

Essential Role of PKC δ in Apoptosis Induction of Mouse Thymocytes

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Abstract: The family of protein kinases C (PKCs) has been implicated in signal transmission leading to apoptosis induction and/or survival. These effects are cell type and tissue dependent. Numerous studies employing phorbol ester, a pleiotropic PKC activator, strongly implicated PKC in apoptosis induction of thymocytes. However, phorbol esters activate both, the conventional PKCs (PKC α , β , γ) as well as the novel PKCs (δ , ϵ , η and θ), the PKC isotype(s) selectively involved in this process have not been established. In this study we used selective pharmacological PKC inhibitors and our established set of PKC knockout mice to define the PKC isotype that is involved in cell death induction of thymocytes. Pharmacological inhibition of nPKCs and in particular gene ablation of PKC δ , results in a profound reduction of p53-dependent as well as independent apoptosis induction. In strict contrast, loss of conventional PKCs as well as loss of two other thymocyte-expressed nPKC family members, PKC ϵ and PKC θ , does not significantly affect thymocyte apoptosis. Taken together, we define an essential and non-redundant pro-apoptotic role of PKC δ in regulating distinct signaling mechanisms that are required to provoke apoptosis of mouse double positive thymocytes *in vitro*.

Key words: Phorbol esters, PKC isotypes, thymocytes, apoptosis induction

INTRODUCTION

Protein kinase Cs (PKCs) are members of the serine/threonine protein kinase family, originally identified by Nishizuka and colleagues in 1977 as cyclic nucleotide-independent kinases^[1]. Members of the PKC family have been implicated in numerous cell responses including cell growth and differentiation, cell cycle control, homeostasis, synaptic transmission, ion fluxes, secretion and tumorigenesis of a wide range of cell types and tissues^[2]. According to their molecular structure and cofactor dependency they are classified into conventional PKCs (cPKCs) PKC α , β , γ , novel PKCs (nPKCs) PKC δ , ϵ , η , θ and atypical PKCs (aPKCs) PKC ζ and PKC i . cPKCs require Ca²⁺ and diacylglycerol for activation, nPKCs are Ca²⁺ independent and aPKCs neither require Ca²⁺ nor diacylglycerol. Some of the isotypes exist as different splice variants^[3]. All PKC isotypes listed, except PKC γ are expressed in T cells. Among them PKC α , PKC δ , PKC ϵ and PKC θ are highly expressed^[4]. PKCs are known to phosphorylate several cellular proteins, leading to modulation of surface antigens, activation of other protein kinases and induction of several key transcription factors, including aberrant signalling responses contributing towards malignant transformation^[5].

Apoptosis or programmed cell death is a physiological process during thymic development.

Thymic selection of immature T cells occurs at CD4⁺CD8⁺ double positive (DP) stage^[6,7]. Only clones that will recognize foreign antigens associated with self-MHC molecules are positively selected to survive and differentiate into CD4⁺ or CD8⁺ single positive (SP) T cells (positive selection)^[8,9]. On the other hand a high avidity T cell receptor (TCR) interaction promotes apoptosis of developing thymocytes (negative selection)^[8-10]. Both, positive as well as negative selection, depend on direct cell-cell interactions between DP thymocytes and thymic stroma cells. However it is still unclear which intracellular signaling pathways are responsible for thymic selection. Deregulation of apoptosis is a hallmark of many diseases such as lymphoid cancers as well as autoimmunity^[11].

The process of apoptosis is regulated at several levels through phosphorylation by many different protein kinases. The PKC family has been shown to exert both inhibitory and stimulatory influences, depending on cell type and stimulus for apoptosis induction. Generally, PKC α , β , ϵ as well as atypical PKC isotypes are considered to be involved in anti-apoptotic pathways, whereas PKC δ and PKC θ are considered to be involved in promotion of cell death^[12]. Previous studies with mouse thymocytes and phorbol esters, pleiotropic PKC activators, however, revealed controversial results: DNA fragmentation induced by calcium ionophore or glucocorticoids in thymocytes

