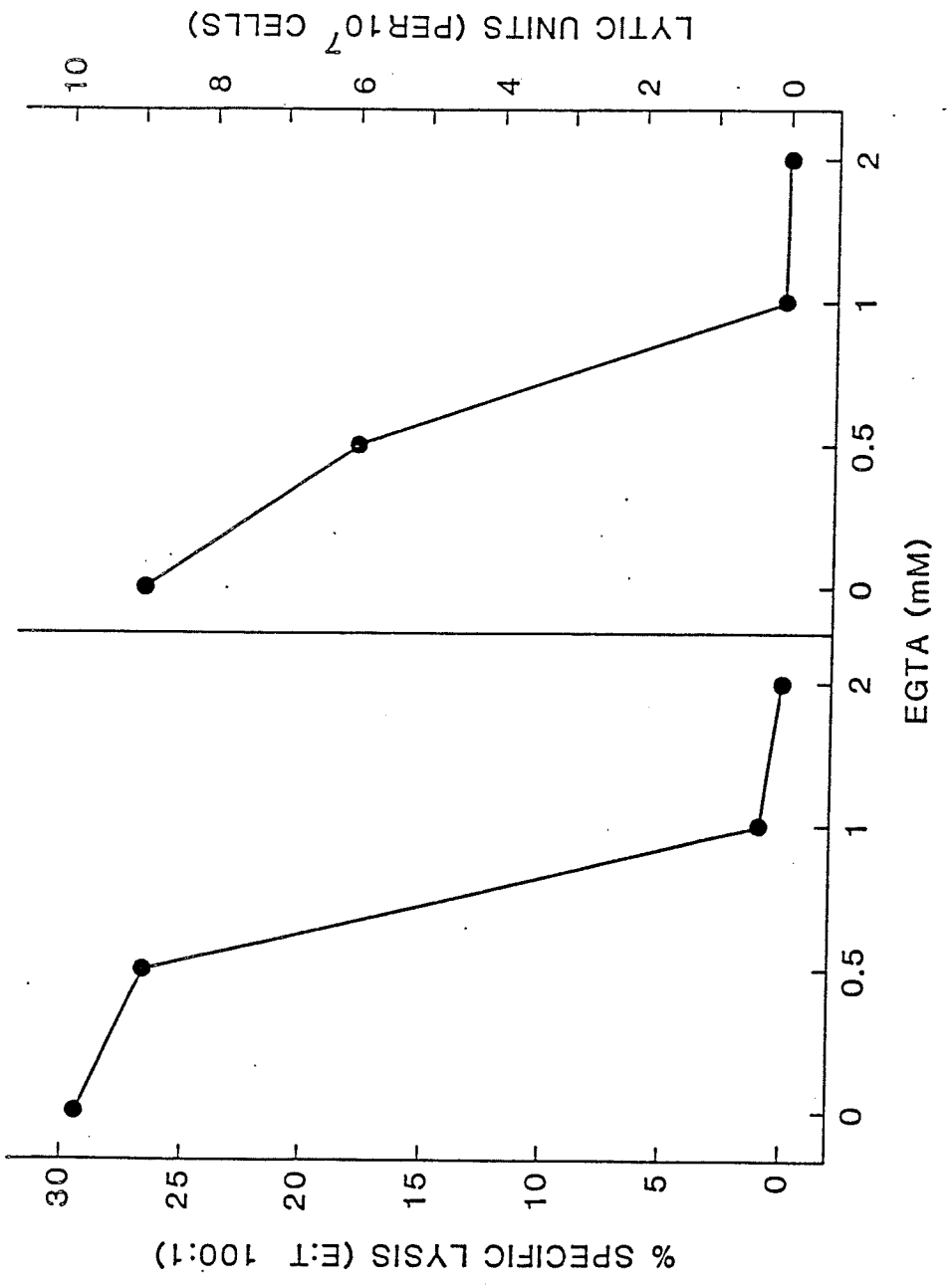

Figure 1: The effect of EGTA on the ability of normal rat nylon woolnonadherant splenocytes to lyse ^{51}Cr-labelled YAC-1 cells. All points are the means of triplicates.

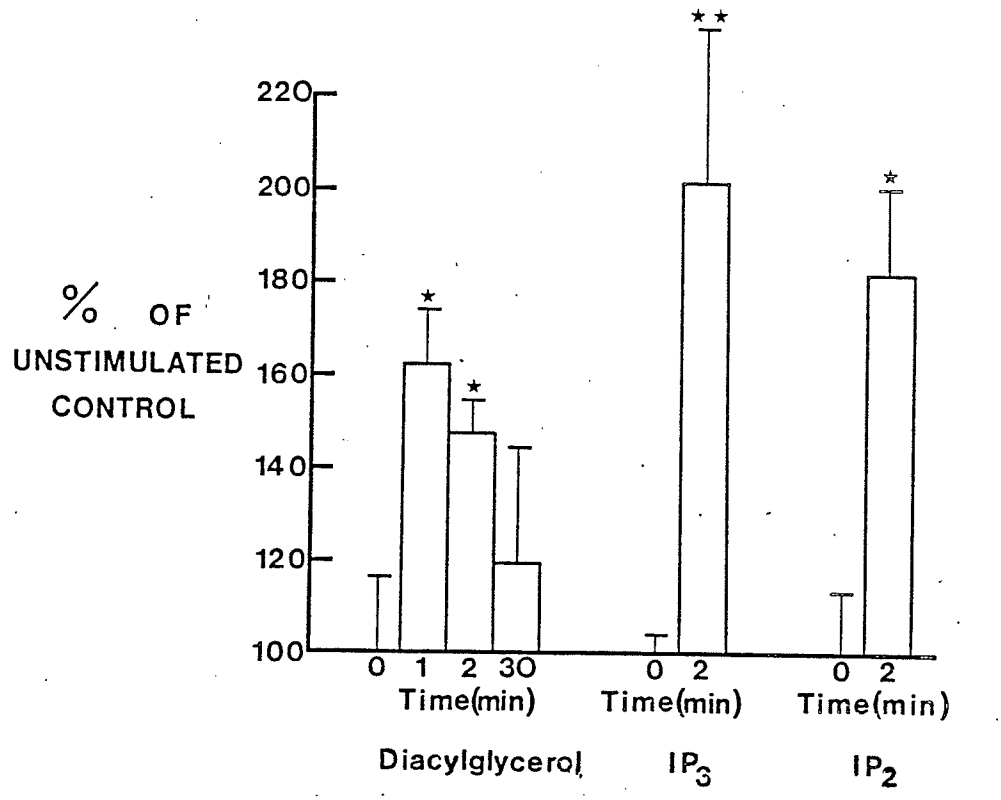

Figure 2: Production of inositol cycle metabolites by RNK-16 cells exposed to YAC-1 cells, compared to unstimulated controls. Label is ^{3}H-inositol for IP_3 and IP_2, and ^{3}H-arachidonic acid for DAG. The data represented are typical of a number of experiments using either RNK-16 or RNK-0.
* indicates $p < 0.005$. ** indicates $p < 0.01$, (T test).

release from the labelled YAC-1 cells (not shown). Killing of YAC-1 by the rat NK cell line RNK also appeared to be abolished by a similar concentration range of EGTA, although the labelled YAC-1 cells leaked some ^{51}Cr in the presence of the highest concentrations of EGTA during the course of this longer assay period (not shown).

When appropriately labelled RNK cells were exposed to unlabelled glutaraldehyde-fixed YAC-1 tumor targets, they responded by generating inositol cycle metabolites (Fig. 2). These metabolites included the second messengers ^{3}H-DAG and ^{3}H-IP_3 as well as the IP_3 catabolite ^{3}H-IP_2. These results were obtained using two separate independently--derived RNK lines, RNK-0 and RNK-16. Production of PA and LPA in RNK-0 cells exposed to YAC-1 was also observed (Fig. 3).

Within a time span consistent with the rise in IP_3 and DAG, we observed a decrease in PIP_2 and PIP in ^{32}P-labelled RNK cells exposed to fixed YAC-1 cells (Fig. 4). PI was unchanged at 5 minutes, but compared to unstimulated control cells, those exposed to tumor cells did show a slight decrease by 15 minutes.

To assess if the production of these second messengers by RNK cells was related to the tumor target's susceptibility to lysis, we tested a number of cell lines in lytic assays with RNK-0 cells. YAC-1 was found to be highly sensitive to RNK-0-mediated lysis, P815 was moderately sensitive, and SL2-5 appeared to be relatively insensitive (Table 1). When these 3 target lines were used to stimulate appropriately-labelled RNK-0 cells, the amount of DAG produced by the RNK cells in response to these targets was proportional to the target's sensitivity to lysis. That is, the most DAG was produced in response

