Identification and Partial Characterization of an EctoATPase Expressed by Human Natural Killer Cells†

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ABSTRACT: An extracellular membrane-associated ectoATPase has been identified on the human natural killer cell line NK3.3. The enzyme is distinct from other classes of ATPases, kinases, and phosphatases. NK3.3 ectoATPase demonstrated a K_m for ATP of 41 μM and a V_{max} of 0.2 μmol/min and required both Ca^{2+} and Mg^{2+} for maximal activity. Purine and pyrimidine nucleotides were competitive inhibitors of the catalytic reaction. Inhibition increased with the addition of increasing negative charge of the phosphate side chain and was also dependent on contributions from the nucleoside. NK3.3 ectoATPase activity was inhibited by reaction with the affinity label [*p*-(fluorosulfonyl)benzoyl]-5′-adenosine (5′-FSBA), which is shown to modify the enzyme at or near the ATP-binding domain. Photoaffinity labeling of intact NK3.3 cells with [α-^{32}P]-8-azidoATP demonstrated an ATP-binding protein of 68–80 kDa unique to NK3.3 cells. A positive correlation was observed between the ability of the various nucleotides to block photoincorporation into the 68–80-kDa protein and their ability to inhibit ectoATPase activity. NK3.3 cells which were made ectoATPase-deficient by reaction with 5′-FSBA demonstrated that this enzyme does not have a major role in the protection of this cytolytic effector cell from the possible lytic effects of extracellular ATP.

Adenine nucleotides are released into the extracellular space in millimolar concentrations from a variety of cells in response to activating stimuli and also as a result of tissue damage or cell death (Haslam & Cusak, 1981; Holmsen, 1985). Extracellular adenine nucleotides cannot cross the cell membrane, but rather mediate their physiologic effects through purinergic receptors (Luthje, 1989; Burnstock, 1990). The nucleotides thus act as signaling molecules to influence physiological processes, cell membrane integrity, and a variety of intracellular biochemical reactions (Ikehara et al., 1981). For example, extracellular ATP stimulates *in vivo* DNA synthesis in bone marrow and thymocytes but inhibits DNA synthesis in spleen, lymph nodes, and peripheral blood lymphocytes (Ikehara et al., 1981). Also, ATP can trigger histamine secretion from mast cells (Diamant & Kruger, 1967; Cockcroft & Gomperts, 1979a,b) and the secretion of specific granules from neutrophils and monocytes (Dubyak et al., 1988; Cockcroft & Stutchfield, 1980). In addition, ATP has been shown to cause both a cytostatic and a cytotoxic effect on some tumor cells (Rapaport, 1990) and to inhibit macrophage-mediated (Blanchard et al., 1991) and natural killer (NK)[1] cell-mediated (Henriksson, 1983; Schmidt et al., 1984) cytotoxicities. Alternatively, extracellular nucleotides can be degraded to the nucleoside by extracellular, cell-surface-located, nucleotide-metabolizing enzymes (ectonucleotidases) and be transported into the cell (Luthje, 1989; Che et al., 1992). The nucleotides can then be salvaged and enter into the regulation of *de novo* purine and pyrimidine biosynthesis or general cell metabolism.

Ectonucleotidases have been described on the cell surface of hepatocytes (Lin, 1990), renal cells (Sabolic et al., 1992), and lymphoid and erythroid cells (Ikehara et al., 1981). These cell types are in contact with the lumen of blood vessels where high local concentrations of extracellular nucleotides may exist. It has been postulated that ectonucleotidases, and more specifically ectoATPases, may provide protection for certain cell populations against the lytic effects of extracellular ATP (Fillipini et al., 1990). However, the role of these enzymes in a cell-mediated event has not been directly demonstrated.

In the immune system, ectoATPases are associated with B-cells (Kragballe & Ellegaard, 1978; Barankiewicz et al., 1989a,b; Barankiewicz & Cohen, 1990), macrophages (Steinberg et al., 1990), and CTL (Fillipini et al., 1990). NK cells, like CTL and macrophages, are cytotoxic effector cells in the immune system. NK cells, unlike CTL, can spontaneously kill certain susceptible tumor target cells in a major histocompatibility complex unrestricted manner and function in the natural resistance against cancer and a variety of infections.

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[1] Abbreviations: NK, natural killer; DMF, *N*,*N*-dimethylformamide; 5′-FSBA, [*p*-(fluorosulfonyl)benzoyl]-5′-adenosine; 8-azidoATP, 8-azidoadenosine 5′-triphosphate; AMPPNP, 5′-adenylyl imidodiphosphate; NEM, *N*-ethylmaleimide; CTL, cytotoxic T-lymphocytes; FBS, fetal bovine serum; BSA, bovine serum albumin; P_i, inorganic phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton(s); I_{50}, concentration of an inhibitor which gives half the maximal velocity.

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In this paper, evidence is presented that the human natural killer cell line NK3.3 possesses an enzymic activity capable of hydrolyzing extracellular ATP that fits the criteria to be classified as an ectoATPase, and possible roles of this enzyme in natural killer cell function are discussed.

MATERIALS AND METHODS

Materials. Nucleotides were purchased as the sodium salts from Sigma Chemical Co. (St. Louis, MO). Activated charcoal, HEPES, glucose, BSA (fraction V), and NEM were purchased from Sigma. [γ-^{32}P]ATP (triethylammonium salt in 50% aqueous ethanol) was purchased from Amersham (Arlington Heights, IL). [α-^{32}P]-8-AzidoATP was purchased from ICN (Irvine, CA). Sodium [^{51}Cr]chromate was purchased from NEN/Dupont Research Products (Boston, MA). RPMI 1640, penicillin/streptomycin, and L-glutamine were purchased from GIBCO BRL (Gaithersburg, MD). Lymphocult-T, a source of cellular growth factors, was purchased from Biotest Diagnostics (Denville, NJ). Rehatuin fetal bovine serum was purchased from Intergen Co. (Purchase, NY). All other chemicals were of reagent-grade purity.