was abrogated by phorbol ester treatment, indicating an anti-apoptotic role of PKCs^[13]. On the other hand, phorbol ester treatment in thymocytes was shown to enhance apoptosis induced by tyrosine-kinase inhibitors, implying that PKC promotes apoptosis^[14]. More recent studies, mostly using PKC specific pharmacological inhibitors, revealed both, an anti-apoptotic and a pro-apoptotic role of PKC isotypes in thymocytes. Simon and co-workers suggested PKC ϵ as a negative regulator of negative selection of DP thymocytes^[15]. Consistently, phorbol ester was also found to prevent immature thymocytes from spontaneous as well as glucocorticoid-induced apoptosis^[16,17]. On the other hand the activation of nPKCs, especially the PKC θ isotype, was suggested to promote a pathway for negative selection^[18,19]. Along this line, a positive involvement of PKCs in glucocorticoid-induced apoptosis^[20] as well as a role of PKCs in peptide-specific cell death^[21] was postulated.

This in part contradictory nature of these results occurred primarily from the use of phorbol esters as PKC activators, because they stimulate both, cPKCs and nPKCs. Additionally, phorbol esters also target receptors other than PKC, a concept that has been largely ignored in the past. In this study we used selective PKC inhibitors and for the first time, our established set of PKC knock out mice to now genetically define the distinct PKC isotypes that are involved in apoptosis induction of thymocytes. To investigate the potential role of PKCs in different apoptosis pathways, we induced apoptosis employing various agents, namely phorbol-12,13-dibutyrate (PDBu) (role in apoptosis^[22-26]), the calcium ionophore ionomycin^[27], staurosporine, an inhibitor of phospholipid/Ca²⁺ dependent kinases mediating mitochondrial dysfunction^[28], dexamethasone, a glucocorticoid regulating T cell survival, growth and differentiation^[29-31], concanavalin A, a mitogenic plant lectin that mimics the effects of high avidity TCR ligation^[32] and two agents inducing DNA damage, camptothecin^[33] and etoposide^[34]. FasL, a protein belonging to the tumor necrosis factor (TNF) family of cytokines was used to induce the extrinsic death receptor apoptotic pathway^[35]. Finally, apoptosis was also induced by TCR crosslinking via anti CD3.

MATERIALS AND METHODS

Mice: C57/Bl6 wild-type mice were obtained from the animal facility of the Medical University of Vienna, Himgberg, Austria. For knock out studies mice lacking a specific PKC isotype, which have been described elsewhere (PKC α ^[36], PKC δ ^[37], PKC θ ^[38] and PKC ϵ ^[39]) were used. p53 knock out mice were provided from A. Villunger.

Genotyping of mice: DNA was extracted from adult tail tissue. Genotyping of the PKC δ , PKC ϵ and

PKC θ alleles was performed by southern blot analysis or PCR was described (PKC δ), (PKC ϵ), (PKC θ)^[37,38,40]. The PKC α allele was genotyped by PCR using the primers PKC α 3' prime E2 (5'-CCT GGT GGC AAT GGG TGA TCT ACA C-3') and PKC α 5' prime E2 (5'-GAG CCC TTG GGT TTC AAG TAT AGA-3') [Pfeifhofer, unpublished data].

Preparation of thymocytes: Thymi were taken from 6-8 week old mice. Cells were singularized by mashing the organ in a strainer and raising the cell suspension through a syringe three times. Cells were washed in RPMI medium (Sigma Aldrich) and used for the assay.