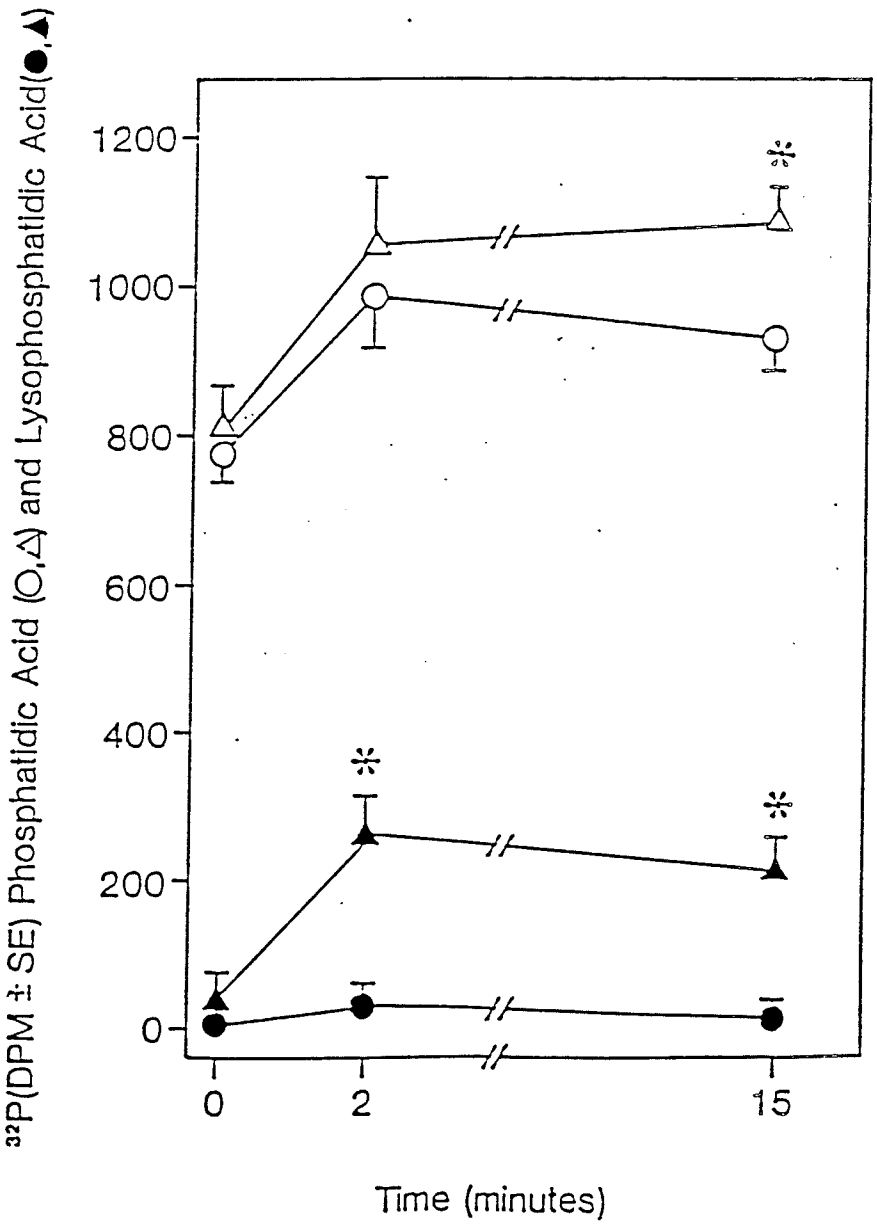

Figure 3. Production of the DAG metabolites PA and LPA in ^{32}P-labelled RNK-0 cells exposed to YAC-1 tumor targets (△,▲) compared to unstimulated controls (○,●). * indicates $p < 0.05$ (T test).

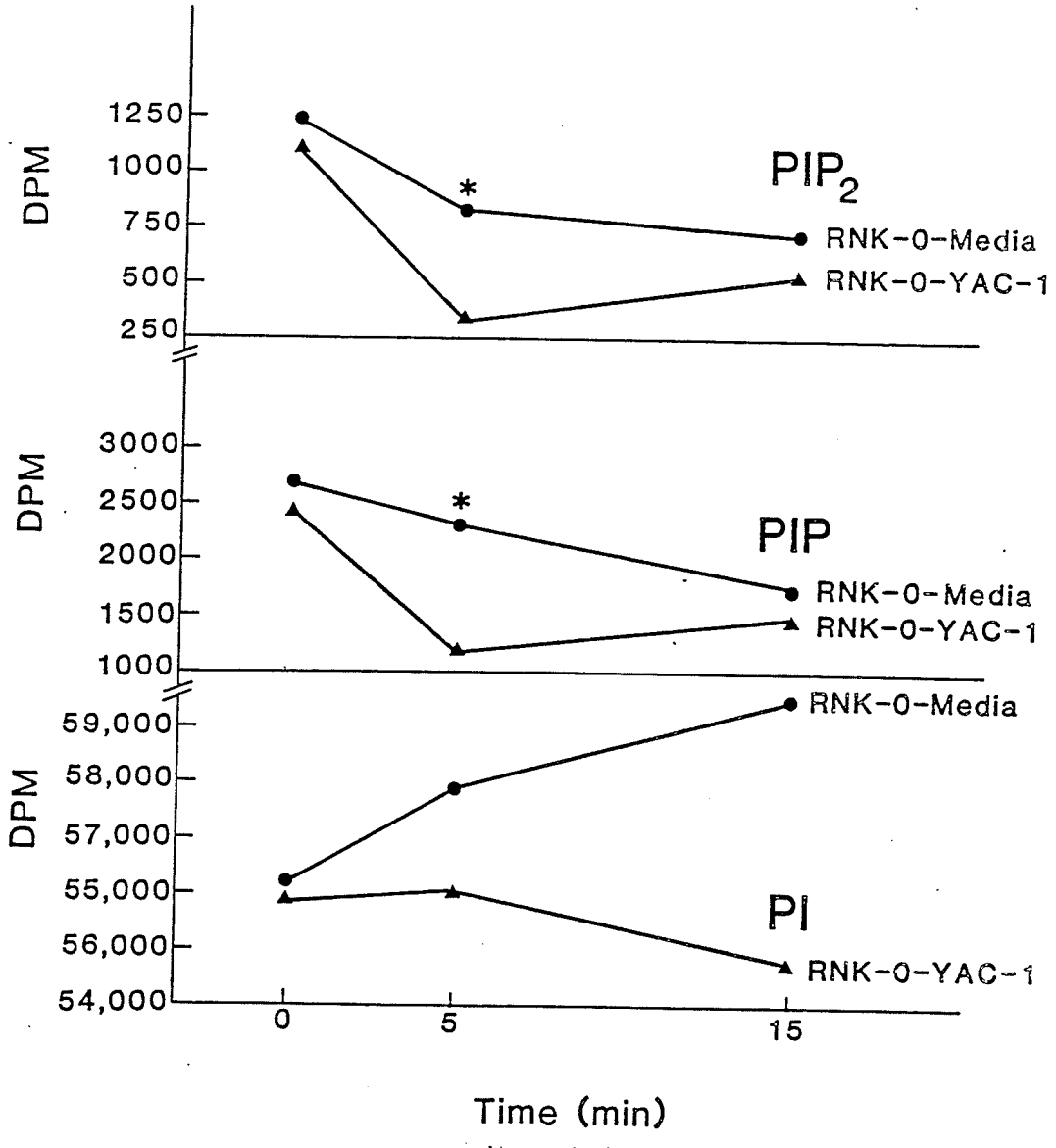

Figure 4: The influence of exposure of RNK-0 cells to YAC-1 cells (▲) or media control (●) on changes in RNK-0 labelling of phosphoinositides. Values are the means of triplicates. * indicates $p < 0.05$ (T test).

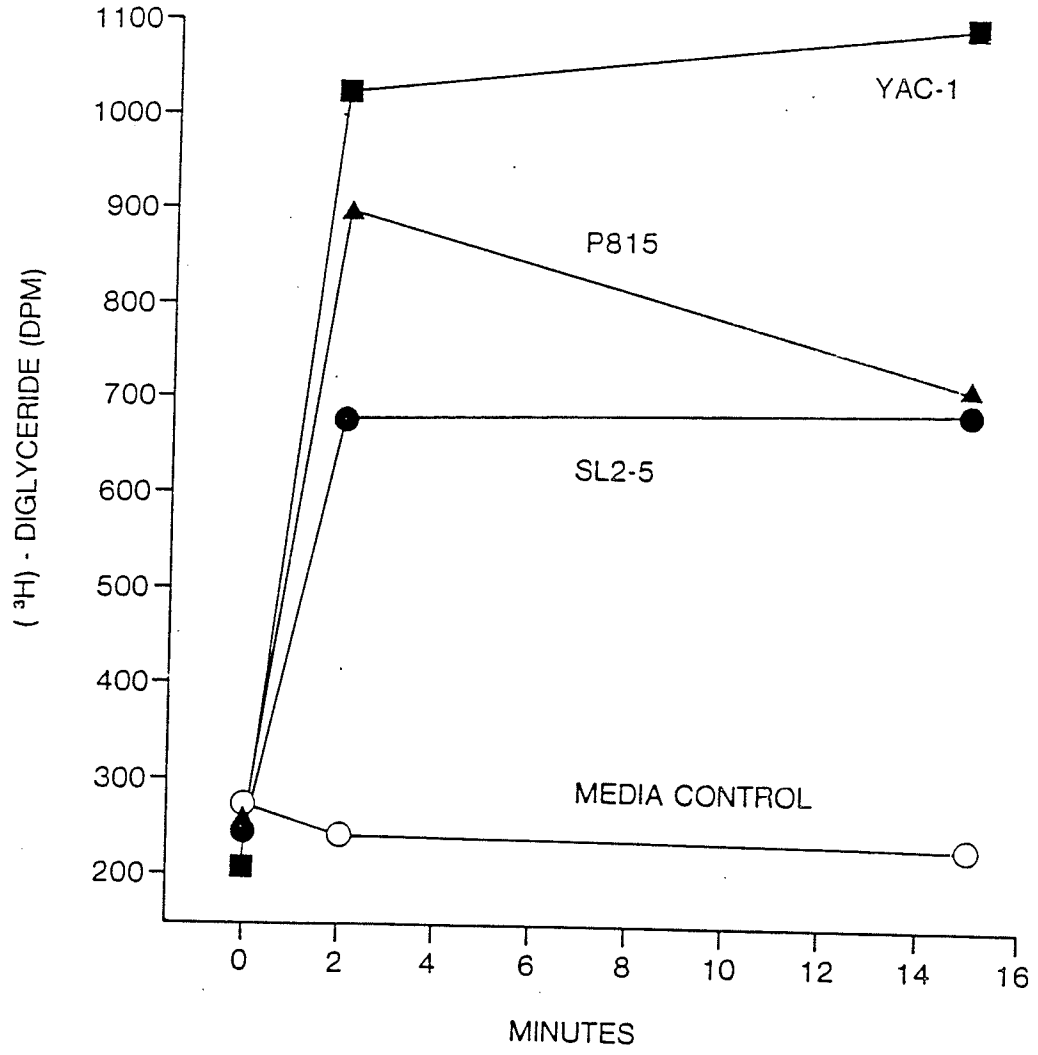

Figure 5: Changes in DAG content of RNK-0 cells following exposure to YAC-1 (■), P815 (▲), SL2-5 (●), or media control (○).

Table I.

