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Involvement of protein kinase C-δ in CD28-triggered cytotoxicity mediated by a human leukaemic cell line YT

F. J. KOS* & H. D. BEAR† *Department of Microbiology and Immunology, †Department of Surgery, and *†Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA

SUMMARY

Ligation of CD28 molecules expressed on the surface of human leukaemic natural killer-like YT cells triggers intracellular signals leading to cytolysis of target cells expressing CD80 or CD86 molecules. Known intracellular events include tyrosine phosphorylation, activation of phosphatidylinositol 3-kinase, and protein kinase C (PKC). In this study, we report that PKC-δ isoenzyme activity is required for CD28-triggered cytotoxicity mediated by YT cells and we also demonstrate that one of the primary targets of bryostatin 1, a modulator of PKC activity, is PKC-δ. Treatment of YT cells with bryostatin 1 caused degradation of PKC-δ, but not other PKC isoenzymes, and completely blocked the cytolytic activity of YT cells. In addition, PKC-δ-specific antibody introduced into YT cells by electroporation inhibited partially the YT cell-mediated cytotoxicity of B-lymphoblastoid cell line JY. This effect was specific, since addition of anti-PKC-δ antibody-blocking peptide in combination with anti-PKC-δ antibody to YT cells for electroporation, neutralized the effect of this antibody. These results demonstrate that YT cell cytolytic activity is dependent on PKC-δ, which is selectively down-regulated by bryostatin 1.

INTRODUCTION

Ligation of CD28 on T cells induces intracellular signals that costimulate T-cell receptor (TCR)-initiated antigen-specific signalling for T-cell activation. Binding of CD28 to its ligands CD80 or CD86 leads to the phosphorylation of the cytoplasmic pYMNM motif, followed by recruitment and activation of the intracellular signalling proteins phosphatidylinositol 3-kinase (PI 3-kinase), growth factor receptor-bound protein-2 (GRB-2), and T-cell-specific protein-tyrosine kinase (Itk).[1] PI 3-kinase influences a variety of functions, including growth factor-dependent mitogenesis, glucose uptake, vesicular trafficking, cytoskeletal organization and apoptosis.[2,3] Accordingly, activation of PI 3-kinase by CD28 implies that this receptor may also play a role in functions that have not previously been ascribed to CD28. Indeed, it has already been shown that CD28-dependent initiation of cytotoxic function of YT cells requires activation of PI 3-kinase.[4,5] A human natural killer (NK)-like leukaemia cell line YT[6] expresses the CD28 membrane glycoprotein and can spontaneously lyse cell lines expressing the CD80[7] or CD86[5] molecules.

PI 3-kinase phosphorylates the inositol ring of phosphatidylinositols PI-3,4-bisphosphate and PI-3,4,5-trisphosphate, which in turn can activate the δ, ε, η,[8] and ζ[9] isoforms of protein kinase C (PKC). Prolonged treatment of YT cells with phorbol-12-myristate-13-acetate (PMA), which depletes PMA-responsive PKC isoenzymes such as α and γ, had no effect on the CD28-triggered cytotoxicity against $CD80^+/CD86^+$ B-lymphoblastoid cell lines and the murine P815 cells transfected with CD80 or CD86.[5] Since PKC inhibitors, such as calphostin C and bisindolylmaleimide (BIM), abolished YT-mediated cytotoxicity, indicating a role for PKC in this process, it is apparent that activation of PMA-non-responsive PKC isoenzymes is likely to be required for CD28-triggered cytotoxicity of YT cells.

Our recent studies on the role of bryostatin 1, a modulator of PKC activity, in T-cell and NK-cell function have shed some light on PKC isoenzymes involved in CD28-triggered cytotoxicity of YT cells. We report here that PKC-δ activity is required for YT-mediated lysis of B-lymphoblastoid cells and that bryostatin 1 selectively depletes PKC-δ and inhibits the cytotoxicity of YT cells.

MATERIALS AND METHODS

Chemical reagents and antibodies

All reagents for lysis buffer and protease inhibitors were purchased from Sigma (St. Louis, MO). Precast gels for sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), SDS, Tween-20, polyvinylidene difluoride (PVDF) membranes, gelatin and detergent compatible protein assay were purchased from Bio-Rad Laboratories (Hercules, CA). Bryostatin 1, manufactured by Ben Venue Laboratories (Bedford, OH), was kindly provided by the National Cancer

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Correspondence: Dr F. J. Kos, Virginia Commonwealth University, Massey Cancer Center, 401 College Street, Richmond, VA 23298-0037, USA.