Cell Culture and Maintenance. The NK3.3 cell line was a generous gift from Dr. Jackie Kornbluth, University of Arkansas, Little Rock, AR. This cell line was derived from an allogeneic mixed lymphocyte culture between normal human donors and is interleukin 2-dependent (Kornbluth et al., 1982; Leiden et al., 1988). These cells were maintained in RPMI 1640 with 100 units/mL penicillin/streptomycin, 2 mM L-glutamine, 15% FBS, and 15% Lymphocult-T at a concentration of 1×10^6 cells/mL at 37 °C in a humidified 5% CO_2/95% air atmosphere. To ensure that these cells function optimally under the above culture conditions, natural cytotoxicity was routinely determined against the NK-sensitive target cell line K562 (an erythromyeloid leukemia cell line purchased from American Type Culture Collection, Rockville, MD) using a ^{51}Cr-release assay (Brunner et al., 1976). Only cell populations exhibiting an acceptable cytotoxic activity of 50–90 lytic units (Pross et al., 1981) were used for experiments. NK3.3 cells were assayed for ectoATPase activity 24 h after feeding.

The human T-cell leukemia line Jurkat (B2.7 clone) has a T-helper surface phenotype and was obtained from Dr. Seth Lederman, Columbia University, College of Surgeons and Physicians. Jurkat cells were grown and maintained in RPMI 1640 containing 10% FBS. Jurkat cells do not possess ectoATPase activity (Barankiewicz & Cohen, 1990). Raji, a B-cell lymphoma deficient in ectoATPase activity (Barankiewicz & Cohen, 1990), was purchased from ATCC and grown in RPMI 1640 containing 10% FBS. Jurkat and Raji were assayed as negative controls.

Assay of ATPase Activity. ATPase activity was measured using [γ-^{32}P]ATP as substrate and counting the amount of [^{32}P]P_i released in supernatants after precipitation of nucleotides with activated charcoal (Reddy et al., 1978). The reaction buffer consisted of 10 mM HEPES (pH 7.4) containing 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 1% BSA. The final concentration of ATP was 0.3 mM and contained 0.3 μCi of [γ-^{32}P]ATP. The final assay volume was 200 μL and contained 1×10^4 cells. Alternatively, the reaction was carried out in RPMI 1640 medium, pH 7.4, without the addition of BSA. Concentrations of divalent metal ions, ligands, and nucleotides were varied accordingly as described in the text. The assay mixture was incubated at 37 °C for 20 min and the reaction stopped by the addition of 0.5 mL of cold 20% (w/v) activated charcoal in 1.0 M HCl. The assay tubes were incubated on ice for 10 min and centrifuged at 10000g for 10 min to pellet the charcoal. Aliquots (0.2 mL) of the supernatant containing the released [^{32}P]P_i were transferred to scintillation vials containing 2.5 mL of Biofluor scintillation fluid (Amersham), and the radioactivity was determined using a Beckman LS 5801 scintillation counter. All assays were performed in triplicate and reported as the mean ± standard deviation.

Before addition to the assay, cells were centrifuged and resuspended either in a 10 mM HEPES buffer, pH 7.4, containing 135 mM NaCl and 5 mM KCl or in RPMI 1640. The viability of NK3.3 cells used for the assay of ectoATPase activity was routinely >98% by trypan blue exclusion, and the spontaneous ^{51}Cr release was <10%.

Modification of NK3.3 with 5′-FSBA. 5′-FSBA was prepared by the method of Pal et al. (1975). NK3.3 cells were suspended in RPMI 1640 medium at a concentration of 5×10^5 cells/mL and modified by 5′-FSBA solubilized in DMF at 37 °C in the presence or absence of various nucleotides as described in the text. The final concentration of DMF was held constant at 2.5%. After incubation, cells were pelleted by centrifugation of the cells and washed once with RPMI 1640 to remove excess 5′-FSBA. Cells were then resuspended in RPMI 1640 and assayed as described above.

Photoaffinity Labeling of NK3.3 Cells and SDS–PAGE. NK3.3 cells (1×10^7 cells/100 μL) or Jurkat cells (1×10^7 cells/100 μL) were suspended in RPMI 1640 medium containing 25 μM 8-azidoATP and 2 μCi of [α-^{32}P]-8-azidoATP and irradiated for 5 min at 366 nm and 1 cm at room temperature using a hand-held UV lamp (Blakray lamp Model UVL-21; Ultraviolet Products, Inc., San Gabriel, CA). The photolysis reaction was quenched by the addition of DTT to a final concentration of 10 mM (Cartwright et al., 1976; Wower et al., 1989). The membrane fraction was isolated (Trevillyan et al., 1985), and proteins were separated on a 10% polyacrylamide gel by SDS–PAGE (Laemmli, 1970). The photolabeled proteins which incorporated the radioactive label were visualized by autoradiography using gas-hypersensitized Kodak Xomat AR film (Phillips et al., 1986).

ATP Cytotoxicity Assays. NK3.3 cells loaded with sodium [^{51}Cr]chromate were modified by 5′-FSBA as described above. Control cells were incubated under identical conditions with the exception that no 5′-FSBA was included. The cells were pelleted by centrifugation, washed, and incubated in RPMI 1640 (without FBS) at a density of 5×10^5 cells/mL in a final volume of 200 μL containing the ATP concentrations listed in the text. The spontaneous ^{51}Cr release was determined as described previously (Brunner et al., 1976).

RESULTS

Identification of Ectonucleotidase Activity on NK3.3 Cells. NK3.3 cells were incubated in the standard reaction buffer or RPMI 1640 medium with [γ-^{32}P]ATP and the extracellular products analyzed. Figure 1 shows that after a 20-min incubation, followed by charcoal precipitation of the nucleotides, a significant amount of radioactivity was contained in the supernatant. The hydrolysis of [γ-^{32}P]ATP was linear for at least 30 min, and no radioactivity was released above background levels in assays containing no cells. In separate experiments, the reaction products were analyzed prior to charcoal precipitation by thin-layer chromatography using EM silica gel 60 F-254 plates (containing a fluorescent indicator) and a solvent system of isobutyric acid/ammonium hydroxide/water, 66/1/33 (v/v). This demonstrated both ADP and ATP in samples which were incubated with NK3.3

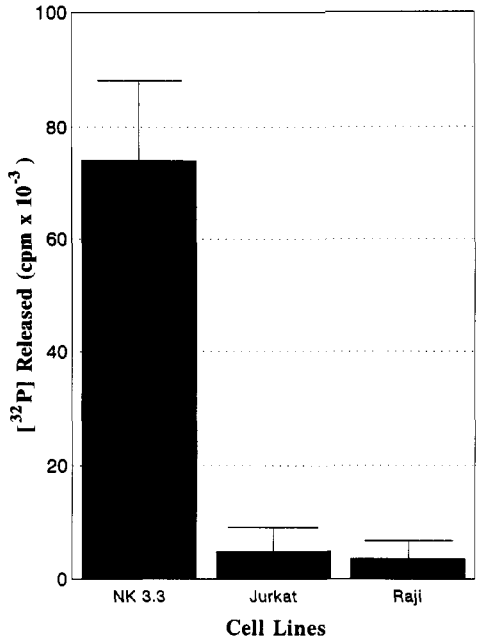

FIGURE 1: EctoATPase activity in NK3.3, Jurkat, and Raji cells. Cells were assayed at a concentration of 5×10^5 cells/mL for ectoATPase activity using the standard assay conditions as described under Materials and Methods. Results are the mean ± standard deviation of at least three experiments performed in triplicate.

cells (data not shown); neither AMP nor adenosine could be detected. Thus, these results suggest the presence of an ATPase activity associated with NK3.3 cells.