SENSITIVITY OF TUMOR TARGETS TO RNK KILLING

Experiment	Tumor Target	Lytic Units Per 10^7 Cells	% Cytolysis (25:1)
1	YAC-1	167	58.1
	P815	20	27.0
	SL2-5	< 0.001	7.2
2	YAC-1	80	36.3
	P815	10	21.3
	SL2-5	< 0.001	2.2

to YAC-1, the least in response to SL2-5, and an intermediate amount of DAG was produced in response to P815 (Fig. 5). This rank order was observed in two separate experiments.

DISCUSSION

Cell-mediated cytotoxicity has long been believed to be calcium-dependent (8,21). Recently, however, a number of groups have reported that in certain target cell-cytotoxic T cell (CTL) combinations, kill is not necessarily dependent upon extracellular calcium, and in these instances, does not appear to be degranulation-dependent (10-12). Other CTL-target combinations follow the classical calcium-dependent pathway. It therefore appears that there may be two separate mechanisms of CTL-mediated killing: one calcium- and degranulation-dependent, the other calcium-independent and employing unknown mediators. Whether this second mechanism is possessed by all or only some CTLs is not clear. It is also not known if other cytotoxic lymphocytes such as NK cells are capable of killing in this manner. By performing lytic assays in the presence of the calcium chelator EGTA, we have verified that our model system of NK cytotoxicity is strictly calcium-dependent.

Granule redistribution and secretion have been implicated in NK cytotoxicity (22-26). This is also a central feature of many cell types that exploit second messengers formed from the inositol phospholipids, IP_3 and DAG, for this purpose. We therefore looked for evidence that this second messenger pathway was activated in RNK cells exposed to susceptible tumor targets. We found that when RNK cells

were brought into contact with YAC-1 cells, the RNK cells rapidly formed significant amounts of IP_3 and DAG, as well as the IP_3 catabolite IP_2, compared to controls. We also observed the formation of the DAG metabolites PA and LPA in RNK cells exposed to YAC-1. In a separate experiment, the parent molecules of these compounds, PIP_2 and PIP, were observed to decrease in RNK cells exposed to YAC-1 within a time span consistent with the observed increases in IP_3 and DAG.

The formation of these potent second messengers in RNK cells exposed to susceptible target cells suggested a possible functional role in the lytic activity of these cells. To examine this further, we exposed RNK cells to three different target cell lines that varied in their susceptibility to lysis by RNK and assayed DAG production in response to these targets. YAC-1, a highly sensitive target, caused a relatively large amount of DAG to be produced. P815, a less sensitive target, induced less DAG production. The least DAG was produced by RNK cells exposed to SL2-5, a relatively resistant target cell line.

It will no doubt be noticed that, while SL2-5 is not very sensitive at all to RNK-mediated killing, it still induced RNK cells to produce some DAG. If production of DAG and IP_3 is of fundamental importance in NK lytic activity, one might have expected that no second messengers would be produced in response to a resistant cell line. However, the lytic assays were performed at a range of effector:target (E:T) ratios, ranging from 80:1 to 1:1, whereas the E:T ratio used in the DAG assays was 1:3, in order to ensure rapid and abundant binding. Conceivably, levels of DAG production that were suboptimal for killing of SL2-5 could have been amplified by the

conditions employed in the DAG assay. Also, NK killing is a complex phenomenon, and may be dependent upon more than simply DAG (and IP_3) formation. In this light, we are rather hesitant to interpret the DAG assays and the lytic assays as being quantitatively equivalent to each other. What is of most importance, we believe, is that for the 3 cell lines tested, the susceptibility to lysis and the ability to cause DAG production in RNK cells followed the same rank order, suggesting that DAG might somehow be involved in NK killing.

Some of our findings reported here have recently been independently confirmed in a report by Seaman et al. (27). This group utilized an RNK line grown in vitro and found that it produced inositol phosphates when mixed with YAC-1 (E:T, 1:2), but not when mixed with a resistant cell line, EL-4, or medium alone. Our results extend the observation that inositol cycle metabolites are produced in RNK upon exposure to a susceptible tumor target to include production of DAG, PA, and LPA, and also demonstrate breakdown of PIP_2 and PIP in RNK cells stimulated similarly.

While this thesis was in preparation a report by Steele and Brahmi (28) was published in which they demonstrated rapid ^{32}P incorporation into PI and PA but not other phospholipids in human NK cells exposed to susceptible targets. This lipid labelling was not observed when NK cells were exposed to resistant targets. An apparent correlation between the degree of lytic susceptibility of the target cells and the extent of PI labelling they induced was also observed by this group, similar to Seaman and colleagues' observation correlating NK susceptibility with IP_3 formation (27), and our observation cor-

relating susceptibility with DAG formation.

Chow et al. (29) have also recently published a paper that demonstrates production of inositol phosphates in human NK cells exposed to susceptible targets. They found that extracellular Ca^{++} was necessary for the activation of the inositol phospholipid pathway, and suggested that the Ca^{++}-dependence of second messenger formation may in part explain the Ca^{++}-dependence of NK cytotoxicity. However, Steele and Brahmi (28) found that PI labelling was not dependent upon extracellular Ca^{++}. It is generally accepted that PI labelling and receptor-mediated depletion of PI are not dependent upon extracellular Ca^{++} (30-34), so the observation to the contrary by Chow et al. must be treated with scepticism pending confirmation.

Chow's group also showed that a synthetic diglyceride and phorbol esters that activate PKC enhanced NK cytotoxicity, while a non-PKC-activating phorbol ester had no such effect. We have observed a similar enhancement of NK cytotoxicity by a PKC-activating phorbol ester (see Chapter II). Also, they reported that sphingosine, a substance which has been shown to inhibit PKC activity, inhibited NK killing, further implicating PKC in this process.

The results of these three recent papers (27-29) all support our findings of inositol cycle activation in NK cells exposed to susceptible target cells. They also support the suggestion that inositol phospholipid-derived second messengers may be important in NK lytic activity. In the next chapter, we will provide additional evidence for this proposal.

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CHAPTER II.

EVIDENCE THAT INOSITOL PHOSPHOLIPID-DERIVED SECOND MESSENGERS MEDIATE NK DEGRANULATION AND KILLING

ABSTRACT

Exocytosis of cytotoxic granule contents towards bound target cells is thought to be of central importance in NK cell-mediated killing. Although cellular cytotoxicity involving degranulation is thought to be calcium-dependent, the biochemical mechanisms which mediate this granule mobilization are unknown. IP_3, which acts to elevate internal calcium levels, and DAG, which activates protein kinase C, are potent second messengers which have been shown to synergistically mediate secretion in other cell types. Generation of these products of inositol phospholipid metabolism was demonstrated in a rat NK cell line, RNK, upon exposure to susceptible tumor targets in Chapter I. We therefore investigated the role of IP_3 and DAG in NK-mediated cytotoxicity, specifically at the level of degranulation. Pretreatment of RNK cells with neomycin, a drug which interferes with the hydrolysis of inositol phospholipids and thus inhibits the formation of second messengers, inhibited RNK cytotoxicity against a susceptible tumor target, and also inhibited RNK production of DAG in response to a similar target. Natural killing exhibited by normal rat nylon wool-nonadherant splenocytes was also inhibited by neomycin. PMA, a phorbol ester which acts like DAG to activate protein kinase C, markedly enhanced lysis of a susceptible target cell by RNK. We evaluated whether modulation of lysis by these drugs was associated with effects on RNK degranulation by assaying the release of a granule-specific serine esterase, BLTE, in response to PMA and the calcium ionophore A23187. These agents synergized to promote the release of

BLTE, and the extent of release was dependent upon the concentrations of both agents. D_2O and cytochalasin B, which enhance secretion in other cells, both enhanced BLTE release from RNK cells, indicating that we were detecting BLTE released via granule secretion, and not due to nonspecific causes such as cell lysis. Our findings lead us to propose that NK cells form IP_3 and DAG in response to susceptible target cells, and a major function of these second messengers is to mediate the exocytosis of cytotoxic granules towards the bound target cells.

INTRODUCTION

In the preceding chapter we demonstrated that the inositol phospholipid second messenger pathway was activated in NK cells upon exposure to susceptible tumor targets. Most significantly, the potent second messengers inositol-1,4,5-trisphosphate (IP_3), an internal calcium mobilizer, and 1,2-diacylglycerol (DAG), an activator of protein kinase C (PKC), were shown to be produced in significant amounts. Results in agreement with ours have recently been reported by others (1-3). This second messenger system has previously been implicated in NK and CTL regulation, since calcium ionophores and phorbol esters, which mimic the effects of IP_3 and DAG, respectively, have modulating effects on these cells. For instance, the calcium ionophores A23187 or ionomycin and the phorbol ester tumor promoter phorbol myristate acetate (PMA or TPA) have been shown to act in synergy to induce IL2 receptor expression and IL2 production in T cells (4,5). These substances also act in concert to stimulate primed CTL precursors to become competent killers (6). With regards to their effects on cytotoxicity, there is still much controversy. Phorbol esters have been reported to exert both inhibitory (7,8) and enhancing (9) effects on the cytotoxicity displayed by CTL and NK cells. In platelets, a model cell system for studying the inositol cycle, both enhancing and inhibitory effects of PKC activation have also been observed (reviewed in 10). It has recently been suggested that inhibition of CTL-mediated cytotoxicity by PMA usually occurs when pretreatment protocols are employed, and is probably due to a transient loss of PKC activity (11).

The production of IP_3 and DAG in response to susceptible target cells suggested that these second messengers might have a possible functional role in NK cytotoxicity. The present study provides evidence which suggests that the second messengers generated from the inositol cycle are intimately involved in both NK cytotoxicity and degranulation, and we propose that these second messengers actually mediate the degranulation event.

MATERIALS AND METHODS

Effector Cells

RNK-O and RNK-16, two independently-derived rat large granular lymphocyte (LGL) tumor lines with functional NK characteristics, were obtained from Dr. C. Reynolds (NCI, Frederick, MD) and grown and prepared as described in Chapter I. An RNK-16 line that had been adapted for growth in vitro (cRNK-16, also supplied by Dr. C. Reynolds), was sometimes employed in BLT-esterase release assays with similar results. The cRNK-16 cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FCS, 1% sodium pyruvate, 1% non-essential amino acids and 2% L-glutamine (all from Gibco). Normal rat spleen cells obtained from 250g male Fischer rats injected intraperitoneally 18 hours previously with 1mg polyinosinic-polycytidylic acid (poly I:C) (Sigma) or saline, were passaged through a nylon wool column as described in Chapter I.