Institute (Bethesda, MD). Ionomycin and the PKC inhibitor BIM[10] were purchased from Calbiochem-Novabiochem (San Diego, CA). Rabbit polyclonal IgG against human BEK (fibroblast growth factor receptor-2), PKC-α, -βII, -δ, -ε and -η, as well as control peptides for PKC-α and -δ, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD28 (CD28.2) and anti-CD3 (UCHT1) monoclonal antibodies (mAb) were purchased from Immunotech (Westbrook, ME). Fluorescein isothiocyanate (FITC)-conjugated goat antimouse $F(ab')_2$ IgG, used for indirect immunofluorescence, was purchased from Caltag Laboratories (Burlingame, CA). Flow cytometry analysis was performed as described previously.[11]

Cell lines and cell culture

The Epstein–Barr virus-transformed human B-lymphoblastoid cell line JY (obtained from Dr Edgar Engleman, Stanford University School of Medicine, Palo Alto, CA) and the human NK-like leukaemia cell line YT[6] (obtained from Dr Eckhard Podack, University of Miami School of Medicine, Miami, FL) were cultured in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated bovine calf serum (HyClone Laboratories, Logan, UT) and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) at 37° in a humidified atmosphere of 5% CO_2 in air. For PKC blocking experiments, YT cells (5×10^5/ml) were cultured from 10 min to 20 hr with bryostatin 1 alone (100 nM) or bryostatin 1 in combination with ionomycin (1 μM). In some experiments, YT cells were incubated with BIM (10 μM). Treated cells were washed twice and used in ^{51}Cr-release cytotoxicity assay and/or for preparation of total cell lysates for Western blot analysis.

Cytotoxicity assay

YT effector cells were assayed in a 4-hr ^{51}Cr-release cytotoxicity assay according to standard procedure.[12] Spontaneous release was always $<10\%$ of the maximum release. Standard error, omitted for clarity of figures, was always $<5\%$ of the mean value of triplicate cultures. In some experiments anti-CD28 (IgG1) or isotype matching control mAb (anti-CD3) were added to the assay.

Western blot analysis of PKC isoenzymes

Total cell lysates from YT cells were prepared by using ice-cold RIPA buffer [phosphate-buffered saline (PBS), 1% nonidet P-40 (NP40), 0·5% sodium deoxycholate, 0·1% SDS] with freshly added protease inhibitors [phenylmethylsulphonyl fluoride (PMSF), aprotinin, sodium orthovanadate]. Cells were further disrupted by passage through 23-gauge needles, incubation on ice and centrifugation at 15 000 ***g***. The supernatant fluid was the total cell lysate. Proteins (20 ng/well, as estimated by Bio-Rad detergent compatible protein assay) from total cell lysates were separated by 7·5% SDS–PAGE and transferred to a PVDF membrane. Non-specific binding sites were blocked with blocking solution (3% gelatin in Tris-buffered saline, TBS) for 1 hr. The PVDF membranes were subsequently incubated with affinity-purified rabbit polyclonal antibodies to specific isoforms of PKC at 0·2 μg/ml for 2 hr. Membranes were washed in TBS solution with 0·05% Tween-20 and incubated for 2 hr with goat anti-rabbit second antibody conjugated to alkaline phosphatase (Bio-Rad). Colour development reagents (Bio-Rad) containing 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium were used to identify specific proteins (detection sensitivity of this system is 100 pg). Images of immunoblots on PVDF membranes were taken with a digital camera and were computer edited.