Apoptosis detection: Freshly isolated thymocytes were plated in 96-well plates at a density of 2.5×10^5 cells per well in a total volume of 200 μ l in RPMI medium (Biochrom) supplemented with 10% heat-inactivated fetal calf serum, 2mM L-glutamine and 50 u mL⁻¹ penicillin/streptomycin (all from Biochrom). For some experiments cells were preincubated with distinct PKC inhibitors for 90 min prior to apoptosis induction. Gö6850 and Gö6976 were obtained from Calbiochem. The pan PKC LMW Inhibitor was obtained from Altana Pharma. Inhibitors were used at a concentration of 500nM. Apoptosis induction was performed by TCR crosslinking (10 μ g mL⁻¹ anti CD3 2C11, precoated) +/- anti CD28 (1 μ g mL⁻¹) or by addition of either concanavalin A (10 μ g mL⁻¹), phorbol-12,13-dibutyrate (1 μ g mL⁻¹), ionomycin (1 μ g mL⁻¹), camptothecin (1 μ M), etoposide (10 μ g mL⁻¹), dexamethasone (1nM), staurosporine (100nM) or FasL (100 ng mL⁻¹) plus enhancer for FasL (1 μ g mL⁻¹) for up to 72 h. FasL and enhancer for FasL were purchased from Alexis, anti CD28 from BD Pharmingen and all other agents were obtained from Sigma-Aldrich. Cells were harvested at time points between 10 and 72 h after apoptosis induction. Late apoptotic and necrotic cells were stained with propidium iodide (5 μ g mL⁻¹ in PBS). Percentage of viable cells was determined by FACS analysis employing the FACScanTM Cytometer (Becton Dickinson) and CellQuestTM software.

RESULTS

PKC inhibition selectively prevents phorbol ester induced apoptosis: In order to investigate the role of distinct PKC isotypes in apoptosis induction of mouse double positive thymocytes we induced apoptosis *ex vivo* in thymocytes derived from wild-type C57/Bl6 mice, which were preincubated with pan PKC LMWI, inhibiting both, the conventional PKCs, PKC α and PKC β , as well as the novel PKCs, PKC δ , PKC ϵ , PKC θ and PKC η or DMSO as buffer control. Apoptosis was induced by various apoptosis inducing agents, as indicated. As result, we observed a strong protection of PDBu induced apoptosis in cells preincubated with the

pan PKC LMWI (Fig. 1A). In contrast, PKC inhibition did not affect apoptosis induced by TCR crosslinking (Fig. 1B), concanavalin A (Fig. 1C), dexamethasone (Fig. 1D), ionomycin (Fig. 1E), staurosporine (Fig. 1F), camptothecin (Fig. 1G) or etoposide (Fig. 1H).

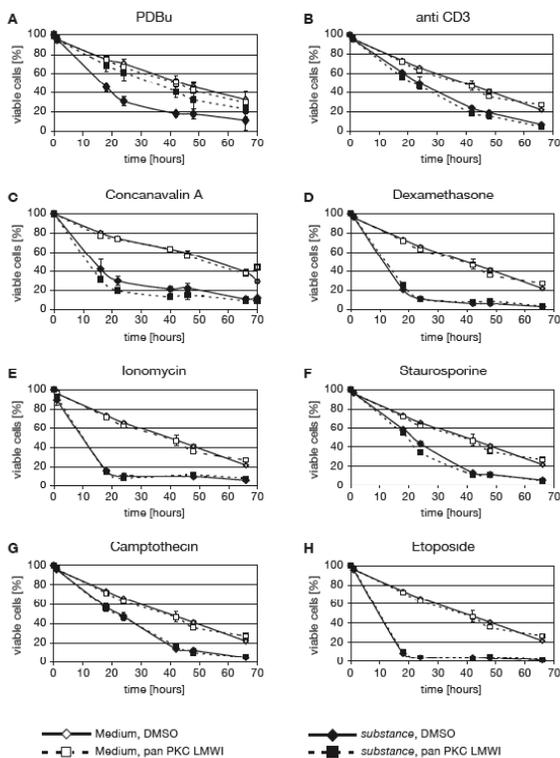


Fig. 1: Pan PKC (cPKC+nPKC) inhibition prevents PDBu induced apoptosis, while apoptosis induced by TCR crosslinking, concanavalin A, dexamethasone, ionomycin, staurosporine, camptothecin and etoposide is not affected. Freshly isolated thymocytes from wild-type mice were plated into 96-well plates at a density of 2.5×10^5 cells per well and preincubated with pan PKC LMWI for 90 min prior to apoptosis induction by PDBu (A), anti CD3 (B), concanavalin A (C), dexamethasone (D), ionomycin (E), staurosporine (F), camptothecin (G) and etoposide (H). Viable cells were determined by FACS analysis at the indicated time points. Results shown are the means \pm SD of four independent experiments