To further characterize the observed ATPase activity, the human T-cell leukemia line Jurkat and the human B-cell lymphoma line Raji [both ectoATPase-deficient (Barankiewicz & Cohen, 1990)] were assayed as negative controls. The release of $[^{32}P]P_i$ from NK3.3 cells was about 15-fold higher than that exhibited by Jurkat and Raji cells, the latter two of which were not statistically significant from background levels (Figure 1). In addition, the cells were intact at the end of the assay as demonstrated by trypan blue exclusion (>90%) and spontaneous ^{51}Cr release from ^{51}Cr-labeled NK3.3 cells (<10%). Furthermore, to examine whether this ATPase activity was due to a secreted enzyme or an intracellular enzyme released through "leaky" membranes, NK3.3 cells were incubated for 20 min in the standard assay buffer without ATP and pelleted. ATPase activity in the supernatant assay buffer following removal of the cells was <8% of the total ATPase activity; assay of the cell pellet demonstrated >92% of the total ATPase activity was associated with this fraction. These assays ensured that the ATPase activity observed did not result from the release of intracellular enzymes.

The extracellular membrane association of ectoATPase was further demonstrated by isolation and assay of the cell membrane fraction. This fraction possessed significant ATPase activity (7500 cpm of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$), whereas membranes from Raji cells did not release any radioactivity from $[\gamma\text{-}^{32}P]ATP$. Thus, these data confirm the extracellular membrane association of the NK3.3 ectoATPase activity.

Characterization of EctoATPase Activity. Table I shows that inhibitors of plasma membrane ATPases (ouabain and VO_4^-), vacuolar ATPases (NO_3^- and NEM) and mitochondrial ATPases (VO_4^- and N_3^-), did not significantly inhibit the NK3.3 ATPase activity when included in assays at concentrations of 0.1–100 mM. Furthermore, 10 mM F^- (a phosphatase inhibitor) was not inhibitory. These data demonstrate that NK3.3 ectoATPase activity is not a result of the activity of other ATPases or phosphatases.

Table I: Characterization of EctoATPase Activity

variable in assay conditions	% control
control[a]	100
ouabain (1 mM)	97 ± 4
VO_4^- (100 μM)	98 ± 9
VO_4^- (500 μM)	87 ± 8
NO_3^- (100 mM)	101 ± 8
NEM (10 μM)	114 ± 6
N_3^- (1 mM)	76 ± 8
F^- (10 mM)	91 ± 12
$-Ca^{2+}$, $-Mg^{2+}$	78 ± 6
$-Mg^{2+}$ only	98 ± 4
$-Ca^{2+}$ only	91 ± 15
RPMI 1640[b]	120 ± 26
$-Ca^{2+}$, $-Mg^{2+}$, +EDTA (2 mM)	47 ± 2
$-Ca^{2+}$, $-Mg^{2+}$, +EGTA (2 mM)	28 ± 5
$-Ca^{2+}$, $-Mg^{2+}$, +EDTA (1 mM), +EGTA (1 mM)	4.7 ± 6.7

[a] Control ectoATPase activity was determined using 1×10^4 cells in the standard assay buffer as described under Materials and Methods which contained 2 mM Ca^{2+} plus 2 mM Mg^{2+}: this activity was considered to be 100%. [b] RPMI 1640 is a phosphate buffer defined medium which contains 0.6 mM Ca^{2+} plus 0.4 mM Mg^{2+}. All assays were performed a minimum of 3 times. Data represents the mean ± the standard deviation.

Dependence of EctoATPase Activity on Divalent Metal Ions. The activity of ectoATPase per 1×10^4 cells using the standard reaction assay which contains 2 mM Ca^{2+} and 2 mM Mg^{2+} was defined as 100% (Table I). Metal dependency of ectoATPase activity was observed when assayed in the absence of exogenous Ca^{2+} or Mg^{2+}. Omission of both Ca^{2+} and Mg^{2+} from the standard assay buffer decreased ectoATPase activity by 22% (Table I). The enzymic activity remained at control levels when either 2 mM Ca^{2+} or 2 mM Mg^{2+} alone was present in the assay (Table 1). To examine alternate assay conditions, the defined medium RPMI 1640 was used as the reaction medium. RPMI 1640 is a phosphate-buffered medium which contains both Ca^{2+}(0.6 mM) and Mg^{2+}(0.4 mM). No significant difference in ectoATPase activity was observed when either assay buffer was used (Table I).

Addition of EDTA to the standard reaction buffer without Ca^{2+} or Mg^{2+} decreased enzymic activity by 53%, while EGTA decreased enzymic activity by 72% (Table I). Furthermore, addition of both metal chelators to the assay mixture resulted in near complete inhibition of enzymic activity (Table 1). These results suggest that the NK3.3 ectoATPase is a (Ca^{2+}–Mg^{2+})ectoATPase.

Dependence of EctoATPase Activity on ATP Concentration. An increase in enzymic activity was observed with increasing substrate concentration which approaches saturation (data not shown), suggesting that NK3.3 ectoATPase activity follows Michaelis–Menten kinetics with respect to ATP. From a double-reciprocal plot of these data, the K_m and V_{max} of this enzyme were calculated to be 41 ± 1 μM and 0.2 ± 0.02 μmol/min, respectively (Table II).

