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Inhibition of a Phosphodiesterase III in the Lysis-Sensitive Target-Induced Elevation of Cyclic AMP (cAMP) in Human Natural Killer Cells

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ABSTRACT. Natural killer (NK) cells are lymphocytes that are capable of destroying tumor cells and virally infected cells (cytolysis) without prior sensitization. When cyclic AMP (cAMP) is elevated artificially in NK cells, it is a potent inhibitor of their cytolytic function. Recently, we have shown that when NK cells are exposed to a range of lysis-sensitive (LS) tumor target cells, there is an increase in intracellular cAMP levels in the NK cells over a 60-min period. There is no increase in NK-cell cAMP in response to lysis-resistant (LR) tumor target cells. We determined that this cAMP elevation is due, in part, to an LS target-induced activation of adenylyl cyclase (AC), and that the AC-activation component appears to require a protein tyrosine kinase (PTK) activity. In the present study, we demonstrated that an LS target-induced inhibition of phosphodiesterase (PDE) is also contributing to the overall elevation of cAMP. Direct measurement of PDE activity showed an inhibition in lymphocytes that were exposed to LS targets but not in those exposed to LR targets. The inhibition of PDE activity was maximal by 30 min. Lymphocytes were exposed to targets and then lysed, so that PDE activity could be measured. Addition of class-selective inhibitors of PDE (at levels sufficient to completely block that class of PDE) to the lysate focused the measurement of PDE activity on those classes of PDE that were unaffected by the selective inhibitor. Using the PDE IV selective inhibitor rolipram and the PDE III selective inhibitors trequinsin and milrinone, we showed that a PDE III is being inhibited in lymphocytes by exposure to LS targets. As PDE III is known to be inhibited by elevated cyclic GMP (cGMP) levels, increased cGMP in NK cells following exposure to LS targets was a possible mechanism by which a PDE III in NK cells might be inhibited. However, when we measured cGMP levels in control and LS target-stimulated lymphocytes, we saw no change. BIOCHEM PHARMACOL **60**;4:499–506, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. NK cell; cAMP; adenylyl cyclase; phosphodiesterase; cGMP

NK† cells are lymphocytes that are capable of killing tumor cells, virally infected cells, and antibody-coated cells. NK cells are the earliest and possibly the predominant defense against tumor cells [1]. They appear to be responsible for limiting the spread of blood-borne metastases as well as limiting the development of primary tumors [2, 3]. NK cells also appear to have a central role in immune defense against viral infection, as evidenced by greatly increased incidence of viral infection in individuals where the NK subset of lymphocytes is completely absent [4, 5]. NK cells are defined by the absence of the T cell receptor/CD3 complex and the presence of CD56 and/or CD16 on their surface. They are able to lyse the above-mentioned target cells without prior sensitization, putting them at the forefront of lymphocyte defense against tumor and virally infected cells [1].

It has been known for many years that increased cAMP levels in NK cells inhibit their ability to lyse tumor target cells [6–11]. In those studies, cAMP was increased by the addition of cAMP analogues, forskolin treatment, or cholera toxin treatment. Physiologically, cAMP synthesis is catalyzed by a class of enzymes called ACs, and cAMP breakdown is catalyzed by PDE [12]. The balance of AC and PDE activities in a cell determines the level of cAMP.

Recently, we demonstrated that cAMP is elevated in NK cells following contact with LS tumor cells but not LR tumor cells [13]. These data showed that LS targets activate AC in NK cells. This AC activation required PTK activity but not PKC activity [13]. PTK activation is the earliest signal known to be generated in the cytolytic response of NK cells [14]. This is consistent with the notion that a signal involved in activation of the cytolytic response is also involved in stimulating the elevation of an inhibitory signal, cAMP (feedback inhibition of the cytolytic response). However, when we inhibited the activation of the AC component of the cAMP elevation by inhibiting PTK activity in the NK cells, there was still an LS target-

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† *Abbreviations*: NK, natural killer; cAMP, cyclic AMP; LS, lysis-sensitive; LR, lysis-resistant; AC, adenylyl cyclase; PDE, phosphodiesterase; IBMX, 3-isobutyl-1-methylxanthine; PTK, protein tyrosine kinase; PKC, protein kinase C; BCS, bovine calf serum; cGMP, cyclic GMP; and PKA, cyclic AMP-dependent protein kinase.

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stimulated increase in cAMP [13]. These findings indicated that LS targets might also induce an inhibition of NK-cell PDE activity as part of the mechanism by which they elevate cAMP and that this PDE inhibition was not dependent on PTK. In the present study, we tested whether PDE activity in NK cells is inhibited following activation of the lytic response by LS targets.

The role(s) for a physiologically stimulated elevation of cAMP in the overall regulation of NK-cell cytolytic function is not clear. However, an increase in an inhibitory signal such as cAMP in response to activation of the lytic pathway would provide a potential feedback mechanism necessary for the turn-off of the NK lytic response. Such a turn-off had already been demonstrated [15–20], but the mechanism by which it occurred was not known.

