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PKCθ is required for the activation of human T lymphocytes induced by CD43 engagement☆

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Abstract

The turnover of phosphoinositides leading to PKC activation constitutes one of the principal axes of intracellular signaling. In T lymphocytes, the enhanced and prolonged PKC activation resulting from the engagement of the TcR and co-receptor molecules ensures a productive T cell response. The CD43 co-receptor promotes activation and proliferation, by inducing IL-2 secretion and CD69 expression. CD43 engagement has been shown to promote phosphoinositide turnover and DAG production. Moreover, PKC activation was found to be required for the activation of the MAP kinase pathway in response to CD43 ligation. Here we show that CD43 engagement led to the membrane translocation and enzymatic activity of specific PKC isoenzymes: cPKC (α/β), nPKC (ε and θ), aPKC (ζ) and PKCμ. We also show that activation of PKCθ resulting from CD43 ligation induced CD69 expression through an ERK-dependent pathway leading to AP-1, NF-κB activation and an ERK independent pathway promoting NFAT activation. Together, these data suggest that PKCθ plays a critical role in the co-stimulatory functions of CD43 in human T cells.
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Keywords: CD43; PKC; T cell activation; NFAT; AP-1; NF-κB; CD69

The interaction between the antigenic peptide presented by the major histocompatibility complex molecules (MHC) on the antigen-presenting cell (APC) and the T cell receptor (TcR)/CD3 complex on the T lymphocyte is the first requirement to induce an antigen-specific T cell response. However, T cell activation depends also on additional signals provided by the association of co-receptor molecules with their counter-receptors on the APC. The fact that those co-stimulatory signals can be replaced by phorbol esters (PMA), which bind and activate most members of the family of protein kinase C (PKC), strongly suggests that some of the signals delivered through these co-receptors depend on PKC activation [1–3].

The CD43 molecule is a heavily glycosylated transmembrane protein expressed on the surface of most hemopoietic cells. Its extracellular domain has an elongated structure that protrudes 45 nm from the cell surface. On T cells, CD43 is differentially glycosylated, generating two major forms: a 113–123 kDa product,

☆ *Abbreviations:* MHC, major histocompatibility complex; APC, antigen presenting cell; TcR, T cell receptor; PKC, protein kinase C; PMA, phorbol myristate acetate; DAG, diacylglycerol; MAP kinase, mitogen-activated protein kinase; AP-1, activator protein-1; NF-κB, nuclear factor κB; NFAT, nuclear factor of activated T cells; mAb, monoclonal antibody; cSMAC, central core of the supramolecular activation complex; Rott, rottlerin; PD98, PD98059; Gö69, Gö6976; MBP, myelin basic protein.

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mainly present on resting $CD4^+$ T lymphocytes, and a 125–135 kDa form expressed mostly on $CD8^+$ T lymphocytes and activated $CD4^+$ T lymphocytes (review in [4]). CD43 engagement has been shown to enhance TcR-mediated cell proliferation and IL-2 production in wild type and $CD28^{-/-}$ T cells [5–7]. Furthermore, CD43-mediated signals were recently shown to enhance HIV-1-dependent gene expression in response to TcR ligation [8].

Engagement of CD43 on T cells leads to the interaction of its cytoplasmic domain with the Src-tyrosine kinase family members Fyn [9] and Lck [10]. This interaction activates both tyrosine kinases resulting in tyrosine phosphorylation of the CD3 complex ζ chain and ZAP-70 [11]. ZAP-70 then phosphorylates downstream molecules allowing the formation of macromolecular complexes containing Shc-GRB2-Vav and Vav-SLP-76 [12]. These early events lead to activation of the MAP kinase pathway resulting in recruitment of AP-1, NF-κB, and NFAT transcription factors, ultimately regulating gene expression [12,13].