Nucleotide Specificity of EctoATPase. EctoATPases possess broad nucleotide substrate specificities. Since other nucleotidases may also exist on the cell surface, direct evidence for the hydrolysis of other nucleotides by ectoATPase cannot be demonstrated unequivocally. However, by competition assay, the relative affinity of ectoATPase for various nucleotides can be assessed. Figure 2 shows that ADP and ITP have strong inhibitory effects against ATP hydrolysis by ectoATPase with an $I_{50} \approx 0.6$ mM and a maximum inhibition of 90% at a 4 mM concentration of either nucleotide. GTP,

Table II: Kinetic Parameters of Nucleotides on NK3.3 EctoATPase Activity[a]

nucleotide	kinetic constant, μM[b]	nucleotide	kinetic constant, μM[b]
ATP	41 ± 1 (K_m)	ITP	69 ± 4 (K_i, competitive)
AMPPNP	50 ± 8 (K_i, competitive)	GTP	319 ± 98 (K_i, competitive)
8-azidoATP	33 ± 13 (K_i, competitive)	UTP	346 ± 95 (K_i, competitive)
ADP	74 ± 30 (K_i, competitive)	CTP	332 ± 131 (K_i, competitive)
AMP	890 (K_i, competitive)[c]		

[a] Assays were performed 3 times in triplicate in the standard assay buffer or RPMI 1640 medium as described under Materials and Methods. [b] K_i's were calculated by Dixon plot with ATP concentrations of 0.075, 0.15, and 0.3 mM. Data represent the mean ± the standard deviation of at least three experiments. Parentheses indicate the type of kinetic constant observed. [c] The K_i for AMP was calculated by Dixon plot as a competitive inhibitor using a single ATP concentration (data shown in Figure 2) and using the K_m listed in this table.

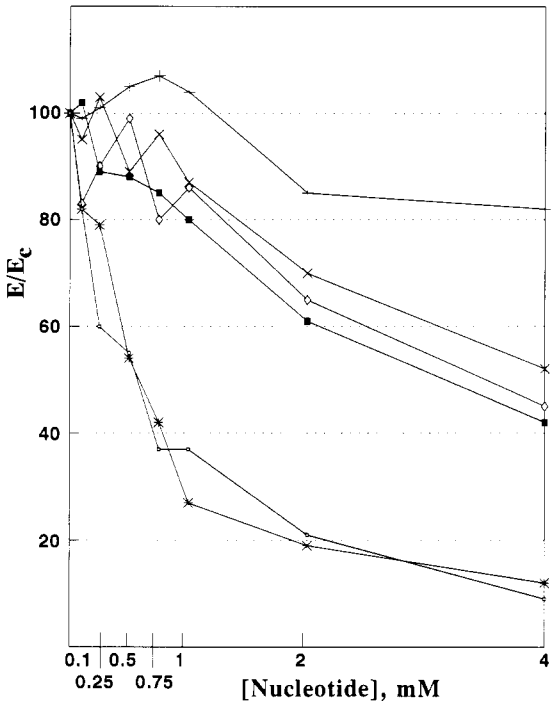

FIGURE 2: Nucleotide inhibition of ectoATPase activity. EctoATPase activity was determined using 1×10^4 NK3.3 cells in the standard assay buffer containing various concentrations of ADP (□), AMP (+), ITP (*), GTP (■), UTP (×), CTP (♦). Activity was assayed using the standard assay conditions as described under Materials and Methods. Residual activity was calculated from the ratio of the measured enzymic activity for the reaction mixture containing the inhibitory nucleotides (E) to the measured enzyme activity for the corresponding control reaction (E_c). Standard errors of the data points were <10%.

CTP, and UTP show a lesser, but significant, inhibitory effect on ATP hydrolysis with an $I_{50} \approx 4$ mM. AMP, in contrast, inhibits enzymic activity poorly (<20% at 4 mM).

Dixon plot analysis demonstrated that AMPPNP, 8-azidoATP, ADP, and ITP were competitive inhibitors with respect to ATP. The K_i's for AMPPNP and 8-azidoATP were approximately equal to the K_m for ATP (Table II). The K_i's for ADP and ITP were approximately 2-fold higher than the K_m for ATP (Table II). GTP, UTP, and CTP were also competitive inhibitors, but with K_i's about 10-fold higher than the K_m for ATP (Table II). In this system, the I_{50} for each nucleotide was an overestimation of the kinetically derived K_i (Naqui, 1983).

Reaction of 5′-FSBA with NK3.3 EctoATPase. The nucleotide affinity label 5′-FSBA was incubated with NK3.3 cells in order to further characterize this enzymic activity. Figure 3 illustrates a time-dependent decrease in enzymic activity upon reaction with 1 mM 5′-FSBA with a maximum inhibition of about 80% after 1 h of incubation. No further increase in activity was observed by prolonging the incubation time to 90 min, by using higher concentrations of the reagent, or by a second addition of the reagent after the initial 1-h incubation. The reaction exhibited a pseudo first-order rate constant of 0.0337 min^{-1} with 1 mM 5′-FSBA (Figure 3 inset). In addition, no loss of ectoATPase activity was observed when NK3.3 cells were incubated under identical conditions in the presence of 2.5% DMF (used for the solubilization of the affinity label). The viability of NK3.3 cells was >90% after incubation with 5′-FSBA or 2.5% DMF alone (control) as assayed by trypan blue exclusion, and spontaneous ^{51}Cr release was <10%. This rules out the possibility that the observed enzymic activity was an intracellular ATPase released through "leaky" membranes.

The rate constant was linearly dependent of the reagent concentration from 0.1 to 1 mM 5′-FSBA (Figure 4) with a second-order rate constant of 0.0334 min^{-1} mM^{-1}. From a double-reciprocal plot of the data in Figure 4 (Figure 4, inset), the K_i for 5′-FSBA can be calculated using the equation:

$$1/k_{obs} = 1/k_{max} + (K_i/k_{obs})(1/R)$$

where $K_i = (k_{-1} + k_{max})/k_1$ and represents the concentration of reagent giving half of the maximal inactivation rate, $1/k_{max}$ is the maximum observed rate constant, and R is the reagent concentration (Huang & Colman, 1984). From the line shown in the Figure 4 inset, k_{max} was calculated to be 0.108 min^{-1}, and the K_i for 5′-FSBA was 2.75 mM.

