

Activation of PKC- ϵ counteracts maturation and apoptosis of HL-60 myeloid leukemic cells in response to TNF family members

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Protein kinase C (PKC)- ϵ , a component of the serine/threonine PKC family, has been shown to influence the survival and differentiation pathways of normal hematopoietic cells. Here, we have modulated the activity of PKC- ϵ with specific small molecule activator or inhibitor peptides. PKC- ϵ inhibitor and activator peptides showed modest effects on HL-60 maturation when added alone, but PKC- ϵ activator peptide significantly counteracted the pro-maturative activity of tumor necrosis factor (TNF)- α towards the monocytic/macrophagic lineage, as evaluated in terms of CD14 surface expression and morphological analyses. Moreover, while PKC- ϵ inhibitor peptide showed a reproducible increase of TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis, PKC- ϵ activator peptide potently counteracted the pro-apoptotic activity of TRAIL. Taken together, the anti-maturative and anti-apoptotic activities of PKC- ϵ envision a potentially important pro-leukemic role of this PKC family member.

Key words: acute myeloid leukemia, surface antigens, HL-60 cells, apoptosis, maturation.

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Activation of all protein kinase C (PKC) family of serine and threonine isoenzymes is associated with binding to the negatively charged phospholipids, phosphatidylserine, while different PKC isozymes have varying sensitivities to Ca^{2+} and lipid-derived second messengers such as diacylglycerol (Gonelli *et al.*, 2009). Upon activation, PKC isozymes translocate from the soluble to the particulate cell fraction, including cell membrane, nucleus and mitochondria (Gonelli *et al.*, 2009). PKC primary sequence can be broadly separated into two domains: the N-terminal regulatory domain and the conserved C-terminal catalytic domain. The regulatory domain of PKC is composed of the C1 and C2 domains that mediate PKC interactions with second messengers, phospholipids, as well as inter and intramolecular protein-protein interactions. Differences in the order and number of copies of signaling domains, as well as sequence differences that affect binding affinities, result in the distinct activity of each PKC isozyme (Gonelli *et al.*, 2009).

In recent years, a series of peptides derived from PKC have been shown to modulate its activity by interfering with critical protein-protein interactions within PKC and between PKC and PKC-binding proteins (Brandman *et al.*, 2007, Souroujon and Mochly-Rosen, 1998). Focusing on PKC- ϵ isozyme and using a rational approach, one C2-derived peptide that acts as an isozyme-selective activator (Dorn *et al.*, 1999) and another that acts as a selective inhibitor (Johnson *et al.*, 1996) of PKC- ϵ , have been identified. These findings are particularly interesting since besides being involved in the physiology of normal cardiac (Braun and Mochly-Rosen, 2003, Johnson *et al.*, 1996, Li *et al.*, 2006), hematopoietic (Gobbi *et al.*, 2009, Mirandola *et al.*, 2006, Racke *et al.*, 2001), and neuronal (Borgatti *et al.*, 1996) cell models, mounting experimental evidences have linked altered PKC- ϵ functions to

solid tumor development (Okhrimenko *et al.*, 2005, Gillespie *et al.*, 2005, Lu *et al.*, 2006). Therefore, taking advantage of the recent availability of small molecule peptides able to activate or inhibit specifically PKC- ϵ by disrupting protein/protein interactions (Dorn *et al.*, 1999, Johnson *et al.*, 1996), which open important therapeutic perspectives, we have investigated the effects of both PKC- ϵ activator and PKC- ϵ inhibitor peptides on the maturation and survival of leukemic cells, using as a model system the HL-60 myeloblastic leukemia cell line, which can be induced to undergo terminal differentiation or apoptotic cell death by a variety of chemical and biological agents (Breitman *et al.*, 1980, Zauli *et al.*, 1996).