The PKC family consists of serine/threonine-specific protein kinases that transduce a plethora of signals mainly derived from the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2). At present, 11 different PKC isoenzymes have been identified and classified according to their structure and cofactor requirements for activation [14]. In addition to the membrane lipid phosphatidylserine, the classical PKC (cPKC) α, βI/βII, and γ isoforms require diacylglycerol (DAG) and Ca^{2+} for activation. In contrast, the novel PKCs (nPKC) δ, ε, η, and θ isoforms, together with the related protein PKCμ (murine homologue protein kinase D, PKD) need DAG but do not require Ca^{2+} [14,15]. Regulation of the atypical PKC (aPKC) ζ and ι isoforms has not been clearly established [16,17]. With the exception of PKCγ, all the PKC isoforms are expressed in T cells [18,19]. However, among them, PKCθ plays a particularly important role in T cell activation. T cell stimulation by TcR ligation induces the translocation of PKCθ to membrane lipid rafts and its localization at the central core of the supramolecular activation complex (cSMAC) formed at the contact region between antigen-specific T cells and APC [20–22]. Studies performed on mature T cells from $PKC\theta^{-/-}$ mice [23,24] and on human T cell lines transfected with constitutive active or inactive forms of PKCθ [2,25–27] have demonstrated that activation of Ras, AP-1, NF-κB, and NFAT are PKCθ-dependent events. In addition, PKCθ induces activation of the c-Jun-N-terminal kinase (JNK), expression of the IL-2 gene and of the leukocyte early activation antigen CD69 [2,27–30].

CD43 engagement has been shown to promote phosphoinositide turnover and DAG production [1]. Recently, we have shown that IL-2 gene expression induced in response to CD43 signaling is a Ca^{2+}- and PKC-dependent event [13] and that PKC activity was required to trigger the MAP kinase pathway after CD43 ligation [12]. However, it is unknown what members of the PKC family of proteins are activated after CD43 engagement. In the present study, we show that CD43 engagement induces the membrane translocation and the catalytic activity of specific PKC isoenzymes: cPKC (α/βI-II), nPKC (ε and θ), aPKC (ζ), and PKCμ in human T lymphocytes. We also show that, by regulating MAP kinase activity and the DNA binding of the AP-1, NF-κB, and NFAT transcription factors PKCθ plays a critical role in the CD43-dependent CD69 expression. Together, our results suggest that CD43 co-receptor functions require PKCθ activity.

Materials and methods

Reagents. L10, an IgG1 mAb that recognizes CD43 [31], was purified from ascites on protein A–Sepharose columns or used as ascites. Rabbit anti-mouse IgG (RαMIg) was generated by repeated immunization with purified mouse IgG, and anti-mouse IgG immunoglobulins were affinity-purified. The anti-CD3 mAb OKT3 (IgG2) was originally obtained from the American Type Culture Collection. The antibodies recognizing different PKC isoforms and anti-STAT1 antibody were from Transduction Laboratories. The anti-actin and anti-c-Fos antibodies were obtained from Santa Cruz Biotechnology. The anti-CD69 antibody was from Caltag. The protein A– or A/G–Sepharose was from Zymed Laboratories. Rottlerin, Gö6976, and PD98059 were from Calbiochem.

Cell culture. Jurkat cells were cultured in RPMI 1640 (Hyclone) supplemented with 5% fetal calf serum (Hyclone) and 5% bovine iron supplemented calf serum (Hyclone), 2 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 50 μM β-mercaptoethanol. Peripheral blood T cells were isolated from healthy adult donors by Ficoll–Hypaque gradient centrifugation, followed by plastic adherence and nylon wool purification, as described [9]. The resultant purified cells were predominantly (>85%) $OKT3^+$ and $L10^+$ (>95%), as determined by FACS analysis. Prior to stimulation, cells were washed and incubated for 2 h in non-supplemented RPMI.

T cell activation. Jurkat cells or purified human T lymphocytes (2×10^7) were incubated in 0.5 ml RPMI for 5 min at room temperature with L10 (4 μg/ml). Crosslinking and activation were achieved by adding RαMIg (4 μg/ml) and further incubating the cells at 37 °C for different times, as indicated in the text. Cells were treated with RαMIg or with isotype control IgG1, as negative controls. As positive control, cells were activated with PMA (50 ng/ml). When indicated, rottlerin (6 μM), PD98059 (50 μM), Gö6976 (0.5 μM) or the solvent (DMSO) was added 15 min prior to activation.