Effect of Adenine Nucleotide Ligands on the Rate Constant for the Reaction of 5′-FSBA. The specificity of 5′-FSBA for NK3.3 ectoATPase was determined by measuring the effect of ligands on the reaction rate of the modification reaction. When AMPPNP was included in the modification reaction [at a concentration at least 10-fold higher than its K_i (Table II)], the rate of inactivation of ectoATPase by modification by 5′-FSBA was completely eliminated (Table III). Similarly, when ADP was included in the modification reaction, ectoATPase activity was not altered when assayed following removal of the affinity label and competing nucleotide. However, AMP caused a reduction in the rate constant of about 2.5-fold. These results are consistent with the nucleotide competition experiments (Figure 2) and suggest that 5′-FSBA is binding at or near the ATP-binding site of NK3.3 ectoATPase.

Identification of a Unique ATP-Binding Protein on NK3.3 Cells by Photoaffinity Labeling. The photoaffinity label 8-azidoATP was determined to be a competitive inhibitor of the enzyme reaction with a K_i of 33 μM (Table II). NK3.3 cells (1×10^7 cells/200 μL) were incubated with 25 μM 8-azidoATP containing 2 μCi [α-^{32}P]-8-azidoATP and irradiated at 366 nm for 5 min at room temperature. Autoradiography of the proteins from the membrane fraction separated by SDS–PAGE demonstrated two major protein bands were photolabeled by [α-^{32}P]-8-azidoATP: a diffuse band of molecular mass ~68–80 kDa and a band at 43 kDa

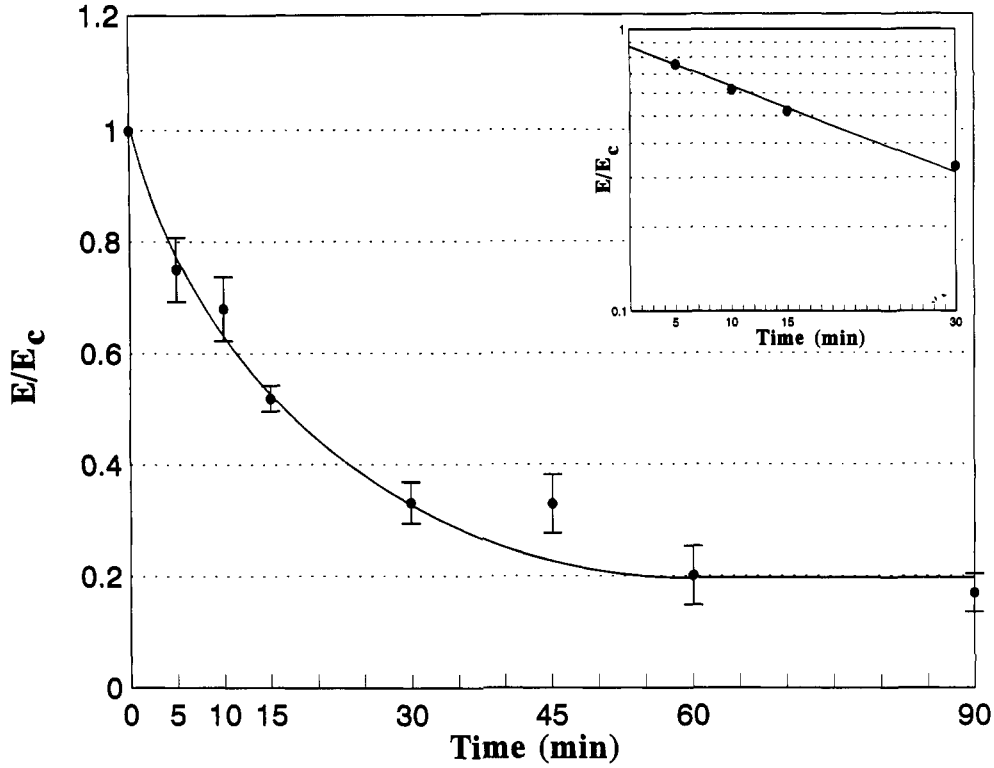

FIGURE 3: 5′-FSBA inhibition of NK3.3 ectoATPase activity. NK3.3 cells (5×10^5 cells/mL) suspended in RPMI 1640 were incubated in the presence or absence (control) of 5′-FSBA, pelleted by centrifugation, and washed with RPMI 1640 medium. EctoATPase activity was assayed as described under Materials and Methods. Residual activity was calculated from the ratio of the measured enzymic activity for the reaction mixture at the time indicated (E) to the measured enzyme activity for the corresponding control reaction (E_c). Inset: Determination of the pseudo-first-order rate constant from the decrease in observed velocity. The rate constant was determined to be 0.0337 min^{-1} from the slope of this semilogarithmic plot for the first 30 min of the modification reaction.

(Figure 5A). In contrast, photolabeling Jurkat cells, which do not possess ectoATPase activity (Figure 1), resulted in the modification of only a 43-kDa major protein (Figure 5A). No incorporation of radioactivity was observed in nonphotolyzed samples (Figure 5B).

Evidence supporting the identity of the 68–80-kDa protein as the ectoATPase is demonstrated by nucleotide competition of the photoincorporation of 8-azidoATP. Figure 5B shows that covalent modification by 5′-FSBA or the presence of the competing nucleotides AMPPNP and ADP all significantly reduce the photoincorporation of radioactivity into the 68–80-kDa band. In contrast, AMP, GTP, UTP, and CTP, which are weak inhibitors of the ectoATPase, do not effectively protect against the photoincorporation of radioactivity (Figure 5B,C). In no experiment was a reduction in the photoincorporation of radioactivity into the 43 kDa band observed.

Quantitation of the photoincorporation of [α-^{32}P]-8-azidoATP into the 68–80-kDa protein band in the presence or absence of nucleotide competitors is shown in Table IV. Modification of NK3.3 cells by 5′-FSBA resulted in a 74% reduction in the [α-^{32}P]-8-azidoATP photolabeling and is consistent with the extent of ectoATPase inhibition observed with 5′-FSBA (Figure 3). Similarly, AMPPNP successfully competed the photoaffinity label, resulting in a 73% reduction of photoincorporation of radioactivity at a concentration 10-fold higher than its K_i, and an 86% decrease at a concentration 40-fold higher than its K_i. The level of inhibition of photoincorporation of [α-^{32}P]-8-azidoATP into the 68–80-kDa protein by 1 mM ADP, AMP, GTP, ITP, UTP, or CTP paralleled the level of inhibition of ectoATPase activity by these different nucleotides at 1 mM concentrations (compare Figure 2 and Table II). No other protein band exhibited competition for the photoaffinity label by the different nucleotides.