Here we report an inhibition of NK-cell PDE in response to LS but not LR target cells. The use of class-selective inhibitors of PDE indicated that a PDE III (cGMP-inhibited PDE) is being inhibited in response to LS targets. This inhibition did not appear to require an elevation of cGMP.

MATERIALS AND METHODS

NK Cell Preparation

Heparin-treated whole blood from healthy human donors was applied to Ficoll–Hypaque (1.077 g/mL) (Sigma) and was centrifuged at 500 *g* for 30 min [21, 22] to remove contaminating red blood cells. Mononuclear cells were collected from the Ficoll–Hypaque and washed twice (250 *g*, 10 min) with PBS (10 mM phosphate, pH 7.2/2.7 mM KCl/140 mM NaCl). To remove platelets, the cells were suspended in a small volume (2.5 to 5 mL) of PBS, layered onto 2.5 to 5 mL of BCS (Biocell Laboratories, Inc.), and centrifuged at 200 *g* for 5 min [23]. The pellet was resuspended in PBS, and the process was repeated. The cells then were suspended in complete medium, which consisted of RPMI 1640 (Mediatech Cellgro, Fisher Scientific) supplemented with 10% heat-inactivated (56°, 30 min) BCS, 2 mM l-glutamine, and 50 U penicillin G with 50 mg streptomycin/mL (Sigma). The cell suspension was applied to glass Petri dishes (150 × 15 mm) and incubated at 37°, 5% CO_2 for 1 hr [24] to remove adherent cells. Nonadherent cells were 30–40% $CD56^+/CD3^-$, 60–70% $CD3^+/CD56^-$, and had undetectable levels of B cells and monocytes.

Cell Lines

Two LS human cell lines were used as target cells in these studies: K562 (chronic myelogenous leukemia) and MOLT-4 (acute lymphoblastic leukemia). One LR cell line was also used as a target cell: Raji (Burkitt's lymphoma). All cell lines were maintained in complete medium.

Inhibitors

IBMX (Sigma) was prepared as a 5 mM stock in complete medium. Rolipram (Sigma) was prepared as a 50 mM stock solution in 95% ethanol. Trequinsin (Sigma) was prepared as a 50 mM stock solution in deionized distilled water. Milrinone (Sigma) was prepared as a 50 mM stock solution in DMSO.

cAMP PDE Assay

Following incubation of lymphocytes with target cells, cAMP PDE activity was assayed using a modification [25] of the procedure developed by Thompson and Appleman [26]. Lysate from 2.5 million lymphocytes was incubated in 100 μL of the following reaction mixture: 40 mM Tris–HCl, pH 8.0, 5 mM $MgSO_4$, 5 μM cAMP with 200,000 cpm [^{3}H]cAMP. Lysis of cells was achieved by 0.01% Triton X-100 in the reaction mixture (protease inhibitors, 0.2 mM phenylmethylsulfonyl fluoride and 10 μM pepstatin, also were present in the reaction mixture). The reaction was initiated by addition of the substrate to NK cell lysate suspended in the Tris buffer. The samples were incubated for 10 min at 37°. The reaction was terminated by boiling the samples for 2.5 min. Then the samples were placed on ice for 5 min. Twenty-five microliters of a 1 mg/mL solution of snake venom (*Ophiophagus hannah*, king cobra) (Sigma) was added to the samples, and they were incubated for 15 min at 37°. The nucleotidase in the snake venom hydrolyzed any 5′-AMP to adenosine. This reaction was terminated by the addition of 250 μL of a 1:3 suspension of AG1X2 anion-exchange resin (Bio-Rad Laboratories) (in deionized water) to the samples. The anion-exchange resin bound all charged nucleotide, leaving the adenosine in solution. The samples were centrifuged to pellet the resin, and a 125 μL aliquot of the supernatant in 5 mL of scintillation fluid was counted in a liquid scintillation counter.

cGMP Assay

The amount of intracellular cGMP in the NK cell preparation was determined using a cGMP assay kit (Amersham Life Science Inc.). The NK cells (2.5×10^6 in 0.5 mL of complete medium) were exposed to target cells (250,000 added in 50 μL of complete medium) for 10, 30, 60, and 120 min. Following the incubation, the cells were pelleted. The supernatant was removed, and the cGMP was extracted from the cells by the addition of 1 mL of 75% ethanol. After addition of the ethanol the assay tubes were vortex-mixed, allowed to sit for 10 min, and then centrifuged at 800 *g* to pellet the insoluble material. The ethanol-insoluble material was re-extracted with 0.5 mL of 75% ethanol, and this extract was combined with the first extract. Controls consisted of the same number of NK cells, to which the target cells were added at the end of the incubation period for each time point (after the reaction

had been terminated by the addition of ethanol). The ethanol extract was evaporated under a stream of N_2, and the dried material was resuspended in assay buffer (0.05 M sodium acetate, pH 5.8). The samples then were acetylated so that cGMP at the 2–128 fmol/tube level could be detected. Anti-cGMP antibody was added to the acetylated samples followed by the addition of ^{125}I-labeled cGMP. The samples were incubated for 16 hr at 2–8°. Following the incubation, a second antibody was added, which bound to the cGMP–first-antibody complex, allowing it to be pelleted by centrifugation at 1500 *g* for 10 min. The pellet was counted in a gamma-radiation counter. The concentration of cGMP in the samples was determined from standard curves, using acetylated cGMP standards; each condition was tested in duplicate.