Essential role of novel PKC isotypes in phorbol ester induced apoptosis: To further investigate which isotype might be involved in phorbol ester induced apoptosis we treated wild-type thymocytes with different PKC inhibitors either inhibiting conventional and novel PKCs (Gö6850 and pan PKC LMWI) or inhibiting selectively the conventional PKCs (Gö6976). As result, both pan PKC LMWI prevented thymocytes from PDBu induced apoptosis while cPKC inhibition did not (Fig. 2). These data suggested a proapoptotic role of at least one of the novel PKCs, PKC δ , PKC ϵ , PKC η or PKC θ . Furthermore and employing thymocytes derived from a p53-deficient mouse line,

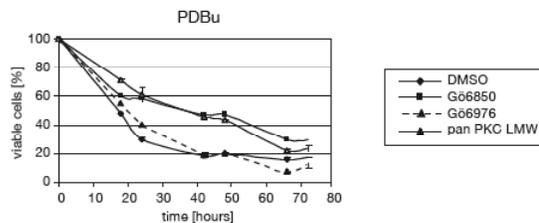


Fig. 2: Inhibition of novel PKCs protects thymocytes from phorbol ester induced apoptosis. Freshly isolated thymocytes from wild-type mice were plated into 96-well plates at a density of 2.5×10^5 cells per well and preincubated with pan PKC inhibitors, pan PKC LMWI or Gö6850, respectively, or Gö6976, the latter an inhibitor of conventional PKCs, for 90 min prior to apoptosis induction by PDBu. Viable cells were determined by FACS analysis at the indicated time points. Results shown are the means \pm SD of three independent experiments

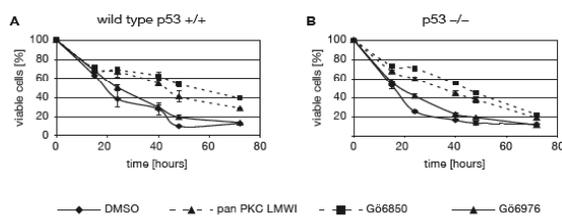


Fig. 3: The proapoptotic role of novel PKCs is independent of p53. Thymocytes from p53 knockout mice have been analyzed in comparison to wild-type littermates. Apoptosis was induced by PDBu. Percentage of viable cells was determined by FACS analysis. Results shown are the means \pm SD of two independent experiments

this proapoptotic action of nPKCs appears independent of p53 function (Fig. 3).

Proapoptotic role of PKC δ in apoptosis of mouse thymocytes: Consistent with the results obtained in studies with pharmacological PKC inhibitors, experiments with our established set of PKC knock out mice revealed a proapoptotic role of PKC δ in phorbol ester induced apoptosis of mouse thymocytes (Fig. 4A). Heterozygous PKC $\delta^{+/-}$ thymocytes demonstrated an intermediate phenotype. Importantly, thymocytes derived from our PKC ϵ or PKC θ deficient mice were indistinguishable from wild-type littermates (Fig. 4B and 4C). PKC ϵ or PKC θ deficiency also did not affect apoptosis induced by ionomycin, concanavalin A, dexamethasone, staurosporine, camptothecin, etoposide or TCR crosslinking (data not shown). As a representative member of the cPKC family, PKC $\alpha^{-/-}$ deficient thymocytes were tested as well. Consistently, with the data collected with experiments employing PKC inhibitors, we did not observe any effects in PKC $\alpha^{-/-}$ thymocytes in respect to apoptosis induction by PDBu (data not shown), as well as ionomycin,