FACS staining. Briefly, cells (1×10^6) were resuspended in 50 μl phosphate-buffered saline containing 2% fetal calf serum and 1% sodium azide (FACS solution) and incubated with anti-CD3 (OKT3), anti-CD43 (L10), anti-CD69 or isotype control mAbs for 20 min at 4 °C. Cells were washed, resuspended in FACS solution, and fixed with 2% paraformaldehyde. Cells were analyzed with a FACSort with the CELLQUEST program (Becton and Dickinson).

Immunoprecipitation and immunoblot analysis. Cells were lysed in 100 μl lysis buffer [25 mM Hepes (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% Triton X-100, 0.5 mM dithiothreitol, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 5 mM NaF, 4 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin] for 20 min at 4 °C. Cell lysates were spun at 13,000*g* for 15 min at 4 °C, and supernatants were precleared with protein A–Sepharose for

at least 2 h at 4 °C prior to immunoprecipitation with the indicated antibody (1 μg/ml) for 2 h at 4 °C and immune complexes were harvested with protein A/G–Sepharose, overnight at 4°C. Proteins from total cell extracts or immunoprecipitates were resolved on SDS–PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% non-fat milk in TBS-T [10 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20], followed by incubation with the indicated antibody diluted in 5% non-fat milk or 3% BSA in TBS-T. After three washes with TBS-T, membranes were incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Biomeda). Proteins were visualized by ECL (Amersham–Pharmacia Biotech), following the manufacturer's instructions.

In vitro kinase assays. Specific PKC isoforms were immunoprecipitated from stimulated or control cell lysates as described above. Immune complexes were washed twice with lysis buffer, twice with kinase buffer [20 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 2 mM Na_3VO_4, and 0.1 mM EGTA (PKCθ) or 0.1 mM $CaCl_2$ (cPKC)] and incubated at 30 °C for 20 min with 25 μl of kinase buffer containing 10 μCi [γ-^{32}P]ATP (3000 Ci/mmol, NEN, Boston, MA), 100 μM ATP, and 2.5 μg myelin basic protein (MBP) as exogenous substrate [30]. The reaction was centrifuged and the supernatant was mixed with an equal volume of 2× SDS–PAGE loading buffer, while the A/G–Sepharose beads were mixed with 20 μl of 2× SDS–PAGE loading buffer. Proteins were separated by SDS–PAGE and radiolabeled MBP was visualized by X-ray film exposure. The amount of precipitated PKC was determined by Western blot analysis and the amount of MBP by Coomassie blue staining.

Subcellular fractionation. Experiments were conducted basically as described [32] with the following modifications. Cells were resuspended in cold hypotonic buffer [42 mM KCl, 10 mM Hepes (pH 7.5), 5 mM $MgCl_2$, and 10 μg/ml each aprotinin and leupeptin] and incubated at 4 °C for 20 min, transferred to a 1 ml syringe, and sheared by being passed 10 times through a 29-gauge needle. Lysates were centrifuged at 200*g* for 10 min at 4 °C to precipitate nuclei, and the supernatant was collected and centrifuged at 100,000*g* for 1 h at 4 °C (cytosolic fraction). The pellet (membrane fraction) was resuspended in 100 μl of lysis buffer and vortexed for 20 min at 4 °C. Samples containing 10^6 cellular equivalents of membrane fraction were separated by SDS–PAGE and immunoblotted with the appropriated Ab.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from stimulated and unstimulated T cells as previously described [33,34]. Binding reactions were carried out using 2 μg nuclear proteins and [^{32}P]dCTP-end labeled double-stranded oligonucleotide probes containing an NFAT binding site from the proximal IL-4 gene promoter [35], an AP-1 binding site from the collagenase gene promoter [36], NF-κB binding site from the mouse κ intron enhancer [37] or a consensus SP-1 binding site. Samples were separated by electrophoresis under non-denaturing conditions and exposed to film for autoradiography.

Transfection. Jurkat cells were transfected with expression vectors encoding for wild type PKCθ or a dominant negative PKCθ mutant [2] using the cell line nucleofector kit V from Amaxa, following Amaxa's optimized protocol. Transfected cells were cultured for 24 h before stimulation; after 8 h stimulation, cells were harvested and nuclear extracts were prepared.