Materials and Methods

Cells and reagents

HL-60 cells, were cultured in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) and seeded at an optimal cell density of $0.8-1.0 \times 10^6$ cells/mL before treatments. A PKC- ϵ -selective agonist octapeptide, $\psi\epsilon$ receptor for activated C-kinase ($\psi\epsilon$ RACK), derived from a PKC- ϵ sequence homologous to its anchoring protein, RACK- ϵ : [HDAPIGYD; PKC- ϵ (85-92)], was synthesized and purified (97%) at Inbios S.r.l. (Napoli, Italy). A PKC- ϵ -selective antagonist peptide, eV1-2 [(EAVSLKPT; PKC- ϵ (14-21)], and a negative control peptide, scrambled eV1-2 (LSETKPAV), were purchased from Calbiochem (Darmstadt, Germany).

HL-60 cells were treated with predetermined optimal concentrations of PKC- ϵ agonist octapeptide (activator), PKC- ϵ antagonist peptide (inhibitor) and scrambled peptide (22 $\mu\text{g}/\text{mL}$); recombinant TNF-related apoptosis inducing ligand (TRAIL) (0.1 $\mu\text{g}/\text{mL}$), prepared as previously described (Zauli *et al.*, 2003), or recombinant tumor necrosis factor (TNF)- α (0.1 $\mu\text{g}/\text{mL}$) (R&D Systems, Minneapolis, MN, USA), used either alone or in combination. In some experiments, HL-60 were treated with 10^{-7} M phorbol myristate acetate (PMA), purchased from Sigma Chemicals (Minneapolis, MN). The PKC- ϵ catalytic activity was evaluated by using the PKC ϵ KinEASE™ FP fluorescein green assay kit, used according to the manufacturer's instructions (Millipore, Billerica, MA, USA).

Assessment of cell maturation and apoptosis

At different times (1-3 days) post-treatment with PKC- ϵ activator and inhibitor peptides, TNF- α or TRAIL, both maturation-inducing activity and cytotoxicity were assessed. In particular, samples were analyzed by: (1) monitoring cell surface antigen expression of CD14 and CD11b myeloid antigens (Todd *et al.*, 1981, Zhang *et al.*, 1994); (2) evaluating the degree of apoptosis by Annexin V/propidium iodide (PI) double staining and flow cytometry analysis and/or poly(ADP)ribose polymerase (PARP) cleavage in Western blot, as previously described (Borgatti *et al.*, 1997, Vitale *et al.*, 1997). For flow cytometry analyses, both the Annexin V-fluorescein isothiocyanate (FITC)/PI apoptosis kit and the CD11b and CD14 phycoerythrin (PE) or FITC-conjugated antibodies (Abs) were purchased from Immunotech (Marseille, France). Unspecific fluorescence was assessed by using isotype-matched controls; (3) examining the morphology of the cells by staining with May-Grunwald-Giemsa solution followed by light microscopy examination (Secchiero *et al.*, 2002, Secchiero *et al.*, 2007); (4) counting the total number of viable cells by trypan blue dye exclusion; (5) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay (Roche Diagnostics Corporation, Indianapolis, IN, USA).

Western blot analysis

Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.5% NP40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 $\mu\text{g}/\text{mL}$ of pepstatin. For Western blot analysis, 50-70 μg of protein were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with monoclonal Abs anti-DNA repair enzyme PARP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-actin (Sigma Aldrich, St. Louis, MO, USA) used for loading control (Milani *et al.*, 2003). Membranes were washed and further incubated for one hour at room temperature with peroxidase-conjugated secondary Ab (Sigma). Detection was then performed using the Renaissance chemiluminescent ECL kit (NEN Dupont, Boston, MA, USA). Protein levels were densitometrically analysed by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Multiple film exposures were used to verify the linearity of the samples analyzed and to avoid saturation of the film.

Statistical analysis

Data were analyzed using the two-tailed Student's t-test. Statistical significance was defined as $p < 0.05$.