RESULTS

Effect of LS Tumor Target Cells on the Overall PDE Activity of NK Cells

Recent studies have shown that cAMP is elevated in NK cells following contact with LS but not LR tumor cells [13]. These data showed that LS targets activate AC in NK cells. This cAMP elevation, which was maximal after a 30-min exposure to LS targets, was diminished greatly when the nonselective PDE inhibitor IBMX was present. This indicated that LS targets may induce an inhibition of NK-cell PDE activity as part of the mechanism by which they elevate cAMP [13]. However, direct measurement of lymphocyte PDE activity is required to determine whether there is a significant PDE-inhibition component of the overall cAMP elevation.

NK-cell PDE activity was measured following exposure to the LS tumor target cells, K562 and MOLT-4. NK cells were exposed to target cells for 0, 3, 10, 20, 30, and 60 min at 37°, 5% CO_2. Following the incubation, the cells were lysed, and PDE activity was measured. Figure 1A shows the time course of inhibition of PDE activity in human NK cells exposed to the LS target, K562 cells. Exposure to K562 targets resulted in a maximal inhibition of overall PDE activity of about 12.5% at about 20 min after exposure to the target cells. Figure 1B shows the time course of inhibition of PDE activity in NK cells in response to MOLT-4 targets. Exposure to MOLT-4 targets also resulted in a maximal inhibition of about 12.5% at about 30 min after exposure to the target cells (the inhibition seen at 10 min with MOLT-4 exposure was not statistically significant, $P > 0.5$). An LR target cell line, Raji cells, produced no effect on NK-cell PDE activity (data not shown).

The lymphocyte preparations utilized in our studies were 60–70% T lymphocytes ($CD3^+/CD56^-$) and 30–40% NK cells ($CD56^+/CD3^-$) with no other cell types detectable, as determined by fluorescence microscopy. Although T lymphocytes are present in the lymphocyte preparations utilized in the studies, the tumor target cells are specific stimuli for the NK cells [27–29]. The use of NK-specific targets allowed us to monitor processes occurring in the NK cell population, while excluding the T lymphocyte population.

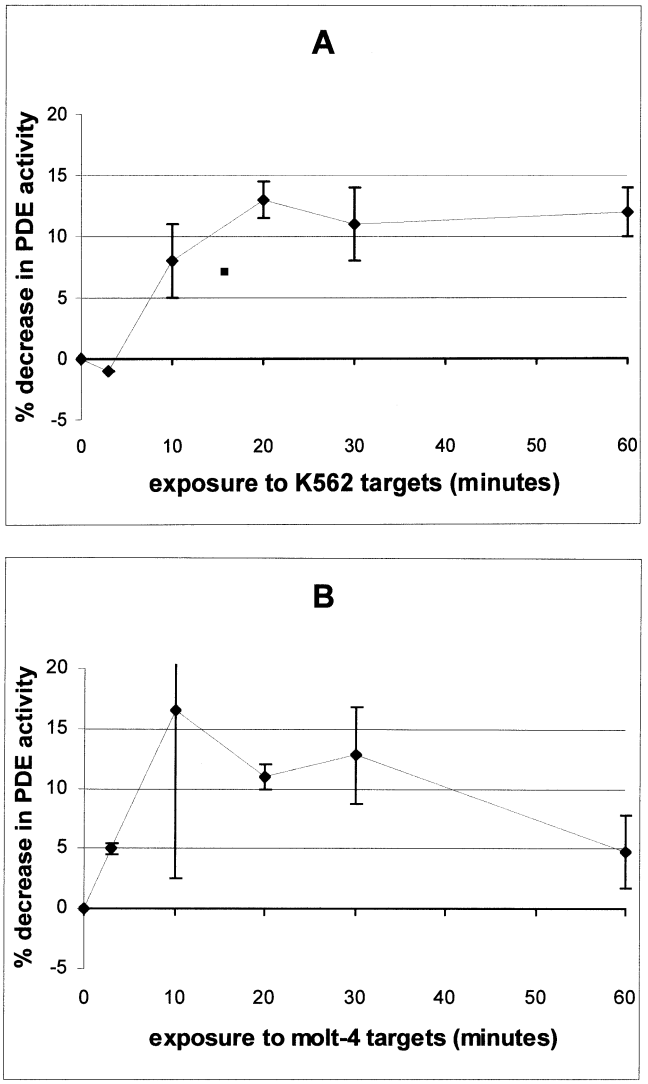

FIG. 1. PDE inhibition in NK cells after 3-, 10-, 20-, 30-, and 60-min exposures to: (A) K562 target cells, and (B) MOLT-4 target cells. Lymphocyte:target cell ratio, 10:1. Baseline PDE activity (0-min time point) was that of lymphocytes and targets that were lysed prior to being combined, approximately 15 pmol 5′-AMP produced per 10 min (lymphocytes ~ 10 pmol/10 min; targets ~ 5 pmol/10 min). Results are the means ± range of duplicate determinations and were repeated in three separate experiments using different donors. $P < 0.05$ for 10-, 20-, 30- and 60-min time points (Fig. 1A); $P < 0.05$ for 20- and 30-min time points (Fig. 1B).