Role of EctoATPase in the Protection of NK3.3 from Lytic Effects of Extracellular ATP. The role of the NK3.3 ectoATPase in protecting this effector cell from the potential lytic effects of extracellular ATP was assessed by modifying ^{51}Cr-loaded NK3.3 cells with 1 mM 5′-FSBA for 1 h. Figure 6 demonstrates that control ^{51}Cr-loaded NK3.3 cells did not release any significant levels of radioactivity (<4%) following addition of up to 10 mM exogenous ATP to the assay and incubation for 4 h at 37 °C. Similarly, modification of the ^{51}Cr-loaded NK3.3 cells by 5′-FSBA inhibited >80% of the ectoATPase activity, and <13% specific ^{51}Cr release was observed with 10 mM exogenous ATP. High concentrations of ATP do not therefore appear to be cytolytic toward NK3.3 cells made ectoATPase-deficient by 5′-FSBA modification. It is concluded that the major role of ectoATPase on the surface of NK3.3 cells is not to protect this effector cell population from the potentially lethal effects of high extracellular ATP concentrations.

DISCUSSION

Characterization of the EctoATPase. Ectoenzymes, by definition, are membrane-bound enzymes with the catalytic site facing the extracellular environment. The ATPase activity expressed by the human natural killer cell line NK3.3 under investigation here fulfills the five interdependent criteria to provide evidence that it is specifically an ectoATPase (Karnovsky, 1986). First, intact cells were used to detect the activity of an ectoenzyme, and the cells remained intact for the length of the assays. Second, the substrate of the reaction, ATP, does not penetrate the cells. Third, the products of the enzymic reaction, ADP and P_i, were detected in the extracellular environment. Fourth, the enzyme activity under investigation was not secreted by the cells or released through "leaky" membranes. Fifth, an impermeant inhibitor, 5′-FSBA (Ben-

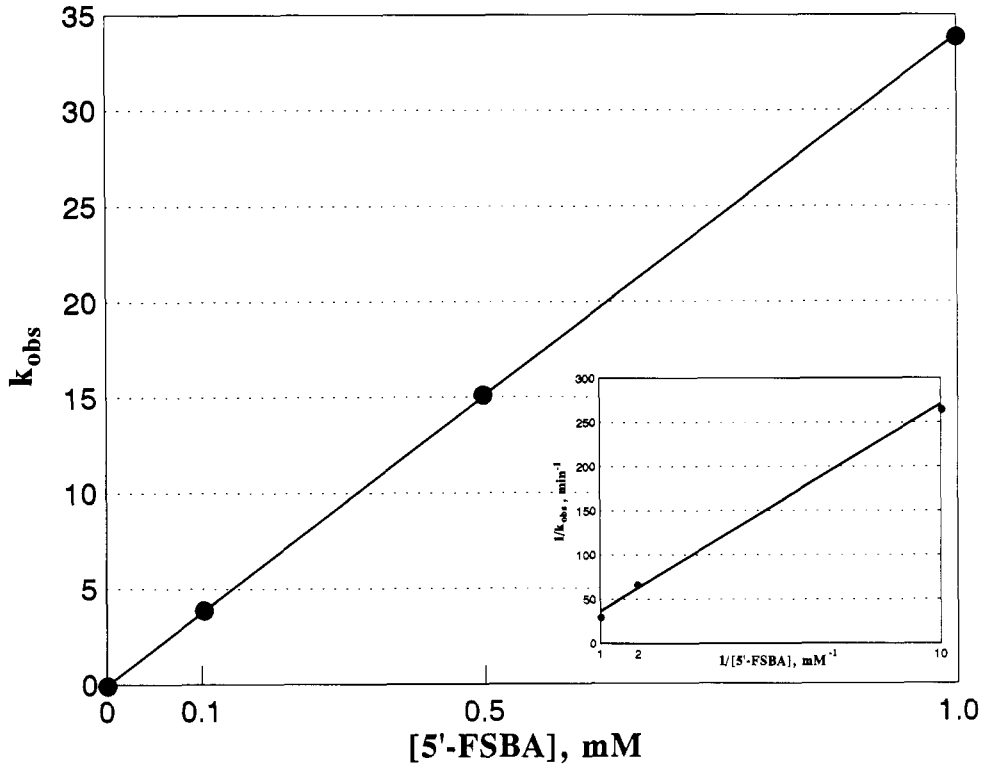

FIGURE 4: Effect of 5′-FSBA concentration on the rate of inactivation of NK3.3 ectoATPase activity. NK3.3 cells (5×10^5/mL) suspended in RPMI 1640 medium were incubated for 1 h at 37 °C with varying concentrations of 5′-FSBA (with a final DMF concentation of 2.5%) as indicated. Control cells were incubated under identical conditions except that no 5′-FSBA was present in the reaction mixture. EctoATPase activity was assayed as described under Materials and Methods. Residual activity was calculated from the ratio of the measured enzymic activity for the reaction mixture containing 5′-FSBA (E) to the measured enzyme activity for the control reaction (E_c), and the k_{obs} was determined as described in the Figure 3 inset. The second-order rate constant was determined to be 0.0334 min^{-1} mM^{-1} from the slope of this plot. Inset: Determination of the K_i of 5′-FSBA. The K_i of 5′-FSBA was determined from this plot to be 2.75 mM as described in the text.

Table III: Effect of Adenine Nucleotides on the Rate Constant for Inactivation by 5′-FSBA[a]

ligand added to modification reaction	k_{obs} ($\times 10^3$), min^{-1}
none	33.7
AMPPNP (0.5 mM)	0
ADP (1 mM)	0
AMP (1 mM)	14.3

[a] NK3.3 cells were incubated with 1 mM 5′-FSBA in the presence or absence of nucleotide ligands as described in Figure 3. Following the modification reaction, the cells were pelleted and assayed for residual ectoATPase activity as described under Materials and Methods. The pseudo-first-order rate constant observed (k_{obs}) was determined as described in the Figure 3 inset.

nett et al., 1978; Colman, 1989), inhibited the enzyme activity on intact cells by modifying the enzyme at or near the ATP-binding domain.