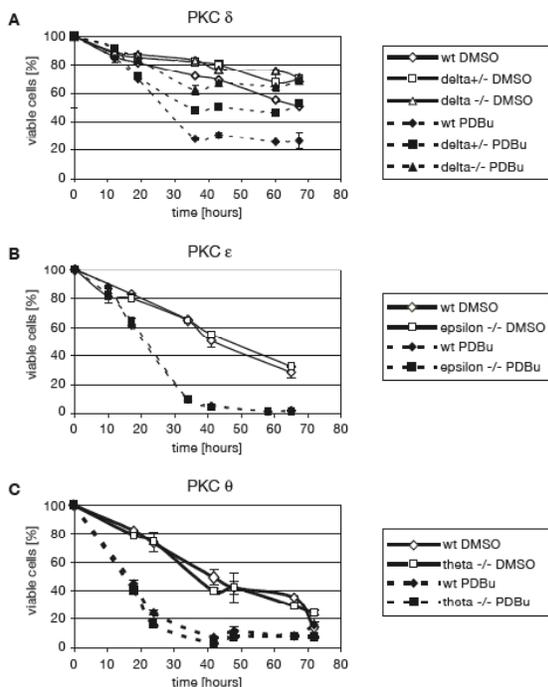


Fig. 4: Proapoptotic role of PKC δ in phorbol ester induced apoptosis versus normal *ex vivo* survival of PKC ϵ deficient and PKC θ deficient thymocytes. Thymocytes from PKC $\delta^{-/-}$, PKC $\epsilon^{-/-}$ and PKC $\theta^{-/-}$ knock out mice and wild-type littermates were plated into 96-well plates at a density of 2.5×10^5 cells per well. Apoptosis was induced by addition of phorbol-12,13-dibutyrate. Percentage of viable cells was determined by FACS analysis. Results shown for PKC $\delta^{-/-}$ (A) are the means \pm SD of two independent experiments. For PKC $\epsilon^{-/-}$ (B) and PKC $\theta^{-/-}$ (C) one representative experiment out of two is shown.

concanavalin A, dexamethasone, staurosporine, camptothecin, etoposide or TCR crosslinking (data not shown).

Interestingly, deficiency of PKC δ additionally protects thymocytes from apoptosis by TCR crosslinking (Fig. 5A and 5B), dexamethasone (Fig. 5C), FasL (Fig. 5D), staurosporine (Fig. 5E) and etoposide (Fig. 5F). No significant effect of PKC δ could be observed in cells treated with concanavalin A (Fig. 5G) and ionomycin (Fig. 5H).

DISCUSSION

Previous studies of the role of the PKC family in apoptosis induction of thymocytes led to very oppositional results due to the application of phorbol esters as PKC activators. Proapoptotic effects^[14,18-21] as well as antiapoptotic effects^[13,16,17] were suggested. Phorbol esters are known to activate both the conventional PKCs (PKC α , β , γ) as well as the novel PKC family members (PKC δ , ϵ , η , θ), making it impossible to define the exact functional roles of distinct PKC isoforms in cell survival and/or apoptosis.

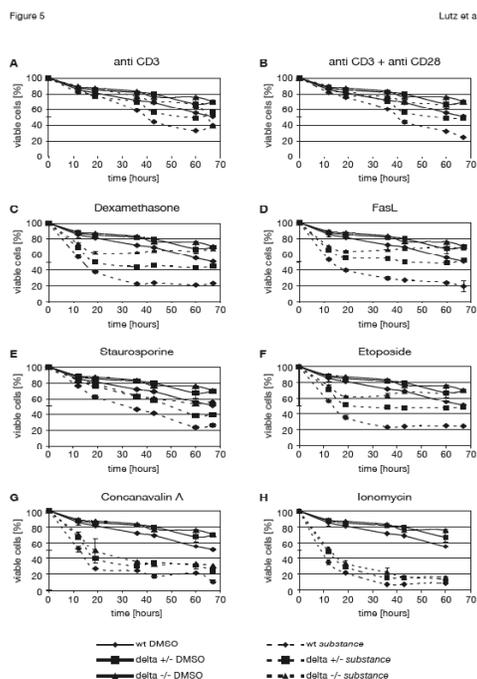


Fig. 5: Pleiotropic proapoptotic role of PKC δ in thymocyte apoptosis. Thymocytes from PKC $\delta^{-/-}$ knock out mice and wild-type littermates were plated into 96-well plates at a density of 2.5×10^5 cells per well. Apoptosis was induced by TCR crosslinking (A & B), dexamethasone (C), FasL (D), staurosporine (E), etoposide (F), concanavalin A (G) and ionomycin (H). Percentage of viable cells was determined by FACS analysis. Results shown are the means \pm SD of two independent experiments