Although target cells remained during the PDE assay, they were present at approximately one-tenth the concentration of lymphocytes. In an effort to determine possible contributions by targets to any changes in cAMP PDE activity, the concentration of lymphocytes in the assay was varied while that of targets was kept constant. The observed inhibition of PDE activity was shown to be dependent on lymphocyte numbers, as there was no measurable inhibition of PDE activity seen when lymphocyte levels were lowered from 2.5 million/assay tube to 1.25 million/assay tube while

TABLE 1. Percent inhibition of total PDE activity in the presence of selective PDE inhibitors

	% Inhibition of total PDE activity
Lymphocytes + 10 μM rolipram	62 ± 7
Lymphocytes + trequinsin (50–150 nM)	36 ± 12
K562 targets + trequinsin (50–150 nM)	0 ± 0
MOLT-4 targets + trequinsin (50–150 nM)	0 ± 0

Values are means ± range of duplicate determinations. Results were repeated in two separate experiments. Total PDE activity: lymphocytes (100% = 12 pmol 5′-AMP/10 min); K562 cells (100% = 4 pmol 5′-AMP/10 min); MOLT-4 cells (100% = 2 pmol 5′-AMP/10 min).

keeping target levels constant at 250,000/assay tube. The percent inhibition after a 20-min exposure to K562 targets was 13% when 2.5 million lymphocytes were exposed to 250,000 K562 targets and 0% when 1.25 million lymphocytes were exposed to 250,000 K562 targets. If the inhibition of PDE activity were occurring primarily in the targets, we would have expected the inhibition to persist, as target levels of PDE were unchanged, and any masking effect due to lymphocyte PDE activity would have been greatly reduced. Further, our earlier studies had shown that there was no significant contribution to the overall elevation of cAMP (of which the PDE inhibition is one component) due to target cells [13].

Effect of LS Tumor Target Cells on the PDE Activity of NK Cells When PDE IV Activity Is Blocked by Rolipram

Multiple isoforms of PDE have been identified [30–32]. Two classes of PDEs, PDE III and PDE IV, have been shown to predominate in lymphocytes [33]. Table 1 shows the effect of treatment with a PDE IV-selective inhibitor, rolipram, as well as a PDE III-selective inhibitor, trequinsin [30], on the measurable PDE activity in the lymphocyte preparations. These results indicated that PDE IV and PDE III account for greater than 98% of the total PDE in our lymphocyte preparations.

We exposed NK cells to LS targets as described above. Following the incubation, the cells were lysed, 10 μM rolipram was added to the lysate (10 μM rolipram maximally blocks PDE IV in our preparations), and PDE activity was measured. Addition of rolipram allowed us to measure only non-PDE IV activity in the lysate. If a PDE IV is being inhibited by exposure to LS targets, then removal of this activity by rolipram treatment should eliminate LS target-induced inhibition of PDE. Figure 2A shows that when 10 μM rolipram was present in the PDE assay, we observed an even greater inhibition of PDE activity in NK cells after exposure to K562 targets. Maximal inhibition of NK-cell PDE (other than PDE IV) was about 35% as compared with about 12.5% when PDE IV activity was present. Figure 2B shows that the MOLT-4-induced inhibition of PDE in NK cells also was not eliminated when PDE IV was blocked.