Results

CD43 induces the translocation to the membrane of PKCα/β, ε, θ, ζ, and μ

Several lines of evidences suggested that members of the PKC family were involved in the CD43-dependent T cell activation [1,13,38]. However, it is presently unknown whether PKC is directly activated in response to CD43 engagement. As a first step towards understanding the role of these enzymes in the CD43 signaling pathway, we initially characterized the profile of PKC isoforms present in Jurkat cells and normal human T lymphocytes. Immunoblot analysis of whole cells extracts with antibodies specific for different PKC isoforms revealed that, consistent with previously published data [39,40], Jurkat cells and normal human T lymphocytes expressed the following PKCs: cPKC: α and βI/βII; nPKC: ε, θ, and δ; aPKC: ι and ζ as well as PKCμ (data not shown).

Taking advantage of the fact that in resting cells, PKCs have preferentially a cytoplasmic distribution and become membrane-associated upon activation [41], we investigated which PKC isoenzymes were recruited to the membrane in response to CD43 engagement. T cells were stimulated with the anti-CD43 L10 mAb for different periods of time, or with PMA as a positive control, and the presence of specific PKC isoforms in the membrane fraction was evaluated. In Jurkat cells, CD43 activation induced a transient translocation to the membrane of a slow migrating form of the cPKCα/β isoforms, peaking at 5 min and decreasing to undetectable levels after 15 min stimulation. Phosphorylation of the membrane-bound form of PKCα/β results in its activation and slow mobility in SDS–PAGE [42,43], suggesting that CD43 engagement results in translocation to the membrane of active cPKCα/β. PMA treatment clearly enhanced the levels of PKCα/β on the membrane fraction. However, in agreement with previously published data PMA treatment did not result in a change in the mobility of PKCα/β [44]. Similar levels of CD43 were found in all lanes indicating equal protein loading (Fig. 1A). CD43 engagement resulted also in activation of novel PKC isoforms. PKCε and PKCθ translocated to the membrane after 1 min stimulation and remained so until 15 min following activation, whereas the distribution of PKCδ was not affected. In contrast, PMA induced the translocation to the membrane of all nPKC isoforms (Fig. 1B). Crosslinking CD43 for 5 min resulted in transient translocation of the aPKCζ isoenzyme to the membrane, while the levels of PKCι remained stable, as compared to control cells. Both aPKC isoenzymes were resistant to PMA-activation (Fig. 1C), as previously reported [45]. Finally, a transient enhancement in the levels of PKCμ detected in the membrane fraction was found as a result of CD43 activation, peaking at 5 min stimulation and decreasing after 15 min to levels lower than those found in control-treated cells. PMA stimulation resulted also in membrane translocation of PKCμ (Fig. 1D). These results suggest that the CD43 signaling pathways involve the activation of cPKCα/β, nPKCθ and ε, aPKCζ, and PKCμ.