In order to identify the exact PKC isoforms positively or negatively involved in apoptosis of primary mouse double positive thymocytes we used selective PKC inhibitors and observed a proapoptotic role of novel PKCs in PDBu induced apoptosis (Fig. 1A and Fig. 2). Beside developmental apoptosis, programmed cell death is necessary as a cellular stress response, for example to agents inducing DNA damage as well as for activation induced cell death. Here and in contrast, pharmacological PKC inhibition neither affected DNA damage induced apoptosis by etoposide or camptothecin nor glucocorticoid induced apoptosis by dexamethasone nor apoptosis induced by the ionophore ionomycin, by staurosporine, by concanavalin A or anti CD3 mediated TCR crosslinking (Fig. 1B-H).

Using thymocytes from all available novel PKC knock out mice we demonstrated the involvement of PKC δ (Fig. 4A and Fig. 5, but neither PKC ϵ (Fig. 4B) nor PKC θ (Fig. 4C) in apoptosis induced by PDBu, dexamethasone, FasL, staurosporine or etoposide. In contrast to the results obtained employing the pan PKC LMWI, knock out studies revealed that PKC δ is

involved in various apoptotic pathways and that the promotion of apoptosis is not restricted to phorbol ester induced apoptosis. Ionomycin and concanavalin A were in fact the only substances where we did not observe reduced apoptosis of PKC $\delta^{-/-}$ and PKC $\delta^{+/-}$ cells (Fig. 5). This difference between inhibitor and knock out studies most likely is due to the fact that there always remains a residual activity of PKC isotypes upon pharmacological inhibition whereas in the knock out studies, it does not.

Investigations of different cell types postulated an essential role of PKC δ in apoptosis induction. For example, PKC δ was found to be activated during spontaneous apoptosis of neutrophils^[41,42], in ventricular myocytes under hypoglycemic conditions^[43], in various cell types in response to H₂O₂^[44,45], manganese containing compounds^[46] and ceramide^[47]. The extrinsic apoptotic pathways mediated by TNF- α and FasL were shown to result in activation of PKC δ , too^[48,49]. Also DNA damage by UV-radiation, ionizing radiation or etoposide is associated with PKC δ activation^[50]. Along this line, inhibition of PKC δ was shown to protect various cell types from various apoptosis inducers, indicating that PKC δ acts as proapoptotic signaling molecule. As potential downstream effectors, proapoptotic BH3-only proteins are likely. Consistently, thymocytes from puma-deficient mice were shown to be resistant to phorbol ester induced apoptosis^[51] and also demonstrated reduced apoptotic responses to stress stimuli as well as to staurosporine or glucocorticoid treatment. DNA damage and oncogenes activate the proapoptotic BH3-only protein puma via the Mdm2-p53 pathway, while PMA, ionomycin, staurosporine and dexamethasone lead to p53 independent activation of puma^[51]. Along this line we demonstrated that the protection of PDBu induced apoptosis in cells preincubated with a pan PKC inhibitor is independent of p53 (Fig. 3).

Taken together, we thus establish a physiological and non-redundant proapoptotic role of PKC δ but not PKC α , ϵ , nor θ in apoptosis induction of mouse DP thymocytes.

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Abbreviations: DMSO, dimethyl sulfoxide; DP, double positive; FACS, fluorescence activated cell sorter; FasL, Fas ligand; aPKCs, atypical PKCs; cPKCs, conventional PKCs; nPKCs, novel PKCs; pan PKC

LMWI, pan PKC low molecular weight inhibitor; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C, SD, standard deviation; SP, single positive; TCR, T cell receptor; TNF, tumor necrosis factor; VP16, Etoposide, wt, wild-type.

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