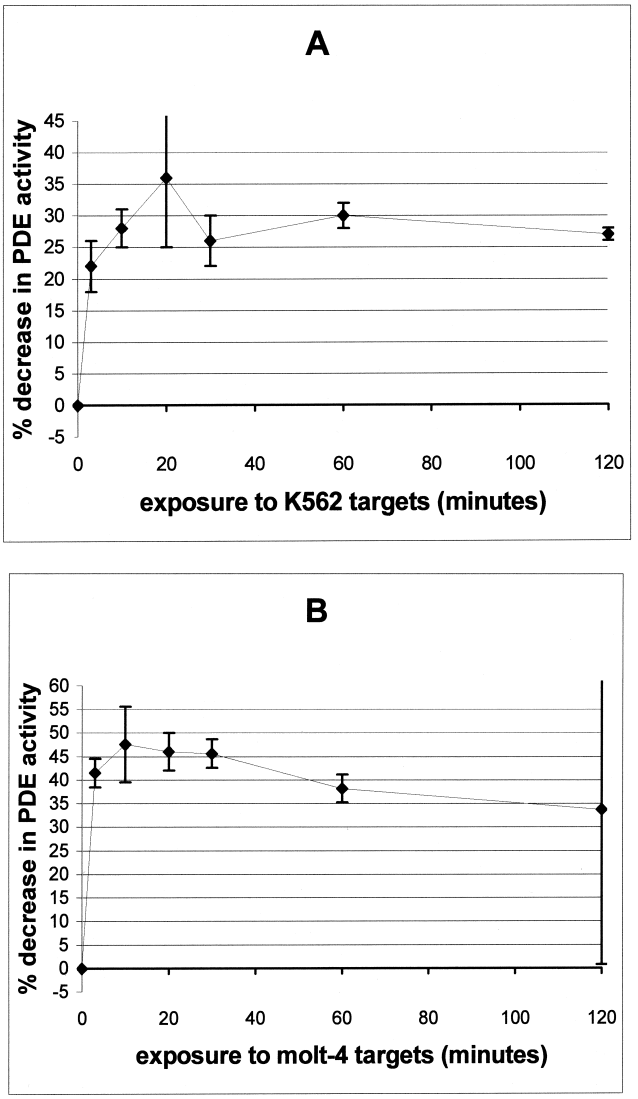

FIG. 2. PDE inhibition in NK cells, when PDE IV activity is blocked by 10 μM rolipram, after 3-, 10-, 20-, 30-, 60-, and 120-min exposures to (A) K562 target cells, and (B) MOLT-4 target cells. Lymphocyte:target cell ratio, 10:1. Baseline PDE activity (0-min time point) was that of lymphocytes and targets that were lysed prior to being combined, approximately 9 pmol 5′-AMP produced per 10 min. Results are the means ± range of duplicate determinations and were repeated in three separate experiments using different donors. $P < 0.05$ for 3-, 10-, 20-, 30-, 60-, and 120-min time points (Fig. 2A). $P < 0.05$ for 3-, 10-, 20-, 30-, and 60-min time points (Fig. 2B).

Maximal inhibition of PDE by MOLT-4 targets was approximately 45% as compared with 12.5% when PDE IV activity was present. These data indicated that PDE IV is not the category of PDE activity in NK cells that is being inhibited in response to LS-target exposure.

Effect of LS Tumor Target Cells on the PDE Activity of NK Cells When PDE III Activity Is Removed by the Selective Inhibitors Trequinsin and Milrinone

As mentioned above, trequinsin is a selective inhibitor of PDE III activity [30]. We exposed NK cells to LS targets as

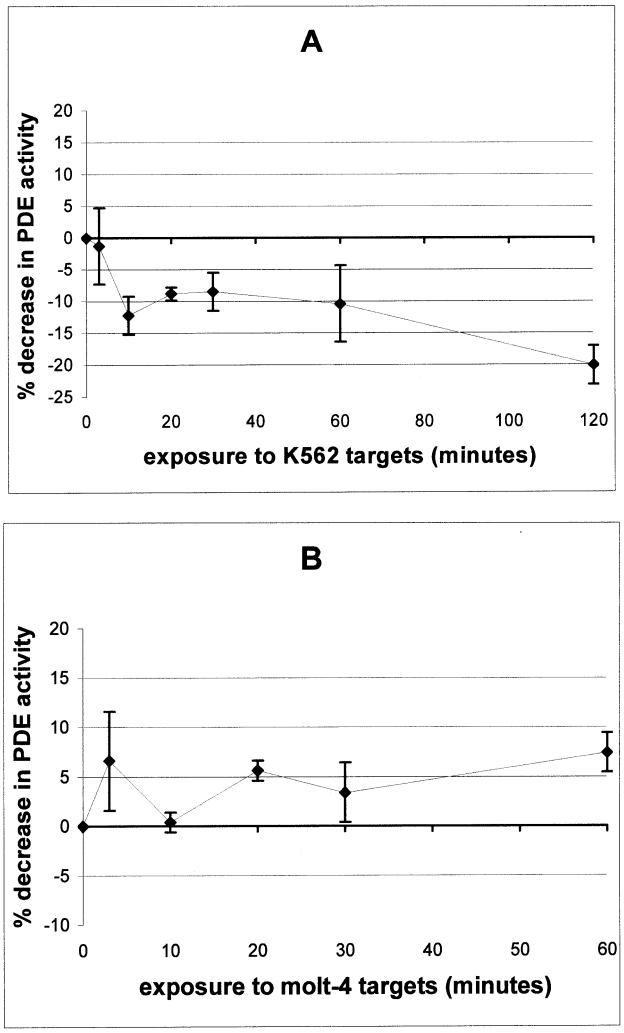

FIG. 3. PDE inhibition in NK cells, when PDE III activity is blocked by 50 nM trequinsin, after (A) 3-, 10-, 20-, 30-, 60-, and 120-min exposures to K562 target cells; and (B) 3-, 10-, 20-, 30-, and 60-min exposures to MOLT-4 target cells. Lymphocyte:target cell ratio, 10:1. Baseline PDE activity (0-min time point) was that of lymphocytes and targets that were lysed prior to being combined, approximately 14 pmol 5′-AMP produced per 10 min. Results are the means ± range of duplicate determinations and were repeated in three separate experiments using different donors. $P < 0.05$ for 10-, 20-, 30-, and 120-min time points (Fig. 3A). $P < 0.05$ for 20- and 60-min time points (Fig. 3B).