In addition to the above criteria, ectoATPases also have other common characteristics that are also shared by the NK3.3 ectoATPase. Namely, ectoATPases are Ca^{2+}- and/or Mg^{2+}-dependent, although the enzyme here functioned with either divalent cation. The fact that the effect of each metal ion was not additive suggests the presence of a single enzyme activity. In addition, ectoATPases are not inhibited by common inhibitors of other known ion-transporting ATPases, phosphatases, and kinases. This rules out the possibility that the [^{32}P]P_i observed in the supernatant was due to an ectokinase/phosphatase mechanism whereby the [^{32}P]P_i would be transferred to a membrane target and then released by an extracellular phosphatase.

The kinetic constants reported for this class of enzymes have been few and variable. The K_m values for ATP of ectoATPases have ranged from 27–28 μM for the enzyme from guinea pig polymorphonuclear leukocytes (DePierre & Karnovsky, 1974) to 210 μM for the rat liver enzyme (Lin, 1985) to 350 μM for the murine macrophage enzyme (Barankiewicz et al., 1989b). The K_m for ATP of NK3.3 ectoATPase is 41 μM and is comparable to the enzyme from polymorphonuclear leukocytes. In addition, the value here is in close agreement with the K_m of other ectonucleotidases which ranges from 10 to 40 μM (DePierre & Karnovsky, 1974).

EctoATPases also have broad nucleotide specificity. The rat liver enzyme has been shown to hydrolyze other nucleotide triphosphates (Lin, 1990). Because of the possibility of other nucleotidases on NK3.3 cells, to conclusively establish the nucleotide substrate specificity, a purified enzyme must be used. However, it was demonstrated here that the NK3.3 ectoATPase also binds ADP and ITP, each with about equal affinity with respect to ATP and serving as competitive inhibitors inhibitors of the reaction. The enzyme is also capable of binding other purine and pyrimidine triphosphates but with a severalfold lower affinity than ATP. The apparent K_i values of the NK3.3 ectoATPase for these nucleotides are consistent with the range of values for the ectoATPase from CTL, 100–500 μM (Fillipini et al., 1990). The kinetic constants of other nucleotidases for these nucleotides have not been reported.

A few generalizations can be made concerning the nature of the active site of NK3.3 ectoATPase. NK3.3 ectoATPase requires a highly charged moiety. This was observed with the nucleotide triphosphates all having an inhibitory effect. In addition, the diphosphate moiety is probably also sufficient since ADP was also a strong inhibitor of the enzyme, but a

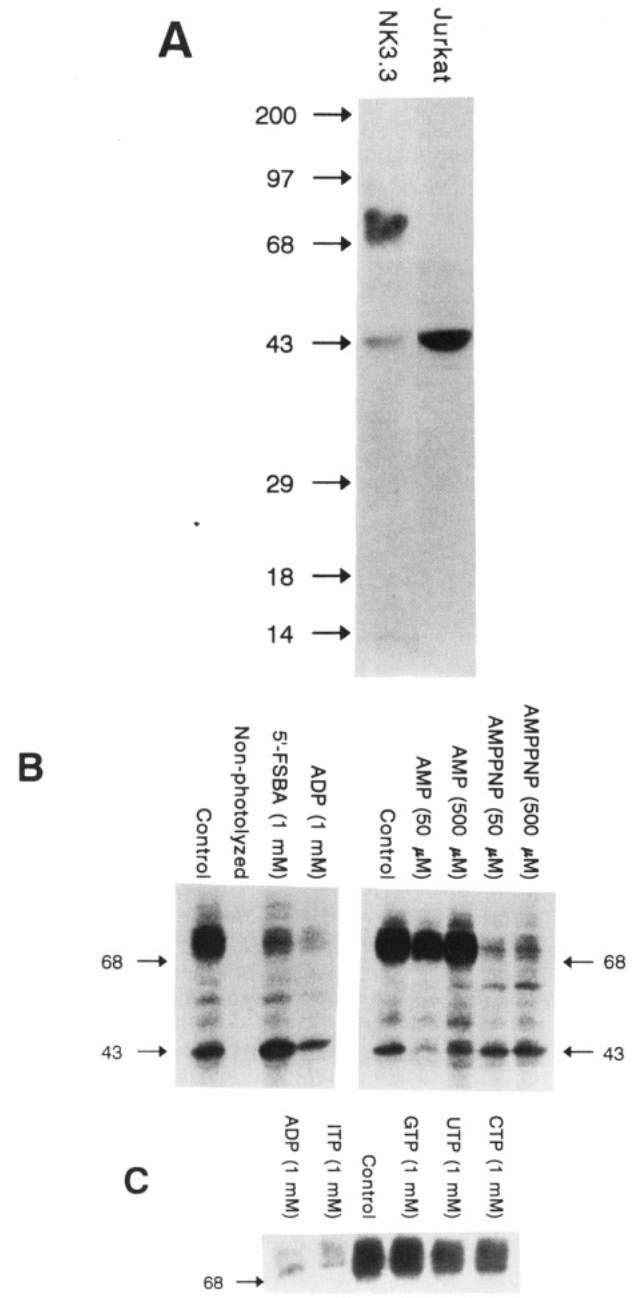

FIGURE 5: Photoaffinity labeling of NK3.3 cells with 8-azidoATP. (A) Identification of an ATP-binding protein unique to NK3.3 cells. NK3.3 cells ($1 \times 10^7/100\ \mu L$) or Jurkat cells ($1 \times 10^7/100\ \mu L$) were suspended in RPMI 1640 medium containing 25 μM 8-azidoATP containing 2 μCi of [α-^{32}P]-8-azidoATP and photolyzed as described under Materials and Methods. Membrane proteins were separated on a 10% polyacrylamide gel by SDS–PAGE (Laemmli, 1970). The photolabeled proteins were visualized by autoradiography using gas-hypersensitized film (Phillips et al., 1986). Molecular mass standards (kDa) label the vertical axes. (B) Adenine nucleotide competition of the photoaffinity probe. NK3.3 cells were preincubated 5 min on ice with the competing nucleotide at the concentrations indicated. The photoaffinity label was then added to the incubation mixture and photolyzed, and the products were analyzed as described above. (C) Purine and pyrimidine nucleotide competition of the photoaffinity probe. The photoaffinity labeling was performed as described above with the competing nucleotide at the concentrations indicated. The products were analyzed as described above.

monophosphate does not provide enough interaction to bind and be a potent inhibitor. The differential inhibition observed here suggests that the cooperation of both the nucleoside and polyphosphate moieties appears to be important in determining the binding specificity of this enzyme.