Results

PKC- ϵ activator peptide counteracts maturation induced by TNF- α

The effect of pre-determined optimal concentrations of specific peptides to modulate the catalytic activity of PKC- ϵ was initially evaluated by using a kit assay specific for PKC- ϵ . As shown in Figure 1, the PKC- ϵ activator peptide significantly ($p < 0.05$) promoted PKC- ϵ catalytic activity. On the other hand, the PKC- ϵ inhibitor peptide depressed the basal levels of PKC- ϵ catalytic activity and profoundly inhibited ($p < 0.05$) the activation of PKC- ϵ induced by PMA. Since an important anti-leukemic strategy is to promote terminal growth arrest and maturation of acute myeloid leukemia (AML) blasts, in the first group of experiments, we have investigated whether the PKC- ϵ activator and inhibitor peptides were able to modulate the phenotypic profile and morphology of HL-60 cells, after treatment with TNF- α , which represents a powerful inducer of maturation along the monocytic/macrophagic lineage (Secchiero *et al.*, 2003). As shown in Figure 2A and 2B, TNF- α markedly up-regulated the surface expression of CD14, which repre-

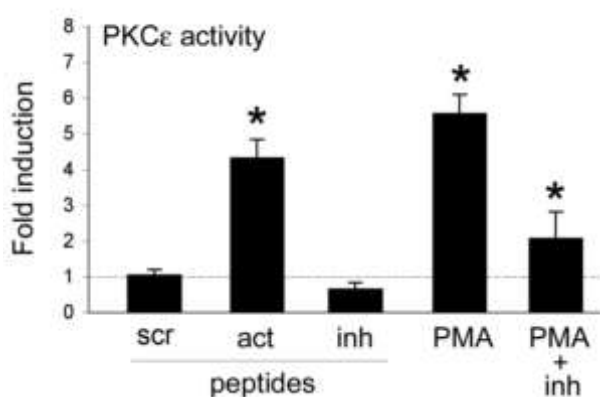


Figure 1. Validation of the PKC- ϵ activator and inhibitor peptides in HL-60 cells. HL-60 cells were incubated with pre-determined optimal concentrations (22 $\mu\text{g}/\text{mL}$) of PKC- ϵ activator (act) or inhibitor (inh) peptides, or with scrambled (scr) peptides used as negative controls for 8 hours. In parallel, PMA was used as positive control, for its ability to activate PKC- ϵ . PKC- ϵ catalytic activity, evaluated as described in Materials and Methods, is expressed as fold of PKC- ϵ activation with respect to control untreated cultures. Data represent the means \pm SD of three independent experiments. * $p < 0.05$.