described above. Following the incubation, the cells were lysed, 50 nM trequinsin was added to the lysate (50 nM trequinsin maximally blocks PDE III), and PDE activity was measured. Addition of trequinsin allowed us to measure only non-PDE III activity in the lysate. Thus, if PDE III were being inhibited by exposure to LS targets, we should see no target-induced PDE inhibition when trequinsin is present in the PDE assay. Figure 3A shows that when 50 nM trequinsin was present in the PDE assay, we no longer observed an inhibition of PDE activity in NK cells after exposure to K562 targets. This indicated that the PDE in NK cells that is being inhibited by exposure to K562 targets is a PDE III. Further, there was a stimulation of PDE activity when PDE III activity was excluded (Fig. 3A). This stimulation was between 10 and 20% above baseline activity.

As lymphocytes predominantly contain PDE III and PDE IV [33] (Table 1), the PDE that is being stimulated by exposure of NK cells to LS targets is most likely a PDE IV, as it is about 60–70% of the total PDE activity (Table 1). Thus, these data indicated that PDE III activity is inhibited in response to LS targets and suggested that a non-PDE III (presumably PDE IV) activity is being activated. The combination of these two events may provide a partial explanation as to why the PDE inhibition seen when we measured total PDE activity was so much smaller than that seen when we measured only the non-PDE IV activity (Fig. 2A).

NK cells were also exposed to MOLT-4 targets, and PDE was measured in the presence of 50 nM trequinsin. Under these conditions, we were again measuring the effect of target-cell stimulation on non-PDE III activity in NK cells. Figure 3B shows that there remained a slight inhibition of PDE activity at several time points even when PDE III activity was excluded from the measurement.

We tested another PDE III-selective inhibitor, milrinone [30]. NK cells were exposed to K562 targets, the cells were lysed, and PDE activity was measured in the presence of 10 μM milrinone (10 μM milrinone maximally blocks PDE III). Figure 4A shows that when PDE III activity was blocked by milrinone, there was no longer any significant inhibition of PDE activity in response to the K562 targets, and again there was a 10–15% activation of PDE activity. As with trequinsin, this indicated that PDE III is the PDE type being inhibited in response to K562 targets. Also as was seen with trequinsin, there appeared to be an activation of other PDEs, presumably PDE IVs, that was occurring simultaneously with the inhibition of PDE IIIs.

NK cells were exposed to MOLT-4 targets, the cells were lysed, and PDE activity was measured in the presence of 10 μM milrinone (Fig. 4B). Although removal of PDE III did not eliminate the MOLT-4-induced inhibition of PDE, it did very significantly reduce it from what was seen when only PDE III and other non-PDE IV activities (to the extent that they were present) were being measured (Fig. 2B). As with K562 targets, it appeared that inhibition of PDE III was a significant part of the overall PDE inhibition seen in response to MOLT-4 targets.

Further evidence that changes in target-cell PDE activity were not contributing significantly to the changes in PDE activity seen in our experiment is provided in Table 1. Table 1 shows that when K562 or MOLT 4 targets were treated with the PDE III selective inhibitor trequinsin (from 50 to 150 nM), there was no inhibition of PDE activity. These results indicated that targets have no PDE III activity. However, when PDE activity was measured in the presence of trequinsin (Fig. 3, A and B), the PDE inhibition no longer was seen. This indicated that the PDE inhibition was due primarily to a PDE III, and as there was