Delivery of anti-PKC antibodies into YT cells by electroporation

YT cells were electroporated in the presence of antibodies according to the procedure described by Szamel *et al.*[13,14] with some modifications. YT cells in 'intracellular buffer' [10 mM piperazine-*N*,*N*′-bis[2-ethanesulphonic acid] (PIPES), pH 7·4, 5 mM $MgCl_2$, 120 mM KCl, 10 mM ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid EGTA, 8·2 mM $CaCl_2$, all from Sigma) were electroporated in the presence of antibodies against PKC izoenzymes (2 μg/0·5 ml/2 × 10^6 YT cells) with or without control peptides against which antibodies had been raised. Rabbit polyclonal IgG antibody against human BEK cells was used as a control antibody. Electroporation optimized for this system was carried out at 1000 μF and 300 V using Electroporator II from Invitrogen (San Diego, CA). After electroporation YT cells were left to recover for 1 hr at 37° in 50% bovine calf serum in IMDM. More than 90% of cells were viable after washing and before ^{51}Cr-release cytotoxicity assay.

RESULTS AND DISCUSSION

YT cell-mediated lysis of JY cells is CD28 dependent

Initially, we confirmed by flow cytometric analysis that YT cells express CD28, but lack CD3, and screening of various cell lines identified $CD80^+$ B-lymphoblastoid cells as the targets for YT-mediated cytotoxicity (data not shown). Among cell lines that were not lysed by YT cells were $CD80^-/CD86^-$ Daudi cells and NK cell-sensitive K562 cells. For further studies attempting to manipulate the cytotoxic activity of YT cells, we chose the $CD80^+$ JY B-lymphoblastoid cell line. JY cell lysis by YT cells was CD28-dependent and addition of anti-CD28 mAb, but not of control anti-CD3 mAb, to the 4-hr ^{51}Cr-release assay abolished the cytolytic activity of YT cells (Fig. 1). These results corroborate the findings of more extensive studies demonstrating that the cytotoxicity of YT cells is dependent on CD28–CD80/CD86 interactions.[5]

Bryostatin 1 selectively depletes PKC-δ and inhibits the cytotoxicity of YT cells

Cross-linking of the CD28 with mAb induced increased tyrosine phosphorylation and activation of a number of proteins in YT cells, including PI 3-kinase and Itk/Emt kinase, resulting in the release of cytolytic granules by YT cells.[5] Since the interaction between CD28 and CD80 induces signals leading to the activation of cytolytic mechanisms in YT cells, YT cell-mediated cytotoxicity might be abolished by interfering with different steps of CD28-triggered signal transduction pathways. Indeed, it has already been shown that two PI 3-kinase inhibitors, wortmannin and LY294002, inhibited the cytolytic activity of YT cells against P815 cells transfected with human CD80 or CD86 and the B-lymphoblastoid cell line MD.[5] In addition, PKC inhibitors BIM and calphostin were found to block cytolytic activity of YT cells, indicating a role for PKC

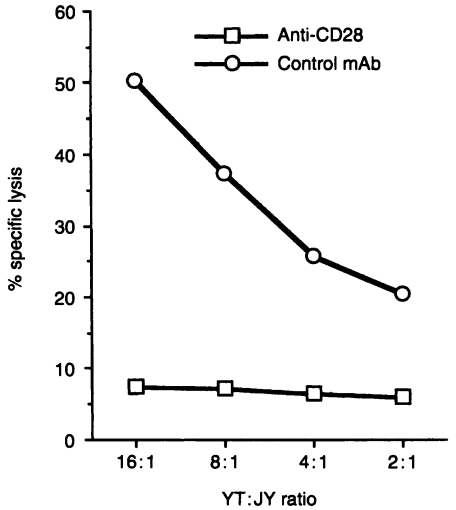

Figure 1. Effect of anti-CD28 mAb on the cytolytic activity of YT cells against JY cells. YT cells were preincubated with anti-CD28 or anti-CD3 mAb (5 μg/2 × 10^6 cells/0·1 ml) on ice for 30 min and tested in the presence of these mAb at 0·5 μg/2·5 × 10^5 cells/0·2 ml in a 4-hr ^{51}Cr-release assay. Mean percentage of specific lysis of triplicate samples (SE < 5%) is shown as a function of effector to target ratio.