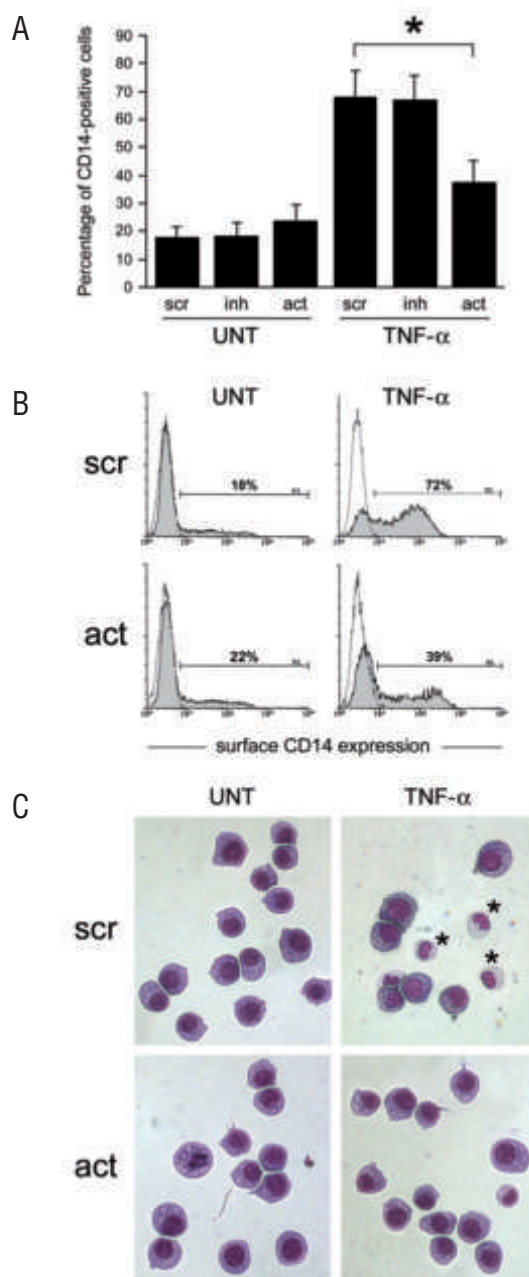


Figure 2. Maturation effect of TNF- α , PKC- ϵ activator and inhibitor peptides on HL-60 cells. HL-60 cells were incubated with control scrambled (scr), PKC- ϵ activator (act) or inhibitor (inh) peptides (22 $\mu\text{g}/\text{mL}$), used alone or in combination with TNF- α (10 ng/mL) for 72 hours and then analyzed for the surface expression of CD14 myeloid antigen, by flow cytometry analysis, and for cell morphology, by microscopic examination. In A, CD14 surface expression is reported as percentage of positive cells, and data are expressed as the mean \pm SD of five independent experiments performed in duplicate. * $p < 0.05$. In B, a representative experiment is shown: shadowed histograms represent cells stained with Abs specific for the indicated surface antigen, whereas unshadowed histograms represent the background fluorescence obtained from the staining of the same cells with isotype-matched control Abs. In C, the effects of TNF- α and PKC- ϵ activator peptide on cell morphology are shown. HL-60 cells were stained with May-Grünwald-Giemsa solution; asterisks indicate cells with monocytic morphology. Representative fields of five separate experiments are shown. Original magnification: X400. UNT = untreated cells.

sents an excellent marker of monocytic maturation since it is undetectable on the surface of monocyte precursors and increases dramatically during their differentiation to monocytes (Todd *et al.*, 1981; Zhang *et al.*, 1994). While PKC- ϵ inhibitor peptide had negligible effects on TNF- α -induced up-regulation of CD14, PKC- ϵ activator peptide significantly ($p < 0.05$) counteracted the TNF- α up-regulation of CD14 (Figure 2A-2B). Similarly, PKC- ϵ activator peptide almost abrogated the TNF- α up-regulation of CD11b, used as additional surface marker of monocytic maturation (Secchiero *et al.*, 2002). To confirm these flow cytometric results by morphological analyses, after 3 days of treatment with TNF- α plus PKC- ϵ peptides, HL60 cells were cytocentrifuged and then stained with May-Grunwald-Giemsa solution before light microscopy examination. As shown in Figure 2C, TNF- α plus PKC- ϵ activator peptide treated cultures showed a decrease of cells with monocytic features, such as condensation and cleavage of the nucleus, with respect to TNF- α alone.

PKC- ϵ activator peptide counteracts the pro-apoptotic activity of recombinant TRAIL

Since the TNF family member TRAIL is a promising therapeutic agent for its ability to induce apoptotic cell death in a variety of tumor cells, including leukemias (Zauli and Secchiero, 2006), we performed additional experiments to evaluate the effects of combined treatments with either PKC- ϵ activator or PKC- ϵ inhibitor peptides plus recombinant TRAIL on the induction of HL-60 cell apoptosis, evaluated by Annexin V/PI double staining and flow cytometry analysis. Increasing doses of recombinant TRAIL were tested, in order to identify the concentration (100 ng/mL) able to promote a significant increase of HL-60 apoptosis without inducing the maximal apoptotic response (Figure 3A). Of note, neither PKC- ϵ activator nor PKC- ϵ inhibitor peptides used alone showed significant effects on HL-60 viability and apoptosis. When used in combination with TRAIL, the PKC- ϵ inhibitor peptide modestly but reproducibly ($p < 0.05$) increased the percentage of apoptosis in response to TRAIL (Figure 3B). Conversely, PKC- ϵ activator peptide significantly ($p < 0.05$) counteracted the ability of TRAIL to induce apoptosis (Figure 3B). The ability of these peptides to modulate the apoptotic activity of TRAIL was also confirmed by Western blot analysis of PARP cleavage, a target of caspase activity (Figure 3C).