Target Cells

YAC-1 lymphoma cells were grown in RPMI-1640 supplemented with 10% FCS and were used in RPMI-FCS-Hepes for lytic assays. For studies of RNK lipid metabolism, the YAC-1 cells were first lightly fixed in RPMI containing 0.52% glutaraldehyde (Fisher Scientific Co., Fairlawn, NJ) at 37°C for 30 min. to ensure that they were not metabolically active and could not be implicated in the lipid changes seen. The fixed tumor cells were washed, incubated for 30 min. in RPMI-FCS, then washed twice in RPMI-FCS and resuspended in RPMI-FCS-Hepes before use. See Chapter I for a more detailed description of this fixation process.

Measurement of Diacylglycerol

This was done as described in Chapter I.

NK Lytic Assays

The details of this ^{51}Cr-release assay has been described in Chapter I. When neomycin was used, RNK cells were preincubated in various concentrations for 30 or 60 min. in a 37°C shaking incubator, then washed twice in Hanks Balanced Salt Solution (HBSS) (Gibco). None of the concentrations of neomycin used were cytotoxic to the RNK cells as determined by trypan blue exclusion staining. Control cells were incubated for a similar period of time with buffer (RPMI--FCS-Hepes) and washed as before. When PMA (Sigma) was used, it was dissolved in DMSO (Fisher) and diluted in buffer to the appropriate concentrations. The PMA solution was added to each well in a constant

volume (40 ul) immediately after centrifuging the microplate for 1 min. at 60 xg so that the effectors and targets had made contact. In this case, the microplates and cell solution were kept at 4°C until the PMA was added, then incubated as usual at 37°C. The final concentration of DMSO in each well (including controls) was constant and less than 0.1%. Each lytic assay was run in triplicate.

Purification of RNK Cytoplasmic Granules

RNK-16 granules were purified by Percoll density gradient centrifugation of homogenates of nitrogen-cavitated cells, as described in (12) with minor modifications. Briefly, RNK-16 cells were harvested as above, and resuspended at 10^8 per ml in disruption buffer (0.25 M sucrose, 0.01 M HEPES, 4 mM EGTA, 200 U/ml heparin, pH 7.4). They were lysed by decompression at 4°C after equilibrating at 450 psi N_2 for 20 minutes. After the addition of $MgCl_2$ to 5 mM, the homogenate was digested with DNAase I (1000 U/ml, 30 minutes, room temp.). Nuclei were removed by filtration through Nucleopore filters of 5 and 3 um, and the resulting homogenate was cooled to 4°C. 5 ml samples were layered on 20 ml of 48% Percoll (in disruption buffer) and centrifuged in a 70 Ti rotor at 20,000 RPM for 10 minutes total with 0 deceleration in a Beckman L2-65B ultracentrifuge. The resulting gradient was fractionated from the bottom by careful insertion of a spinal tap needle from the top and the removal of 1 ml fractions via attached polyethylene tubing.

Analysis of Cytolysin Activity

Cytolysin activity of each fraction obtained was determined as a

function of sheep erythrocyte hemolysis by the ELISA reader technique described in (13). Briefly, 1-5 x 10^5 SRBC in HBSS containing 4mM Ca^{++} and 2mg/ml BSA were added in 100 ul to each well of a V-bottom 96 well plate containing 100 ul of granule fractions serially diluted in Ca^{++}-free PBS. The plate was then incubated at 37° C for 15-20 minutes and then centrifuged at 1500 RPM for 5 minutes. 150 ul aliquots were transfered to wells of a flat bottom 96 well plate and read on a Titertek Multiskan ELISA reader with a 414 filter. % Hemolysis was calculated from a total obtained by incubating SRBC with 1% Triton X-100.

Analysis of β-Glucuronidase Activity

β-glucuronidase activity of each fraction was determined using a fluorometric microassay, as described in (14). Briefly, 100 ul of diluted granule fractions were added to 50 ul substrate/fluorogen complex (4-MU-β-D-Glucurnonide, final concentration 10mM) and 50 ul citrate-phosphate buffer (pH 4.5) and incubated for 2 hours at 37° C. 2ml stopping buffer (glycine buffer, pH 10.3) was added, and relative fluorescence measured on a fluorimeter (excitation 360 nm; emission 446 nm).

BLT-Esterase Assay

RNK-16 cells grown *in vivo* and *in vitro* (cRNK) or normal nylon wool-nonadherant rat spleen cells were prepared as before and resuspended in phenol red-free HBSS containing 1 mg/ml BSA (Sigma) and 10 mM Hepes (HBSS-BSA-Hepes). PMA and A23187, both dissolved in DMSO,

were diluted in this same buffer. 5 x 10^5 cells were added to V-bottom microtitre plates in 100 ul, and PMA, A23187, or DMSO control solutions were added to the wells in aliquots of 50 ul, such that the final volume per well was 200 ul, and the final DMSO concentration was constant throughout the experiment, including controls (0.03%). The plates were then lightly mixed on an IKA-Schuttler MTS 4 plate shaker for 2 min. and incubated at 37°C for 4 hours. After this incubation, the cells were pelleted by centrifuging the plates at 300 xg for 10 min. BLT-esterase was quantitatively analysed by a colorimetric microassay based on the assay described by Green and Shaw (15). BLT (Nα-CBZ-L-lysine thiobenzyl ester) was dissolved in H_2O (10 mM) and diluted to 1 mM in HBSS-Hepes (no BSA). DTNB (5,5-dithiobis(2-Nitro-benzoic acid)) was dissolved in dimethyl formamide (10 mM) and diluted to 0.5 mM in HBSS-Hepes. The assay was carried out in roundbottomed microtitre plates (96 wells/plate). Each well received 100 ul of the diluted DTNB solution. Reaction wells received 50 ul of the diluted BLT solution and blank wells received 50 ul of HBSS-Hepes. To each well, 100 ul of the cell supernatant was added, the plates were mixed by shaking on a plate shaker, and held overnight at room temperature in the dark. The next day, BLT-esterase activity was assessed by observing the absorbance at 414 on a Titertek Multiskan plate reader. Reported BLT-esterase release was equal to:
(esterase release from stimulated NK cells) - (esterase release from NK cells cultured in the absence of stimulators).

When cytochalasin B (Sigma) was used, RNK cells were incubated in a 5 ug/ml solution for 30 min. in a 37°C shaking incubator. Control

cells were incubated in a similar concentration of vehicle. Cells were then washed twice by centrifugation and resuspended in assay buffer. In experiments utilizing D_2O (Sigma), the cells were resuspended in HBSS-BSA-Hepes that had been prepared with D_2O, such that the final concentration of D_2O per well after all solutions were added was 30%. Neither cytochalasin B nor D_2O were toxic to the RNK cells when used together with the other agents in these assays, as determined by trypan blue exclusion staining. By using trypsin as a source of protease in the place of the RNK supernatants, we determined that saturation of this assay system did not occur in any of the experiments performed.

RESULTS

Modulation of RNK Lysis By Agonists and Antagonists of the Inositol Cycle.

To study the intriguing possibility that there was a functional relationship between the lytic activity of RNK cells and their production of inositol phospholipid-derived second messengers which we demonstrated in Chapter I, we first examined the effects of neomycin, an agent which interacts with polyphosphoinositides and inhibits their hydrolysis and the subsequent formation of second messengers (16). Neomycin inhibited the lytic activity of both RNK-0 and RNK-16 in a dose-dependent fashion (Fig. 1, top and middle panels). Similar inhibition of natural killer activity was observed using nylon wool-nonadherant rat splenocytes (Fig. 1, bottom panel) and an RNK line grown *in vitro*, cRNK (data not shown). Neomycin also inhibited