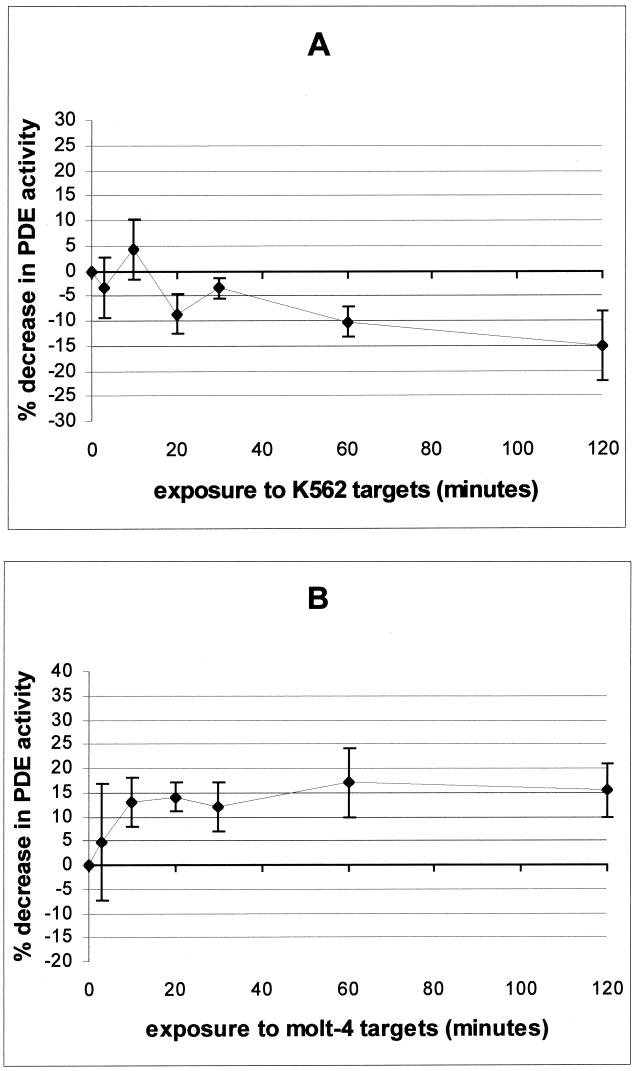

FIG. 4. PDE inhibition in NK cells, when PDE III activity is blocked by 10 μM milrinone, after 3-, 10-, 20-, 30-, 60-, and 120-min exposures to (A) K562 target cells, and (B) MOLT-4 target cells. Lymphocyte:target cell ratio, 10:1. Results are the means ± range of duplicate determinations and were repeated in three separate experiments using different donors. $P < 0.05$ for 20- and 60-min time points (Fig. 4A). $P < 0.05$ for 10-, 20-, 60- and 120-min time points (Fig. 4B).

no measurable PDE III in targets, it must be occurring in the lymphocytes.

Effect of LS Target on cGMP Levels in NK Cells

Our data indicated that a PDE III is primarily responsible for the K562-induced inhibition of PDE activity in NK cells. PDE III inhibition also appeared to be a significant aspect of the MOLT-4-induced inhibition of PDE activity. PDE IIIs are known to be inhibited by increased concentrations of cGMP, which is able to compete with cAMP for binding at the active site [30]. Thus, one mechanism by which LS target-induced inhibition of PDE III might be occurring is via an LS target-stimulated increase in NK-cell cGMP levels. Using a radioimmunoassay, we measured

TABLE 2. cGMP levels in lymphocytes

	cGMP (fmol/2.5 million cells)		
	Length of exposure to targets		
	5 min	10 min	20 min
Control lymphocytes	19.3 ± 2.8	16.1 ± 0.6	19.3 ± 2.8
Lymphocytes exposed to K562 targets	17.3 ± 0	14.8 ± 0	16.1 ± 1.8

Values are means ± range of duplicate determinations. Results were repeated in two separate experiments.

cGMP levels in NK cells in response to K562 targets. NK cells were exposed to K562 targets for 5, 10, and 20 min, and cGMP levels were compared with those of appropriately matched controls (Table 2). These time points were chosen because the cGMP elevation would need to be maximal at the point at which maximal PDE inhibition was seen (Fig. 2A) if this was the mechanism by which it was occurring. There was no significant alteration of cGMP levels upon exposure to K562 targets at any of the time points (Table 2). We also measured cGMP levels in NK cells where PDE activity was inhibited by the nonselective PDE inhibitor IBMX. This was done to verify that a cGMP elevation was not occurring. It is possible that the enzyme that catalyzes the production of cGMP, guanylyl cyclase, might have become activated and cGMP levels elevated, but that PDE activity was destroying the cGMP before it could be measured in the assay. By measuring cGMP levels in cells where the total PDE activity was blocked by IBMX, we should see any cGMP elevation that occurred. However, in the presence of 0.5 mM IBMX, we still were unable to see any elevation of cGMP in response to K562 targets (data not shown). We also measured cGMP levels in NK cells exposed to MOLT-4 targets but were unable to see any elevation (data not shown). Thus, while PDE III activity in NK cells clearly was being inhibited in response to LS targets (Figs. 3 and 4), the mechanism by which this inhibition occurs did not appear to involve elevation of NK-cell cGMP.

DISCUSSION

Recently, we showed that cAMP is elevated in NK cells following contact with LS tumor cells but not LR tumor cells [13]. These data showed that LS targets activate AC in NK cells. This AC activation requires PTK activity but not PKC activity [13]. PTK activation is the earliest signal known to be generated in the cytolytic response of NK cells [14]. This is consistent with the notion that a signal involved in activation of the cytolytic response also is involved in stimulating the elevation of an inhibitory signal, cAMP (feedback inhibition of the cytolytic response). However, when we inhibited the AC component of the cAMP elevation by inhibiting PTK activity in the NK cells, there was still an LS target-induced increase in cAMP [13]. These findings indicated that LS targets might

also induce an inhibition of NK-cell PDE activity as part of the mechanism by which they elevate cAMP and that this PDE inhibition is not dependent on PTK activity. Here we show that LS targets do induce an inhibition of PDE in NK cells.