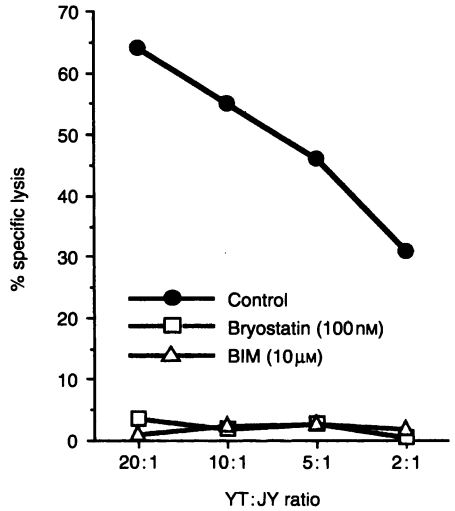

Figure 2. Effect of PKC inhibitors on YT cell cytotoxicity. YT cells were preincubated with BIM (10 μM) or bryostatin 1 (100 nM) in combination with ionomycin (1 μM) for 20 hr. Then, ^{51}Cr-labelled JY cells were added and incubated for another 4 hr at 37°.

in CD28-triggered cytotoxicity.[5] PKC isoenzymes involved in this process have not been identified.

Bryostatins, a class of natural products found in marine bryozoans,[15] constitute a unique group of modulators with poorly defined isotype-selective effects on PKC. Our interest in potential therapeutic T-cell-mediated effects of the bryostatin 1[16,17] prompted us to analyse also some aspects of PKC regulation by bryostatin 1. Since bryostatin 1 has been shown to down-regulate PKC (i.e. decrease the total amount of the enzyme in the cell), we analysed the effect of bryostatin 1 treatment of YT cells on their cytotoxicity and on expression of PKC isoenzymes. Incubation of YT cells for 20 hr with bryostatin 1 and ionomycin (or bryostatin 1 alone) completely blocked cytolytic activity of YT cells (Fig. 2). The blocking effect of bryostatin 1 was as potent as the effect of BIM treatment (Fig. 2). Bryostatin 1 at 100 nM (or 20 nM) did not affect the viability of YT cells within 24–48 hr. Higher concentrations, e.g. 1 μM, caused 30–40% loss of cell viability within 20 hr. The BIM concentration used in these experiments was not toxic to YT cells within 20 hr.

Treatment of YT cells with bryostatin 1 for 20 hr did not induce CD28 down-regulation that would impair target cell recognition and subsequent YT cell-mediated cytotoxicity. Flow cytometric analysis of CD28 expression levels on YT cells before (Fig. 3a) and after (Fig. 3b) treatment with bryostatin 1 showed no major changes. Thus, this result excludes CD28 down-regulation as the cause of functional inhibition of YT cells after exposure to bryostatin 1.

To examine whether bryostatin 1 treatment of YT cells leads to selective down-regulation of certain PKC isoforms, we analysed the expression of individual PKC isoenzymes by immunoblotting. Our screening procedure revealed that 20-hr treatment of YT cells with 100 nM bryostatin 1 had a profound effect on PKC-δ expression; no PKC-δ was detected in total cell lysates of YT cells treated with bryostatin 1 plus ionomycin (Fig. 4) or with bryostatin 1 alone (not shown). In contrast,

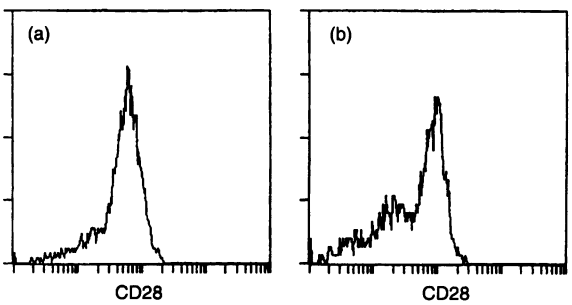

Figure 3. Comparison of CD28 expression on YT cells before (a) and after (b) treatment with bryostatin 1. Cells (5 × 10^5/ml) were incubated without or with bryostatin 1 (100 nM) for 20 hr. After washing, YT cells were stained for indirect immunofluorescence with anti-CD28 mAb followed by FITC-conjugated goat antimouse F(ab')$_2$ IgG.

the levels of PKC-α, PKC-βII, PKC-ε and PKC-η expression were not affected by the treatment (Fig. 4). PKC-α was detected on immunoblots as a doublet (Fig. 4). It is possible that the two bands are differentially phosphorylated forms of PKC-α. Normal untreated YT cells did not express PKC-βI, PKC-γ and PKC-λ and bryostatin 1 treatment did not induce their appearance.