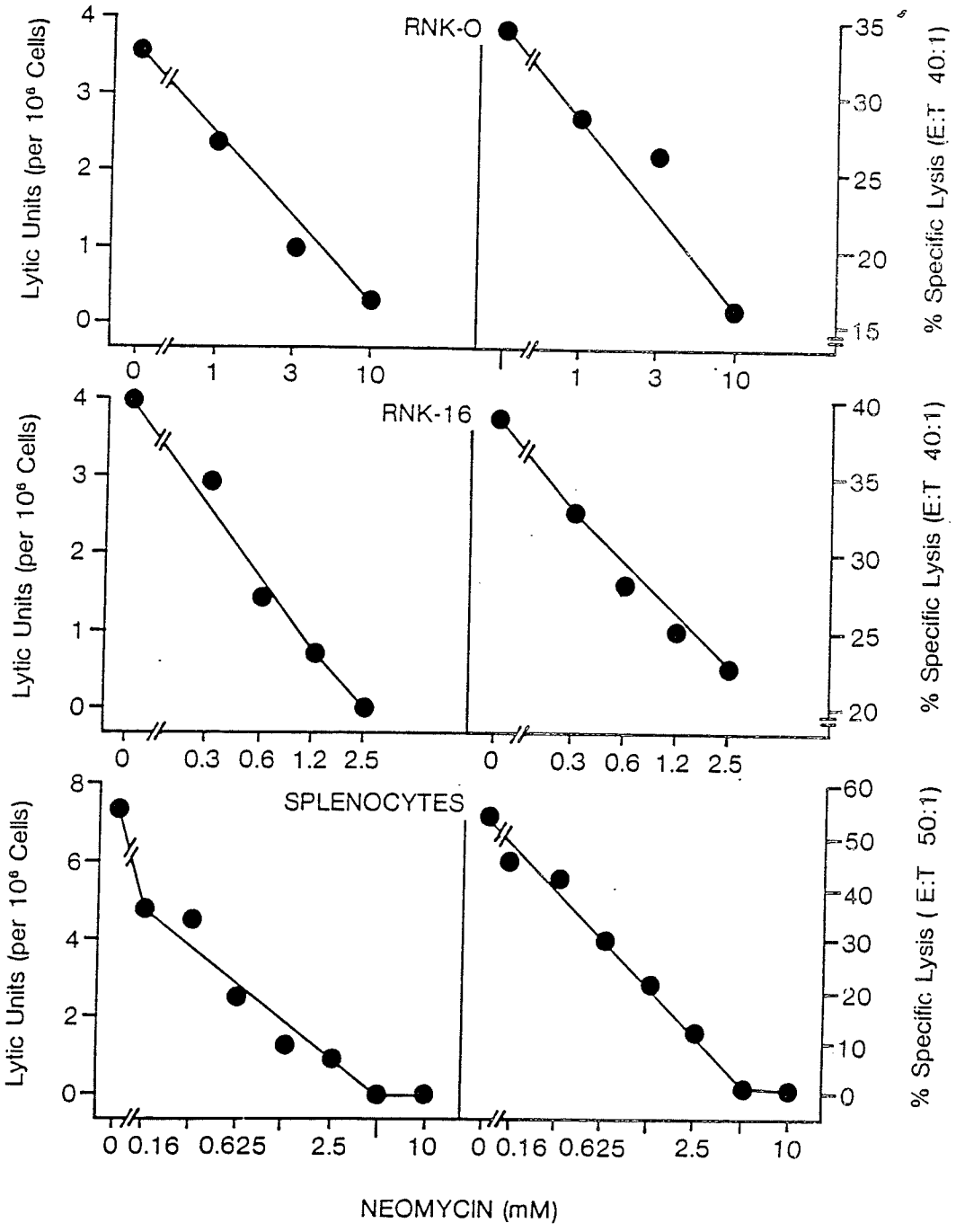

Figure 1: Dose-response of neomycin inhibition of cytotoxicity against YAC-1 tumor cells by RNK-0, RNK-16, and nylon wool-nonadherant rat splenocytes. RNK-0 cells were preincubated at 37°C in the indicated doses of neomycin for 30 min. RNK-16 cells and the splenocytes were preincubated with neomycin for 60 minutes. Effector cells were then washed twice with buffer before being used in the assay. All points are the means of triplicates.

the production of DAG in RNK cells exposed to YAC-1 targets (Fig. 2), consistent with the idea that the inhibition of cytotoxicity was mediated through inhibition of inositol cycle metabolite formation.

Since the effects of DAG on other cells are generally believed to be through activation of PKC (17) and PMA also binds and activates this enzyme (18), we next assessed whether PMA affects RNK killing of tumor cells. As shown in Fig. 3, PMA enhanced RNK lysis of YAC-1 cells 9-fold, over the concentration range examined. PMA had no effect on YAC-1 lysis in the absence of effector cells, and did not alter lysis induced by isolated RNK granules, ruling out a direct target cell effect (data not shown). Although PMA has been reported to exert an inhibitory effect on cellular cytotoxicity when used in pretreatment protocols (7,8), we were able to consistently demonstrate an enhancement of lysis by adding it directly to the lytic assay immediately after the effector and target cells were brought into contact with each other by light centrifugation.

Synergistic Effects of PMA and A23187 in Inducing RNK Degranulation.

Recent reports indicate that NK cells and CTL possess a number of serine esterase enzymes that appear to be unique to these cells (19, 20). Cytochemical and fractionation techniques have localized these enzymes, including one having a specificity for the synthetic substrate BLT, to the cytotoxic granules (21,22). By employing Percoll density gradient centrifugation of RNK homogenates and assaying each fraction for activity of the granule markers cytolysin and β-glucuronidase, we have verified that in this cell line, the majority of the BLT-esterase (BLTE) activity is also located in the granule fractions,

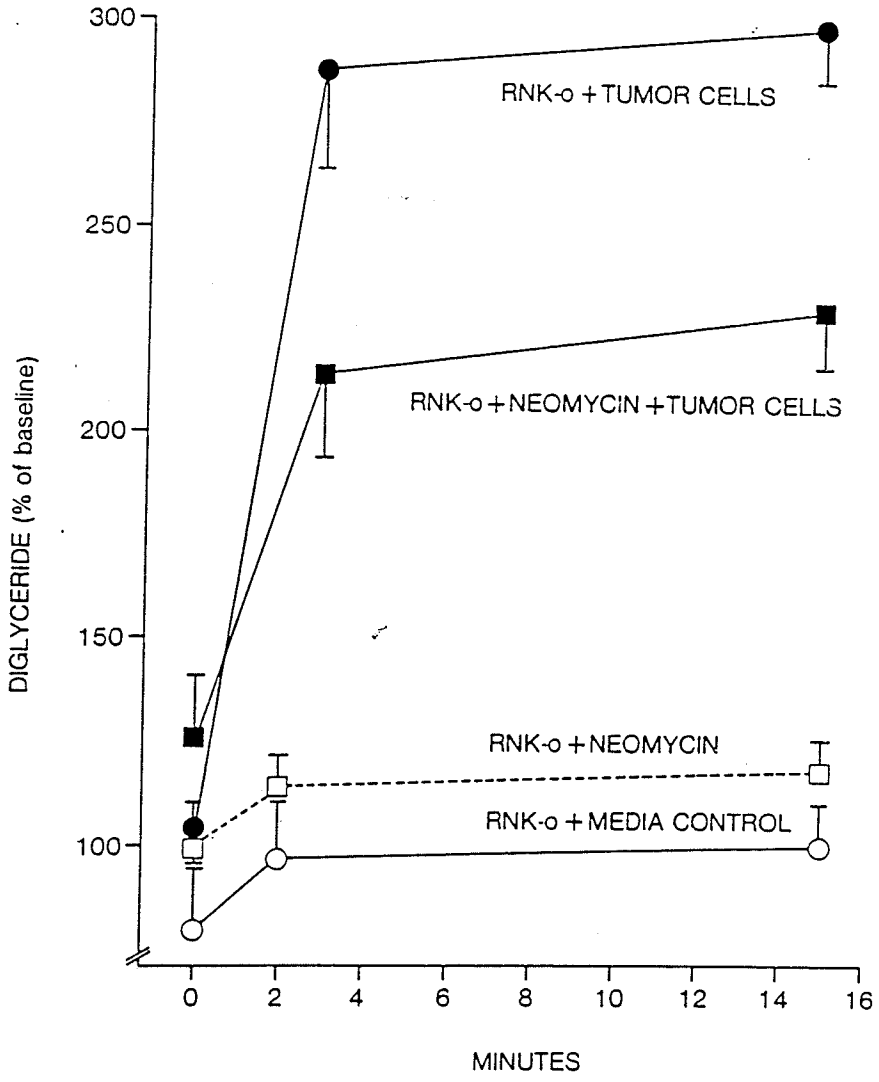

Figure 2: Pretreatment of RNK-0 cells with 10mM neomycin for 30 minutes inhibits diacylglycerol production induced by YAC-1 cells. ^{3}H-arachidonic acid-labelled RNK-0 cells were either preincubated with neomycin and then exposed to YAC-1 (■) or medium control (□), or preincubated with buffer control and then exposed to YAC-1 (●) or medium control (○).

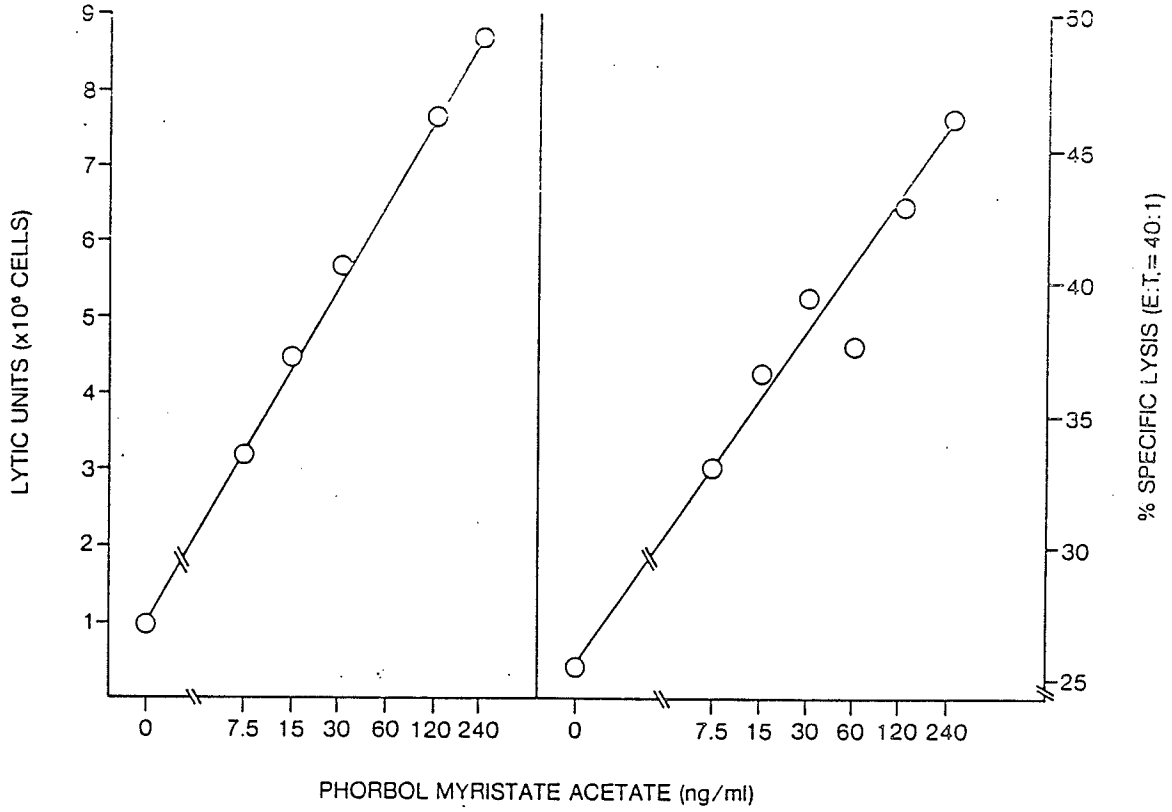

Figure 3: Dose-dependent enhancement of RNK-0 mediated lysis of ^{51}Cr-labelled YAC-1 tumor cells by phorbol myristate acetate. Targets and effectors were combined and lightly centrifuged to initiate contact with each other all at 4°C, before adding PMA or DMSO. This figure is representative of a number of experiments. All points are the means of triplicates.

Table I.