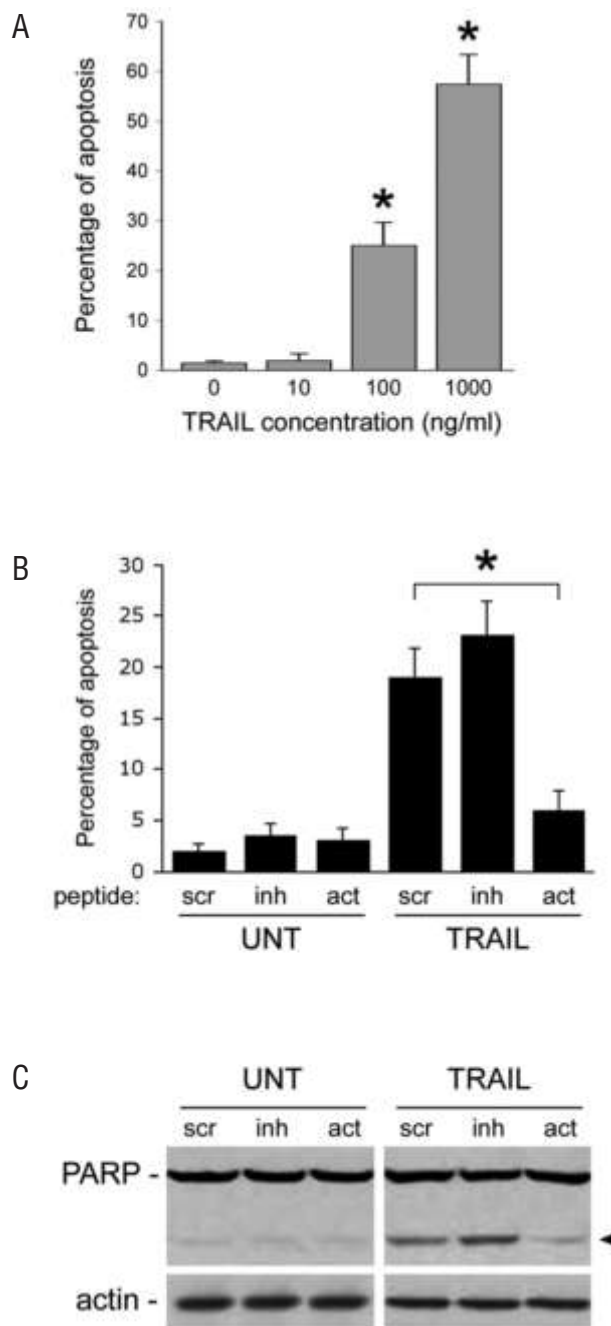


Figure 3. Effect of PKC- ϵ activator and inhibitor peptides on TRAIL-mediated cytotoxicity in HL-60 cells. A: Dose-dependent effect of TRAIL on apoptosis quantitatively evaluated by flow cytometry after Annexin V/PI staining in HL-60 cells. Cells were cultured for 24 hours with the indicated concentrations of TRAIL. B: HL-60 cells were incubated with optimal concentrations (22 $\mu\text{g/mL}$) of PKC- ϵ activator (act) or inhibitor (inh) peptides, used alone or in combination with recombinant TRAIL (0.1 $\mu\text{g/mL}$) and the percentage of apoptosis was assayed by counting the number of Annexin V positive cells. In A and B, data represent the means \pm SD of five independent experiments performed in duplicate. * $p < 0.05$. C: PARP cleavage was also analyzed as apoptotic marker. The full-length (115 kDa) and the cleaved forms (80 kDa; arrowhead) of PARP of a representative experiment are shown. Actin staining is shown as loading control. For both flow cytometry (B) and Western blot analysis (C) scrambled (scr) peptides were used as negative controls. UNT = untreated cells.