The time-courses of down-regulation for both PKC-δ expression and cytolytic activity of YT cells pretreated with bryostatin 1 are shown in Fig. 5. Pretreatment of YT cells with bryostatin 1 caused gradual down-regulation of PKC-δ and parallel inhibition of the cytolytic function of YT cells (Fig. 5). These results suggest, again, that the expression of PKC-δ correlates with the cytolytic activity of YT cells, and that bryostatin 1 treatment inhibits both phenomena.

PKC-δ-specific antibody inhibits the cytolytic function of YT cells

It is believed that PKC isoenzymes mediate separate biological effects in some cases.[18,19] To demonstrate these PKC isotype-

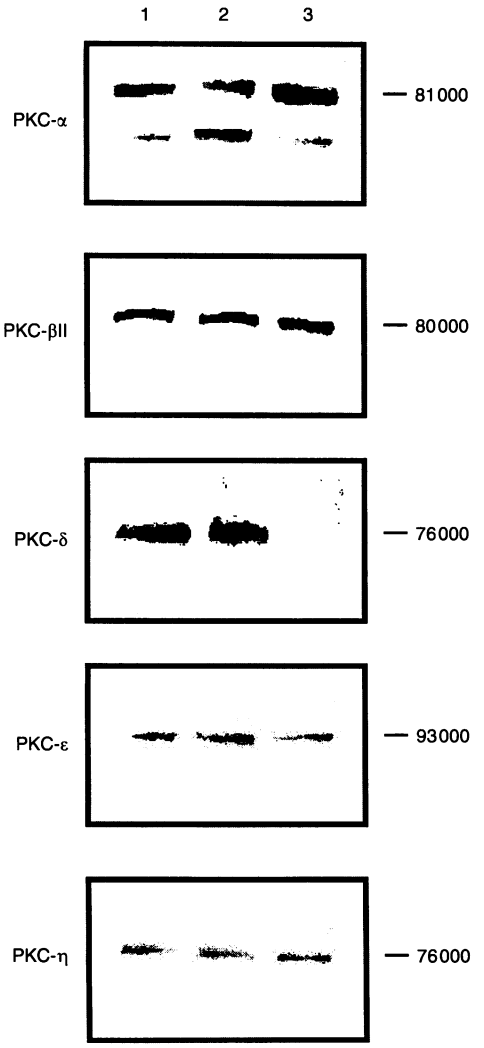

Figure 4. Western blot analysis of PKC-α, PKC-βII, PKC-δ, PKC-ε and PKC-η expression in YT cells after treatment with bryostatin 1. YT cells were exposed to bryostatin 1 (100 nM) and ionomycin (1 μM) in culture for 10 min (lane 2), 20 hr (lane 3), or were untreated (lane 1). Total cell lysates were prepared and analysed as described in the Materials and Methods.

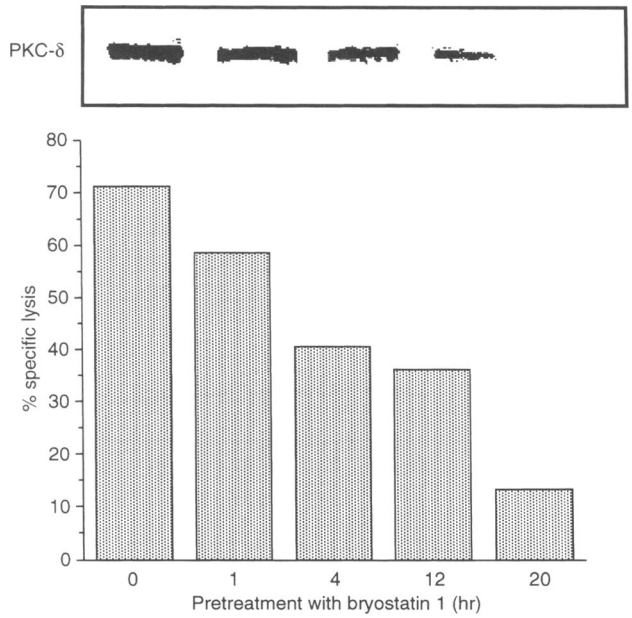

Figure 5. Down-regulation of PKC-δ expression and parallel inhibition of cytolytic activity of YT cells as a result of pretreatment of YT cells with bryostatin 1. YT cells were pretreated with bryostatin 1 (100 nM) for the indicated period of time and tested in a 4-hr ^{51}Cr-release cytotoxicity assay against JY cells. Percentage of specific lysis is shown for a YT:JY ratio of 20:1. PKC-δ expression in total cell lysates was detected by immunoblotting as in Fig. 4.