Comparison of Strontium-Induced BLTE Release With Lytic Efficiency of RNK-16 Cells

RNK-16 Population	Lytic Units Per 10^7 Cells	% Specific Lysis (E:T = 20:1)	% BLTE Released Over Background (100%) by 25mM $SrCl_2$
A	87.0	42.1	193.33 ± 12.89 P<0.0025
B	3.3	8.3	109.74 ± 8.34 P<0.10

fractions 1-3 (Fig. 4). Additional BLTE and β-glucuronidase activity between fractions 17 and 20 most likely represents either lighter immature granules, or enzyme released from granules ruptured during the isolation process. RNK cells caused to degranulate by exposing them to 25 mM SrCl released BLTE into the supernatant, and the amount of BLTE released appeared to coincide with their cytotoxic potential (Table I). This indicates that this assay may be employed with confidence to measure cytotoxic granule secretion.

To study whether or not inositol cycle metabolites might have a role in promoting the degranulation event in RNK cells, the effects of the PKC activator PMA and the calcium ionophore A23187, which theoretically would supply the signals normally provided by DAG and IP_3, were assessed in a BLTE-release assay in the absence of target cells. As shown in Fig. 5, A23187 alone caused a small amount of BLT-esterase to be released from RNK cells when concentrations in excess of 18.5 ng/ml were employed. This is consistent with a recent report that mouse CTL lines can be induced to release perforin by treating them with A23187 (23). A single dose of PMA (100 ng/ml) synergized with all the doses of A23187 tested, including low concentrations which by themselves had no effect, inducing a greater degree of esterase release than could be accounted for by a simple additive effect. This BLTE release was enhanced by preincubating the RNK cells in cytochalasin B, an agent which disrupts microfilaments and has been shown to enhance secretion in other leukocyte systems (24,25). A dose-dependent effect of PMA in promoting esterase release from RNK cells was observed by employing a constant concentration of A23187 and varying the amount of PMA (Fig. 6, top).

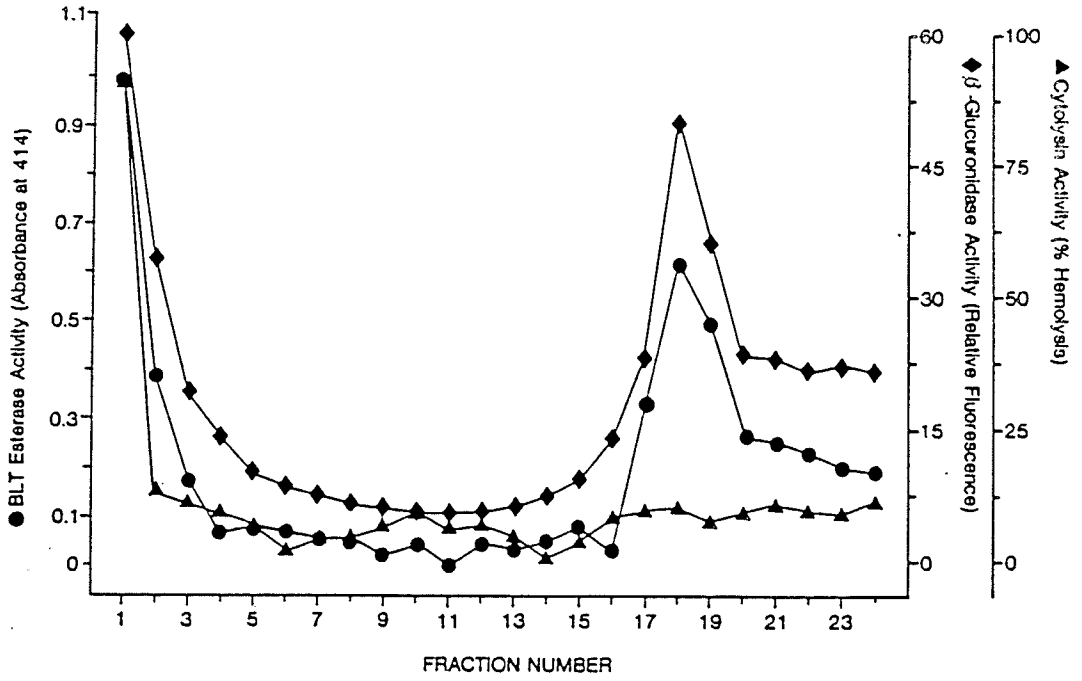

Figure 4: Percol density gradient fractions of nitrogen-cavitated RNK-16 cells showing cosedimentation of BLT-esterase activity (●) with the granule markers cytolysin (▲) and β-glucuronidase (◆) in fractions 1-3. Additional BLTE and β-glucuronidase activity between fractions 17-20 is likely the result of lighter immature granules and granule rupture during the purification procedure.

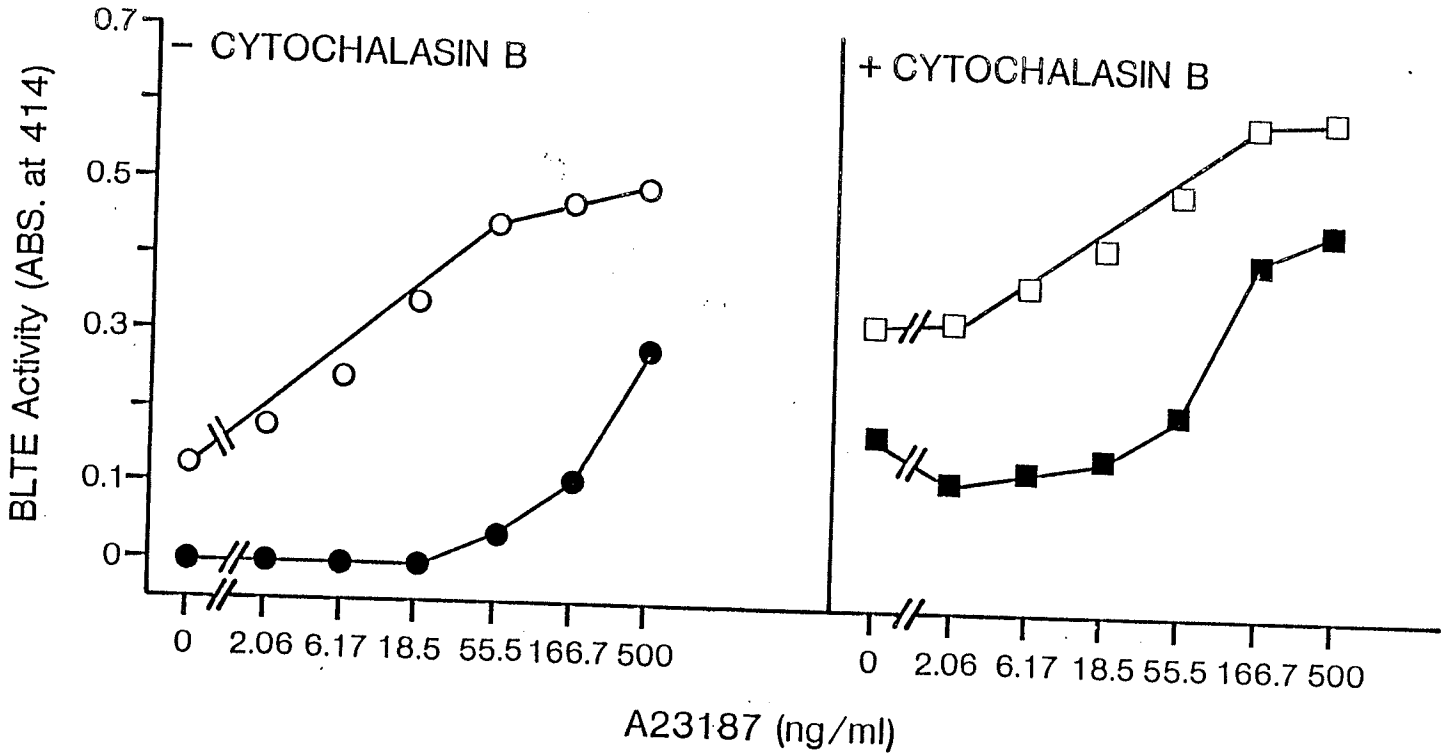

Figure 5: Dose-response of A23187-induced release of BLT-esterase (BLTE) activity from RNK-16 cells in the presence (○,□) or absence (●,■) of 100 ng/ml PMA. Pretreatment of the RNK-16 cells with 5 ug/ml cytochalasin B for 30 min. at 37°C (□,■) resulted in enhanced release of BLTE compared to RNK-16 cells similarly pretreated with control buffer (○,●). All points are the means of triplicates.

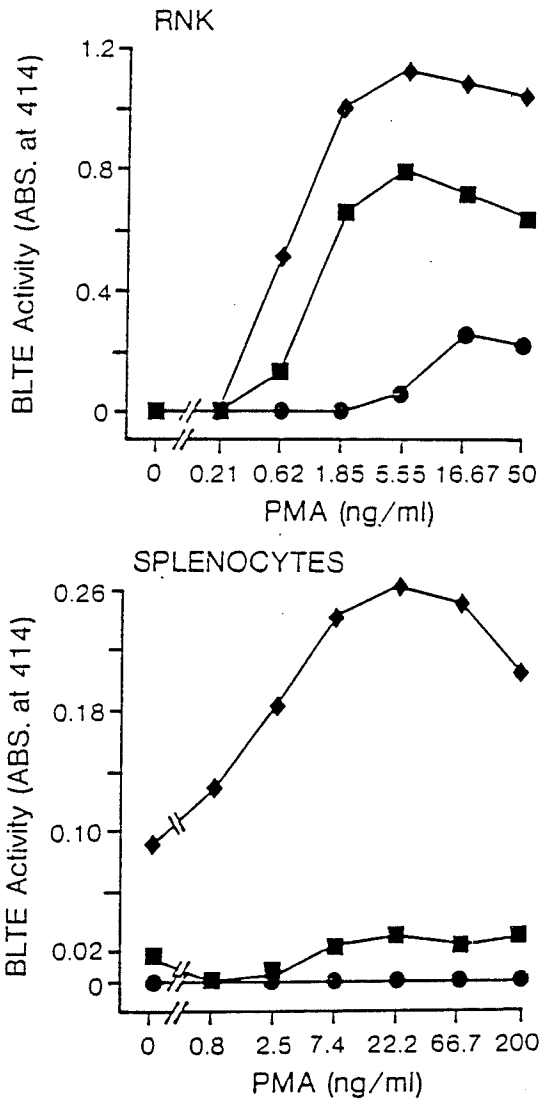

Figure 6: Dose-response of PMA-induced release of BLTE activity from cRNK-16 cells and nylon wool-nonadherant rat splenocytes.

top) cRNK-16 cells were incubated with the concentrations of PMA indicated and 30% D_2O (●), 166.67 ng/ml A23187 (■), or both (◆).

bottom) nylon wool-nonadherant splenocytes from poly I:C treated rats were incubated with the concentrations of PMA indicated only (●), or PMA in addition with 8.33 ng/ml A23187 (■) or 166.67 ng/ml A23187 (◆). All points are the means of triplicates.