Table IV: Effect of Competing Nucleotides on the Photoincorporation of [α-^{32}P]-8-AzidoATP[a]

ligand added	^{32}P incorporated (% control)	ligand added	^{32}P incorporated (% control)
none	100	AMP (1 mM)	59
none (nonphotolyzed)	0	adenosine (1 mM)	81
5'-FSBA (1 mM)	26	ITP (1 mM)	47
AMPPNP (500 μM)	27	GTP (1 mM)	99
AMPPNP (2 mM)	14	UTP (1 mM)	75
ADP (1 mM)	49	CTP (1 mM)	74
AMP (500 μM)	109		

[a] NK3.3 cells (10^7) suspended in 200 μL of RPMI 1640 were photolabeled with [α-^{32}P]-8-azidoATP and photolyzed as described under Materials and Methods and in Figure 5. Membrane proteins were separated by SDS–PAGE on a 10% polyacrylamide gel. ATP-binding proteins were identified by autoradiography using gas-hypersensitized film (Phillips et al., 1986). The 68–80-kDa protein band of each lane was cut out and counted. The 43-kDa protein band was cut out and counted and used as an internal standard. The incorporation was normalized to the radioactivity contained in each respective 43-kDa band. The sample containing no added ligands was considered to have an efficiency of photoincorporation of 100%. 100% ^{32}P incorporation in noncompeted control cells varied between experiments with a range of 1200–1500 disintegrations/20 min. Data represent an average of two determinations.

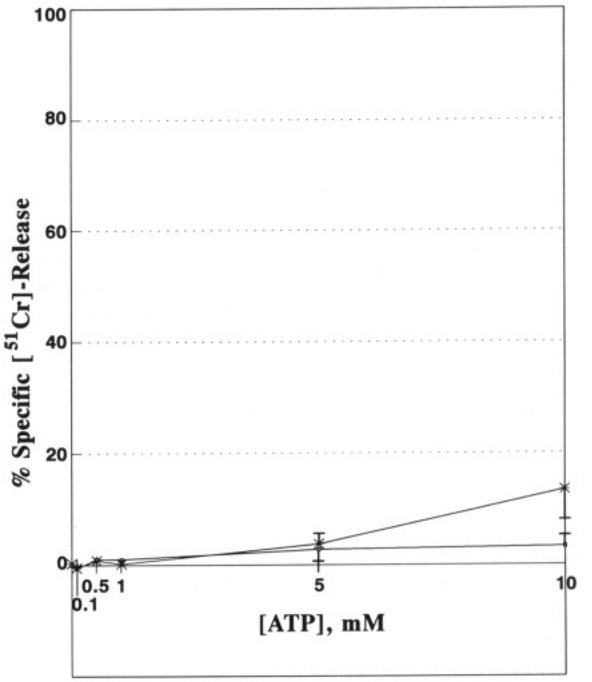

FIGURE 6: Effect of exogenous ATP concentration on the specific ^{51}Cr release from NK3.3 cells modified by 5'-FSBA. ^{51}Cr-loaded NK3.3 cells were modified by 5'-FSBA as described under Materials and Methods. 5'-FSBA-modified (*) and unmodified (□) cells were incubated for 4 h at 37 °C at a density of 1×10^4 cells/200 μL containing the ATP concentrations indicated. Cells were pelleted by centrifugation, and the radioactivity released into the supernatant was quantitated by γ counting. Data points are the mean of two determinations each performed in triplicate.

A putative NK3.3 ectoATPase protein was identified herein by photoaffinity labeling with 8-azidoATP migrated on SDS–PAGE as a diffuse band with a similar molecular mass of 68–80 kDa. Photoincorporation of radioactivity into only this band was reduced by competition with the nucleotide inhibitors of ectoATPase activity, supporting the identity of this protein band as the NK3.3 ectoATPase.

Role of EctoATPase in NK Cell Function. The role of ectonucleotidases in cell function has yet to be directly demonstrated, although several hypotheses have been sug-

gested. For example, the rat liver canalicular membrane ectoATPase has been suggested to degrade the extracellular nucleotide to adenosine for intracellular transport and conservation of the nucleoside (Che et al., 1992). If the above were a potential role of this enzyme in the NK system, one would expect to find products of an ectoADPase and ecto-5′-nucleotidase, namely, AMP and adenosine, respectively. Since neither of these two products could be detected, it is unlikely that NK3.3 ectoATPase functions in a nucleotide salvage pathway.

In thymocytes (Lin, 1985) and smooth muscle (Gerwins & Fredholm, 1992), extracellular ATP increased the intracellular Ca^{2+} level. This correlated with the degradation of ATP, and nonhydrolyzable ATP analogues did not stimulate Ca^{2+} uptake. There, however, was no direct characterization of the ATP degrading activity as an ectoATPase. In this regard, no effect on Ca^{2+} mobilization could be observed when NK3.3 cells were incubated with concentrations of exogenous ATP $\leq$1 mM (data not shown). This result demonstrates that ectoATPase does not appear to function to modulate ligands for a purinergic P_{2y} receptor (Burnstock, 1990). Furthermore, NK3.3 ectoATPase does not appear to function as a P_{2z} receptor [i.e., a receptor forming transmembrane pores allowing solutes <900 daltons to pass (Burnstock, 1990)] since the cells were not permeable to trypan blue (M_r < 900) following the ATPase assay.

An attractive hypothesis has been set forth for lymphoid effector cells in which ATP has been implicated to be part of the lethal hit that is delivered by effector cells (Fillipini et al., 1990). In this regard, indirect evidence has been used to suggest that CTL ectoATPase protects this effector cell population from the potentially lytic effects of extracellular ATP. Through affinity labeling studies, it was demonstrated herein that a major a role of this enzyme in this capacity is not feasible for NK3.3 cells since cells made ectoATPase-deficient by reaction with 5′-FSBA did not result in altered membrane permeabilization when challenged with high concentrations of extracellular ATP. Furthermore, ATP has not been found in lytic granules isolated from NK cells (Z. Brahmi, personal communication).

EctoATPase activity may function in mediating the effects of ATP on human NK lytic activity. In this regard, we have preliminary evidence that 5′-FSBA inhibits NK3.3 natural cytotoxicity with a similar dose dependency as observed for inhibition of NK3.3 ectoATPase (K. E. Dombrowski, unpublished results). The system described here will allow the elucidation of such a mechanism of ectoATPase action in a defined cell system.

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