There are multiple isoforms of PDE, which are regulated by different mechanisms [30–32]: Ca^{2+}/CaM-stimulated PDE (PDE I), cGMP-stimulated PDE (PDE II), cGMP-inhibited PDE (PDE III), cAMP-specific PDE (PDE IV), cGMP-specific PDE (PDE V), photoreceptor-like PDE (PDE VI), and high-affinity cAMP-specific PDE (PDE VII). These isoforms, except for those that are cGMP-specific (V and VI), are capable of hydrolyzing cAMP. To determine the mechanism by which LS targets induce an inhibition of NK-cell PDE activity, it is necessary to determine which of the several types of PDE isoforms is being inhibited in response to LS targets. Two types of cAMP-PDE isoforms are known to predominate in lymphocytes, PDE IV and PDE III (cGMP-inhibited PDE) [33, 34] (Table 1). A particular subtype of PDE III, PDE IIIB, has been shown to be the predominant form of PDE III in lymphocytes [33]. PDE III can be inhibited by elevation of cGMP, and PDE III and IV are activated by a PKA-induced phosphorylation [30, 35].

If a PDE IV were being inhibited in response to LS targets, then removal of this activity by rolipram treatment should eliminate the observation of LS target-induced inhibition of PDE. Figure 2 shows that when rolipram completely blocked PDE IV (in the PDE assay), we observed a greater inhibition of PDE activity in NK cells after exposure to LS targets. These data indicated that PDE IV is not the PDE activity that is inhibited in response to LS targets. Further, since PDE IV was a majority of the total lymphocyte PDE activity, but was not inhibited in response to LS targets, to some extent it was masking the PDE inhibition in the measurement of overall PDE activity. By removing the PDE IV activity, we could see the inhibition of the class of PDE that was affected in response to LS targets (between 30–45%, Fig. 2) more clearly.

When we selectively inhibited the other class of PDE activity that predominates in lymphocytes [33] (Table 1), PDE III, we did not observe a significant inhibition of PDE activity in NK cells after exposure to K562 targets at any time point (Figs. 3 and 4). MOLT-4-induced inhibition of PDE in NK cells also was diminished greatly when PDE III was removed from the overall PDE activity by a selective inhibitor (Figs. 3 and 4). However, MOLT-4-induced inhibition of PDE did not appear to be entirely dependent upon a PDE III activity, as there remained some significant inhibition in the presence of the PDE III-selective inhibitors. This was consistent with the fact that activation of the cytotoxic response by a particular target cell is likely to be dependent on the activation of more than one activating receptor [36], as well as the extent to which they have structures on their surface that bind killer cell inhibitory receptors (KIR) [36]. LS targets, such as MOLT-4 and K562, are not equally susceptible to lysis by NK cells [9, 11]. This is likely due to differing patterns of structures on their surfaces that are capable of interacting with receptors that activate the cytotoxic response as well as KIRs; these differences could possibly account for some differences in the PDE inhibition induced by a particular target cell.

When PDE III activity was removed but PDE IV activity was still present, we saw a significant increase in PDE activity after about 10 min in response to K562 targets. This suggested that a PDE (presumably PDE IV) possibly was being activated in response to LS targets. The maximum cAMP elevation in NK cells stimulated by LS targets occurs between 10 and 30 min [13]. There is evidence for PDE IV being activated by a PKA-catalyzed phosphorylation [30, 35]. Thus, one possible scenario is that the increase in cAMP produced by LS target-induced activation of AC and inhibition of PDE III could activate PKA, which then would activate PDE IV. This presents a possible feedback mechanism, by which the elevation of cAMP stimulated by LS targets could be involved in returning cAMP levels to their resting level.

Our data indicated that a PDE III is being inhibited in response to LS targets. As PDE III is known to be inhibited by cGMP, it was possible that an LS target-induced elevation of cGMP was a consequence of the activation of the cytolytic response in NK cells. However, we were unable to measure a significant increase in NK-cell cGMP levels upon exposure to LS targets (Table 2). Thus, while PDE III activity in NK cells clearly was being inhibited in response to LS targets (Figs. 3 and 4), the mechanism by which this inhibition occurred did not appear to involve elevation of NK-cell cGMP. An alternative mechanism by which a PDE III in NK cells could be inhibited is via a receptor-linked heterotrimeric guanine nucleotide binding (G) protein. There is precedence for receptor-linked G protein-induced inhibition of a PDE III in ventricular myocytes [37]. This ventricular myocyte G protein is pertussis toxin (PTX) sensitive in that its function can be blocked by PTX treatment. In previous studies we have shown the presence of a PTX-sensitive G protein in NK cells [22]. Our future studies will investigate the role of this G protein in the LS target-induced inhibition of PDE III.

The fact that target-induced inhibition of PDE persists in NK cell lysate (where disruption of noncovalent physiological inhibitors could occur due to detergent and dilution effects) may indicate that it is the result of a covalent modification of the PDE, such as dephosphorylation of a phosphorylated (activated) PDE. Thus, a possible mechanism for PDE inhibition is that LS targets activate a phosphatase in NK cells that changes PDE III from a phosphorylated (active) to a dephosphorylated (less active) state. PDE III is activated by PKA- (and possibly other protein kinase-) catalyzed phosphorylation [30, 35]. We will look for LS target-induced phosphatase activation as we pursue the mechanism by which LS targets induce inhibition of NK-cell PDE.

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