PMA by itself appeared to have a negligible effect in this assay. Addition of 30% heavy water (D_2O) to the cells for the duration of their incubation with PMA and A23187 also markedly enhanced the amount of BLTE released. D_2O has been used in mast cell systems to enhance the secretion of histamine (26), most likely due to a stabilizing effect on microtubules (27). The fact that D_2O and cytochalasin B, two agents that have been used in other systems to enhance secretion, enhanced the release of BLTE from RNK cells provides additional evidence that this assay is detecting a secretory event. That is, it is very unlikely that secretion enhancers would enhance BLTE release if the majority of the enzyme was released due to reasons other than active secretion, such as toxic or lytic effects of A23187 and/or PMA. Furthermore, PMA and A23187, alone or in combination, were determined to be nontoxic at the concentrations and conditions used in the assay by trypan blue exclusion staining (not shown), further ruling out the possibility that cell lysis and subsequent non-specific release of granule components was occuring.

In order to verify that these mimetics of IP_3 and DAG could mediate degranulation of a cell population containing normal LGL, rat nylon wool- nonadherant spleen cells were examined. A similar synergism between A23187 and PMA in promoting the release of granule esterases was also observed with this cell preparation (Fig. 6, bottom).

DISCUSSION

Experimental evidence has been obtained in recent years which supports the contention that NK cells kill via a calcium-dependent

process that involves exocytosing their granules towards the bound target cells, releasing a preformed molecule which delivers the lethal hit. This evidence includes the observations that: 1) pretreatment of NK cells with strontium chloride causes degranulation, resulting in inhibition of cytotoxic activity, and recovery is thought to coincide with granule regeneration (28,29); 2) the carboxylic ionophore monensin, an agent which inhibits secretory processes, causes large vesicles to accumulate in the cytoplasm and irreversibly inhibits NK cell-mediated cytotoxicity (30,31); 3) purified NK granules exhibit potent cytotoxicity towards a large number of cell types, whereas granules isolated from noncytotoxic cells do not (32); and 4) antibodies directed towards LGL cytoplasmic granules inhibit natural killing (33).

It was believed that CTL kill their targets by similar means, but recently a number of groups have published data which suggests that killing in certain CTL-target combinations is not necessarily calcium-dependent, and in these instances does not appear to be degranulation-dependent (34-36). Other CTL-target combinations follow the classical calcium-dependent cytotoxic pathway. It therefore appears that there may be two separate mechanisms of CTL-mediated killing: one calcium- and degranulation-dependent, the other calcium-independent and employing unknown mediators. Whether this second mechanism is possessed by all or only some CTLs is not clear. It is also not known if other cytotoxic lymphocytes such as NK cells are capable of killing in this manner, though we have verified that RNK killing of YAC-1 cells is strictly calcium-dependent (see Chapter I). Here, we have established a link between the second messengers formed from inositol-

containing phospholipids and the exocytosis of RNK granules and cytotoxicity.

RNK cells form inositol cycle metabolites immediately following contact with susceptible tumor targets (Chapter I). The production of potent second messengers concomitant with the binding of a susceptible target cell suggests a role for these messengers in the cytotoxic process. Evidence in support of this idea was obtained by employing modulators of the inositol cycle in lytic assays.

Inhibition of the generation of inositol phospholipid-derived second messengers by neomycin significantly reduced RNK killing in a dose-dependent manner. Cytotoxic cells obtained from normal rat spleens by passage through a nylon wool column were similary inhibited in their cytotoxicity by neomycin. Neomycin also inhibited DAG formation in RNK cells, a finding which is consistent with the idea that the effect of neomycin on cytotoxicity was being mediated through effects on the inositol cycle. While it may initially appear that the inhibition neomycin exerted on cytotoxicity was much greater that that exerted on DAG production, it should be realized that these two assays are not necessarily quantitatively equivalent. For instance, the effector:target (E:T) ratios in the cytotoxicity assays ranged from 100:1 or 80:1 to 1:1, whereas the E:T ratio used in the DAG assays was 1:3 in order to promote rapid and abundant binding. Qualitatively, we interpret these results as indicating that a dose of neomycin which is effective in inhibiting RNK cytotoxicity against YAC-1 cells is also effective in significantly inhibiting RNK production of DAG in reponse to YAC-1. A similar concomitant inhibition of NK cytotoxicity and PI

metabolism by the same agents has recently been reported by Steele and Brahmi (2). These investigators showed that quercetin and dibutyryl-adenosine-cAMP plus theophylline inhibited both NK cytotoxicity and ^{32}P incorporation into PI.

Because neomycin may have been exerting other effects not specific to inhibition of the inositol cycle, another complementary stategy was deemed necessary to confirm the functional importance of this pathway in NK cytotoxicity. In addition to simultaneously inhibiting the inositol cycle and cytotoxicity, we decided to try to enhance the effects of this second messenger system and determine if cytotoxicity was likewise enhanced. We therefore used the phorbol ester tumor promoter PMA, which like DAG can activate PKC, in a lytic assay. If PKC activation is indeed important in NK cytotoxicity, one would expect to enhance cytotoxicity by increasing PKC activity with PMA. This was in fact what we were able to demonstrate by adding PMA into lytic assays immediately after target and effector cells had been brought into contact with each other. PMA was added in this way to ensure that any potential inhibitory effects of PKC activation by this agent, such as degranulation before conjugate formation, down-regulation of surface receptors, PMA-induced degradation of PKC, or other nonspecific inhibitory effects, did not occur prior to the cytotoxic activation of the RNK cells by the tumor targets.

This enhancement of NK cytotoxic activity by PMA has recently been confirmed by Chow et al. (3). These investigators demonstrated enhanced cytotoxic activity of human NK cells exposed to PKC-activating phorbol esters and a synthetic diglyceride, but not by a non-

PKC-activating phorbol ester. They further implicated PKC activity in NK-mediated cytolysis by demonstrating that the PKC inhibitor sphingosine could inhibit killing of a susceptible target cell.

Though either the neomycin experiments or the PMA data alone could conceivably have different interpretations based on possible nonspecific effects, we felt that taken together they strongly suggested that inositol cycle second messengers are in some way involved in RNK cytotoxicity. However, at this point it was not yet possible to distinguish at what stage in the killing process these messengers had the most profound effects. It seemed possible that they could be acting at the binding stage, perhaps to increase the efficiency of effector-target cell conjugate formation. Alternatively, these messengers may have influenced the actual delivery of cytotoxic molecules, whether they be cytolysins or natural killer cytotoxic factors (NKCF), from the NK cell to the target cell. It has recently been reported that calcium ionophores and phorbol esters synergize to promote NKCF release (37). However, NKCF appears to kill with much slower kinetics than cytolysin (38,39), and our lytic assays were run only for the duration necessary to detect cytolysin-mediated kill. Therefore, though NKCF release may be mediated through these messengers, it is unlikely that this fully accounts for our results. It has also recently been reported that treatment of NK cells and CTL with phorbol esters alters their adherance properties, causing NK cells to bind to plastic (40) and promoting conjugate formation of CTLs with antigen-nonbearing cells (41). Increasing the effectiveness of binding between cytotoxic cells and their targets could conceivably

lead to more efficient killing, and may be a partial explanation of the observed effects of PMA on cytotoxicity. It seems more probable, however, that if second messengers having the potency of IP_3 and DAG are being produced in response to tumor cells, they will have a more central role in the cytotoxic process. The most important stage in NK-mediated cytoysis is arguably the degranulation event, allowing the delivery of granule cytolysins to the target cell. Since increased internal calcium and PKC activation have been shown to be important in granule mobilization and secretion in other cell systems such as platelets (42), mast cells (43), and neutrophils (44), the participation of IP_3 and DAG in NK degranulation appeared to be a good possibility.

To more directly test the hypothesis that degranulation is affected by inositol cycle products, we examined the effects of the IP_3 and DAG mimetics, A23187 and PMA, respectively, on granule exocytosis in RNK cells. For this purpose, we employed an assay that detects released BLT-esterase activity, an enzyme normally sequestered in NK and CTL cytotoxic granules, as a measure of degranulation. We first verified that BLTE was present in the granules of RNK cells, and that degranulating RNK cells with SrCl caused measurable quantities of BLTE to be released into the supernatant. Not only was this the case, but the amount of BLTE released from RNK cells exposed to SrCl seemed to coincide with the lytic potential of the RNK cell preparation tested. This gives support to the contention that assaying BLTE release provides a usefull measurement not only of NK degranulation, but also of the potential of granule-mediated killing.

PMA and A23187 synergistically promoted the release of BLTE from RNK-16 and cRNK cells in the absence of target cells. Similar results were obtained when nylon wool-nonadherant rat spleen cells were used, indicating that our results with RNK cells represent characteristics of true NK cells, and are not an artefact of the transformed cell line. In addition, by using the cRNK cells grown *in vitro*, we have eliminated the possibility that our results could be attributable to contaminating non-NK cells in the RNK lines grown *in vivo*. While this thesis was in preparation, Nishimura *et al*. (11) published data which shows a similar synergistic effect of PMA and the calcium ionophore ionomycin in causing serine esterase release from mouse CTL clones. This agrees with our data using RNK cells, which shows that doses of A23187 and PMA that alone are relatively ineffective at triggering BLTE release can synergize to cause substantial degranulation. Our findings are also in agreement with the now classic study of Kaibuchi *et al*. (45) which first showed that suboptimal concentrations of A23187 and the synthetic diacylglycerol OAG, which can activate PKC, could synergize to cause secretion from human platelets.