Discussion

In this study, we have demonstrated for the first time that PKC- ϵ activation significantly counteracted the differentiation program induced by TNF- α along the monocytic lineage. This aspect of PKC- ϵ activity was not described before and it has important therapeutic implications since induction of mortality by terminal differentiation represents an alternative approach to cytodestruction of cancer cells by conventional antineoplastic agents, and has important biological implications. In this respect, retinoid acids are well known inducers of granulocytic differentiation of primary acute promyelocytic leukemia (APL) blasts and leukemic cell lines (Chambon, 1994). However, while some retinoids are currently used in the treatment of the M3-type of AML, agonists such as vitamin D₃, able to induce monocytic differentiation in other subtypes of AML and in particular in M4- and M5-types, did not demonstrate efficacy in clinical trials performed in AML patients, mainly due to a secondary hypercalcemia thus limiting the dose of vitamin D₃ that could be administered (Bar-Shavit *et al.*, 1983).

While the PKC- ϵ inhibitor peptide showed negligible effects on the TNF- α -mediated induction of leukemic maturation, this does not exclude that the association of PKC- ϵ inhibitor peptide and pro-maturative drugs might represent an important therapeutic combination. In this respect, it is also noteworthy that both PKC- ϵ inhibitor and activator peptides were able to significantly modulate the degree of apoptosis induced by TRAIL. The resistance of leukemic cells to currently used therapies occurs in part because leukemic cells safeguard their survival through mechanisms that allow them to escape death receptor-mediated apoptosis (Hanahan and Weinberg, 2000). Much attention has recently attracted the TNF family member TRAIL for its ability to overcome resistance to apoptosis in several tumors, including hematological malignancies (Secchiero and Zauli, 2008). While several studies have demonstrated that TRAIL resistance in a variety of hematological malignancies is mainly due to constitutively high levels of c-FLIP or low levels of TRAIL receptors (Secchiero and Zauli, 2008), we have here demonstrated that also the selective activation of the PKC- ϵ family member can markedly counteract the susceptibility to TRAIL cytotoxicity in the sensitive HL-60 cell line. Our current findings are in line with

those recently described by Gobbi *et al.* (2009), who demonstrated that PKC- ϵ activation by phorbol esters confers resistance to apoptosis induction in the K562 leukemic cell line. However, an important difference between the study of Gobbi *et al.* (2009) and our current data is that we have used inhibitor and stimulatory peptides which might have important future clinical applications. In fact, Yonezawa *et al.* (2009) have recently reviewed the potential use of PKC- ϵ specific peptides developed by important pharmaceutical companies, which specifically inhibit PKC- ϵ and ameliorate pathological conditions in a rodent insulin resistance model.

In summary, we propose that the ability of PKC- ϵ to promote leukemogenesis might be twofold: on one hand, it protects from TRAIL mediated apoptosis, perhaps by down-regulating Bid as demonstrated in other cancer cell models (Sivaprasad *et al.*, 2007) and, on the other hand, it counteracts maturation along the monocytic lineage. Although the physiological stimuli able to activate PKC- ϵ in the bone marrow context are unknown, it is noteworthy that leukemic cells, like normal hemopoietic cells, survive and proliferate in the context of bone marrow niches (Li and Neaves, 2006), it will be of interest to investigate the effect of cell-to-cell contact between bone marrow stromal cells and leukemic cells on the activation state of PKC- ϵ . Thus, while a potential role of PKC isoforms and in particular of PKC- ϵ in promoting tumorigenesis has been previously proposed, the novelty represented by this study is that we have demonstrated that PKC- ϵ not only affect leukemic cell survival but also leukemic maturation. Moreover, a clinical implication of our study is that selective inhibition of PKC- ϵ with inhibitor peptides, either to drugs able to induce maturation or to death inducing ligands or to combination thereof, should be taken into account in order to improve their therapeutic potential.

Acknowledgments

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