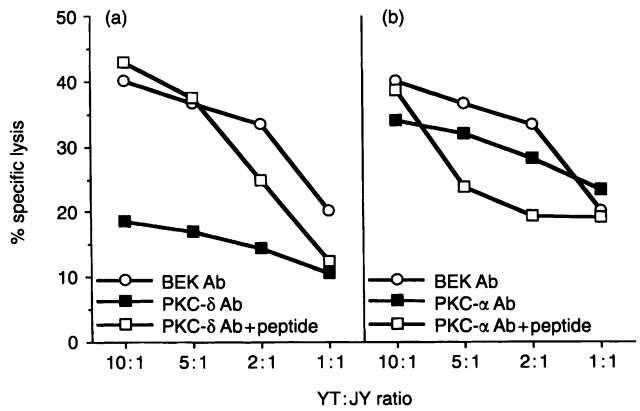

Figure 6. Inhibition of YT cell-mediated cytotoxicity of JY cells by anti-PKC-δ (a) but not anti-PKC-α (b) antibodies. Anti-PKC antibodies without or with control blocking peptides as well as anti-BEK control antibody were introduced into YT cells by electroporation as described in the Materials and Methods. Mean percentage of specific lysis of JY cells is shown as a function of effector to target ratio.

specific effects, several different experimental approaches can be undertaken, including the use of selective agents to activate or block the individual PKC isoenzymes or the use of antisense oligonucleotides that are complementary to the mRNA of specific isoenzymes. None of these approaches have proven to be fully satisfactory in addressing these issues, and we have therefore chosen another approach that is very simple. To confirm our conclusions from experiments correlating the lack of cytotoxicity of YT cells with the loss of PKC-δ induced by bryostatin 1, we attempted to inhibit PKC-δ activity selectively in YT cells by introducing PKC-δ-specific antibody into YT cells by electroporation. The results in Fig. 6 show that anti-PKC-δ (Fig. 6a) but not anti-PKC-α (Fig. 6b) antibodies delivered to YT cells by electroporation reduced YT cell-mediated killing of JY cells. Cytolysis of JY cells was inhibited up to 60% in anti-PKC-δ-treated YT cells. This effect was specific, since addition of a peptide blocking anti-PKC-δ in combination with anti-PKC-δ antibody to YT cells for electroporation, neutralized the effect of this antibody (Fig. 6a).

A similar experimental approach has already been successfully applied to demonstrate that antibody against PKC-β reduced the potential of human lymphocytes to secrete interleukin-2 (IL-2) and proliferate in response to the stimulation with anti-CD3 mAb[13] and antibodies against PKC-α and PKC-θ introduced into human lymphocytes by electroporation completely inhibited IL-2 receptor expression.[14] It is worth noting that the latter study also provided direct evidence that

introduction of PKC isoform-specific antibodies by electroporation effectively neutralizes cytoplasmic PKC activity in T lymphocytes. It has been shown that different anti-PKC antibodies, introduced into T cells by electroporation, partially inhibited specific PKC activity and to a similar extent as in the cytosolic fraction after direct addition of the respective antibodies.[14] These results revealed that sufficient amounts of anti-PKC antibodies were introduced into the cells to inhibit selective PKC activities.

YT cells mediate their cytotoxic activity primarily through exocytosis of cytolytic granules, containing perforin and granzyme B,[20] which can be released after CD28 cross-linking.[5] It is interesting to mention here that secretion of granules from another cell type, namely rat basophilic cells RBL-2H3, required PKC-β and PKC-δ isoenzymes.[21] Thus, PKC-δ may be involved in the process of granule release by YT cells. These data do not exclude, however, the role of PKC-δ in other pathways, for example, activation of tyrosine kinases.[22]

The results presented in this study extend previous reports and suggest that CD28-triggered cytotoxicity of YT cells requires the involvement of PKC-δ isoenzyme. In addition, this study demonstrates that one of the primary targets of bryostatin 1 is PKC-δ, which was selectively down-regulated by prolonged treatment with this agent.

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