We were concerned that some of the concentrations of PMA and A23187 used in the BLTE assays might possibly be toxic to the cells, resulting in nonspecific release of BLTE. To verify that this was not the case, trypan blue exclusion staining was performed on cells treated indentically to those employed in these assays, and no toxic effects of these agents were noticeable. To confirm that nonspecific release of BLTE via cell lysis was not occuring, we employed cyto-

chalasin B and D_2O, two agents which have been shown to enhance secretion in other cells (24-26). Both agents, when used in BLTE-release assays employing RNK cells stimulated with A23187 and PMA, enhanced the amount of esterase released. It seems quite unlikely that this would be the case if RNK lysis was occuring, and we therefore interpret this as indicating that the BLT-esterase we assayed was released via secretion.

Further evidence for the proposal that phosphoinositide catalysis is involved in RNK cytotoxicity is found in a recent report (1) that OX-34, a monoclonal antibody reactive with a ≈52 kDa molecule on the surface of NK cells, including RNK-16, can block RNK-mediated killing and production of IP_3. Crosslinking OX-34 bound to RNK-16 cells increased the concentration of cytoplasmic free calcium and inositol phosphates. Also, IP_3 production by RNK-16 was reported to be proportional to the lytic susceptibility of the target cell used as a stimulant. We have observed similar trends in the levels of DAG produced by RNK cells exposed to different targets with varying sensitivities to RNK-mediated lysis (see Chapter I). These results and those of Steele and Brahmi (2) and Chow et al. (3) are all consistent with our earlier report that the inositol cycle is activated in RNK cells exposed to YAC-1 (Chapter I), and support our present findings that 1) neomycin inhibits both RNK-mediated cytotoxicity and DAG production in RNK cells exposed to a susceptible target, 2) PMA, a DAG mimetic, enhances kill, and 3) PMA and A23187 can synergize to induce degranulation in RNK cells, presumably by supplying the triggering signals normally provided by DAG and IP_3.

Our results do not argue against potential effects of IP_3 and DAG on RNK-target cell binding or NKCF release. Rather, we interpret them as fitting into a more comprehensive model of lytic activation, in which the second messengers generated from the inositol phospholipids are formed upon contact with a target cell, and are of importance to cytotoxic lymphocytes on a number of different levels. These molecules may regulate T cell activity and proliferation by controlling IL2 receptor expression (4) and IL2 production (5) and can stimulate CTL precursors to become competent killers (6). They may have an influence on the adhesive properties of NK cells and CTLs (40,41) which could play a role in increasing the efficiency of effector-target cell binding and stabilization of these conjugates. They may also be important in mediating the release of NKCF (37). The results reported here lead us to propose that IP_3 and DAG also directly influence NK cytotoxicity by mediating the exocytosis of cytotoxic granules. Taken together, our observations link inositol phospholipid-derived second messengers with cytotoxicity and degranulation, and lend further support to the stimulus-secretion model of cellular cytotoxicity.

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CONCLUDING REMARKS

1. Introduction

The last decade has seen enormous advances in understanding the signal transduction mechanisms of many calcium-dependent cellular responses. The inositol phospholipid pathway has come to be accepted as providing potent second messengers that participate in many such responses in a wide range of cell types (see ref. 1). In this thesis we have shown that inositol phospholipid metabolites - inositol phosphates, DAG, PA, and LPA - are formed in natural killer cells upon exposure to susceptible tumor targets. We have also demonstrated the breakdown of the inositol phospholipid parent molecules of these metabolites in response to a susceptible target cell. In addition, we have provided evidence which suggests that the two second messengers generated from this pathway, IP_3 and DAG, may be intimately involved in NK lytic activity at the level of degranulation.

Shortly after this project was initiated, the first reports implicating second messengers derived from the inositol phospholipids in CTL reactivity were published (2-4. Reviewed in ref. 5). However, to our knowledge only three papers have been published pertaining to the inositol phospholipid pathway in NK cells (6-8), none of which have addressed possible specific functions for the generated second messengers in as much detail as this study.

2. Future Research Possibilities

If, as we have proposed, IP_3 and DAG cooperate in NK cytotoxic activity by mediating degranulation, a number of future research areas

present themselves.

2.1 Electron Microscopic Analysis

We have carried out preliminary experiments utilizing electron microscopy to visually assess RNK granule mobilization and fusion in response to either target cells or A23187 and PMA. As yet no firm conclusions can be made, but additional experiments with carefull quantitation of the induced responses will be illuminating, especially if the morphological effects can be correlated with results obtained from parallel lytic and BLTE-release assays.

2.2 Protein Phosphorylation

In other systems where PKC- and Ca^{++}-activated pathways have been implicated, protein phosphorylation studies have provided information as to what response systems are turned on as a result of cellular activation. For example, in platelets, 20 Kd myosin light chain is phosphorylated by calcium/calmodulin-dependent myosin light chain kinase, and a 40 Kd protein of unknown function is primarily phosphorylated by PKC (reviewed in ref. 9). Quantification of similar protein phosphorylations in stimulated NK cells could provide insights into the control of secretory events and attempts could be made to correlate phosphorylation with granule mobilization and fusion, secretion, and lytic activity, as has been done with other cells, including platelets (9).

We attempted some protein phosphorylation studies during the course of this project, but our RNK model system did not prove to be amenable to this type of assay. An excessive background phosphor-

ylation activity was present, perhaps due to the possibility that this tumor line is already in a partially stimulated state. Other cell lines or freshly-isolated normal NK cells might be better suited to these studies.

2.3 Molecular Biology

It seems unlikely that inositol cycle-derived second messengers formed upon target cell recognition would have activity limited to only immediate functions such as degranulation, NKCF release, or adhesiveness. Rather, these messengers might also have more far-reaching effects, such as gene activation. Support for this idea comes from the CTL system, where calcium ionophores and phorbol esters have been shown to synergize not only in causing serine esterase release from granules (10), but also in inducing IL2 receptor expression and IL2 production (3,4).

Once degranulation has been triggered, the NK cell must regenerate its granule supply. The genes encoding the granule components must be activated for transcription. It would be evolutionarily efficient for the cell to utilize the same signals for granule exocytosis and regeneration. Analysis of which genes are turned on in the NK cell immediately following activation by target cells or PMA and A23187 might provide additional clues as to which proteins are involved in NK reactivity.

2.4 Search for the NK Receptor

Identification of the inositol phospholipid pathway as a/the

signal transduction mechanism in NK cells could also prove useful in the search for the elusive NK receptor. Since the minimal requirements for a NK receptor include signal transduction and initiation of degranulation, monoclonal antibodies (mAbs) raised against NK cell surface antigens could be screened for their ability to cause IP_3 and DAG formation and the consequent calcium flux and degranulation. In fact, the recent report by Seaman et al demonstrated that cross-linked mAB OX-34 could cause production of inositol phosphates and a calcium flux in RNK cells (6). No degranulation data was supplied, but a role in recognition of target cells was suggested by the fact that this mAb could block IP_3 formation and cytotoxicity when RNK cells were exposed to YAC-1 cells. Since the OX-34 determinant is homologous to human CD2 (6,11) and CD2 has already been implicated in NK recognition and triggering (12,13), further research into the role of this molecule in NK recognition and signal transduction should be considered.

Ortaldo and colleagues have raised a mAb against human NK cells that does not appear to recognize CD2, CD8, CD11, CD16, or Leu19 (14). They have proposed that it may recognize a NK receptor on the basis of cytotoxicity blocking and enhancement experiments. It will be interesting to see if this antibody can cause second messenger formation and degranulation.

None of the NK surface molecules presently identified by mAbs may be the actual NK receptor. However, some of them may prove to be important associated molecules. In any case, demonstration of inositol cycle activation should be considered a crucial test of any proposed NK receptor.

It is possible that exceptions to this suggestion may arise, however. There have been recent indications that there may be a Ca^{++}- and degranulation-independent lytic mechanism in CTL (15-17). It is conceivable that some cells with NK activity may possess similar abilities, in which case activation of the inositol phsopholipid pathway may prove not to be universally essential for NK lytic activity. However, it should be noted that there have been no reports of agranular NK cells with cytolytic capabilities, as there have been of agranular CTL (see ref. 18), indicating that NK cells may not possess a granule-independent lytic mechanism.

3. Clinical Significance

The clinical significance of our findings may include the possibility that defects in NK phosphoinositide metabolism or protein phosphorylation could occur, leading to depressed NK activity. As a result of the association of the phosphoinositide pathway with platelet activity, many laboratories began searching for patients with bleeding disorders that could be explained in these terms. To date, these investigations have proved largely unsuccessful (Dr. J.M. Gerrard, personal communication). However, a recent report has described a new platelet disorder, and the authors suggest that it may be due to the impairment of an unspecified part of the signal transduction process (19). Although this conclusion has yet to be confirmed, it does indicate that these types of defects could be clinically relevant. The paucity of examples of these disorders in platelets might indicate that these processess are so biologically

fundamental that their impairment may prove to be lethal.

A recent paper has reported the generation of a somatic cell mutant of the T-cell line Jurkat, that expresses a structurally normal, but functionally deficient, CD3/Ti complex (20). The mutant receptor complex appears to be inadequately coupled to the inositol phospholipid pathway, resulting in functional defects in some receptor-dependent processes. These types of mutants may provide additional means of studying receptor signal transduction processes in lymphocytes. They also point to the possibility of this type of defect occuring in a clinical setting.

4. Conclusion

In conclusion, we have demonstrated that inositol phospholipid metabolites are formed in a rat NK cell line upon exposure to a susceptible tumor targets. We have also provided evidence that the two known second messengers generated from this pathway, IP_3 and DAG, are intimately involved in NK lytic activity at the level of degranulation. This conclusion was made based on studies of neomycin inhibition of cytotoxicity and DAG formation, PMA enhancement of cytotoxicity, and PMA and A23187 synergism of granule-localized BLTE release.

The results of this study also recommend several potentially interesting areas of future research which may have implications in other studies of NK cells, including basic biology and clinical science.

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