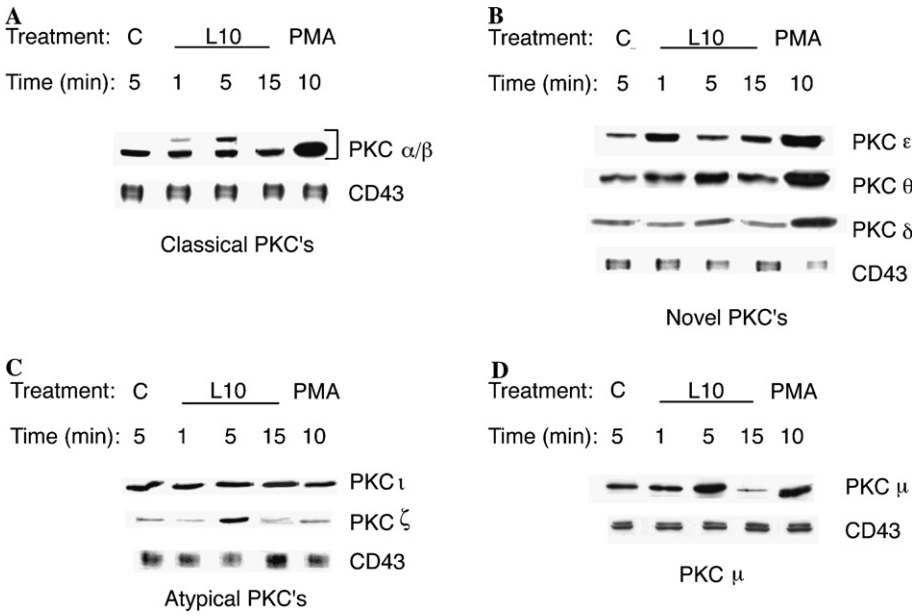

Fig. 1. CD43 induces the translocation to the membrane of PKCα/β, ε, θ, ζ, and μ. Jurkat cells were activated as described under Materials and methods with RαMIg only ("C"), the anti-CD43 L10 mAb or PMA. Membrane fractions were prepared and the levels of (A) PKCα/β, (B) PKCε, PKCθ, and PKCδ, (C) PKCι and PKCζ, and (D) PKCμ were determined by Western blot analysis. CD43 levels were determined as loading control (bottom panel). Results are representative of three independent experiments.

CD43 engagement promotes PKCθ and PKCα/β catalytic activity

PKCθ and PKCα/β have been shown to play a role in Ag-dependent T cell activation [23,24,46–48]. Since we had found that the CD43-dependent signals resulted in membrane translocation of PKCθ and PKCα/β, we evaluated whether CD43 engagement promoted also their kinase activity. In vitro kinase assays were performed on PKCθ and PKCα/β immunoprecipitates obtained from Jurkat cells or human peripheral blood T lymphocytes activated with the anti-CD43 L10 mAb. PKCθ activity resulting of crosslinking CD43 on Jurkat cells (Fig. 2A) or normal T lymphocytes (Fig. 2B) followed a very similar kinetics, with maximum enzymatic activity at 1 and 5 min after activation. PKCθ kinase activity after CD43 engagement was approximately 3-fold above the levels observed in RαMIg-treated cells. Whereas, PKCα/β kinase activity was increased 2-fold in Jurkat cells (Fig. 2C) as well as in human T lymphocytes (Fig. 2D), 1 min after CD43 engagement. As expected, PMA treatment enhanced PKCθ and PKCα/β catalytic activity (Figs. 2A–D). Thus, engagement of the CD43 molecule in human T cells induces PKCθ and PKCα/β enzymatic activity.

Activation of the MAP kinase pathway induced through CD43-mediated signals is PKCθ-dependent

We have previously shown that crosslinking CD43 on the T cell surface leads to activation of the ERK MAP kinase [12]. To further delineate the role of members of the PKC family in the CD43-mediated signals, we investigated the participation of PKCθ and/or PKCα/β in ERK 1/2 MAP kinase activation by using rottlerin, a selective PKCθ inhibitor, or Gö6976, a cPKC isoenzyme inhibitor [49,50]. As previously reported [12], engagement of CD43 on Jurkat cells resulted in a time-dependent ERK phosphorylation (Fig. 3). However, when cells were pre-incubated with rottlerin, ERK activation was abrogated. Similarly, PMA-mediated ERK phosphorylation was reduced in the presence of rottlerin (Fig. 3A). In contrast, the selective cPKC inhibitor, Gö6976, had no negative effect on the CD43-induced ERK activation, yet it reduced ERK phosphorylation levels induced in response to PMA stimulation (Fig. 3B). Equivalent amounts of protein were present in all lanes as determined by blotting the same membranes with anti-actin antibody. At the concentration used, rottlerin (6 μM) and Gö6976 (0.5 μM) selectively inhibited PKCθ and PKCα/β, respectively, as determined by in vitro kinase assays (data not shown) in agreement with previously published data [51]. Thus, these results suggest that although CD43 can activate PKCθ and PKCα/β, PKCθ but not PKCα/β is involved in the early activation of ERK resulting from CD43 engagement.

PKCθ is required to induce AP-1, NF-κB, and NFAT DNA binding in response to CD43 engagement

Receptor-induced AP-1, NF-κB, and NFAT activation has been shown to be deficient in PKCθ knockout T lymphocytes [23,24]. Since we had previously shown

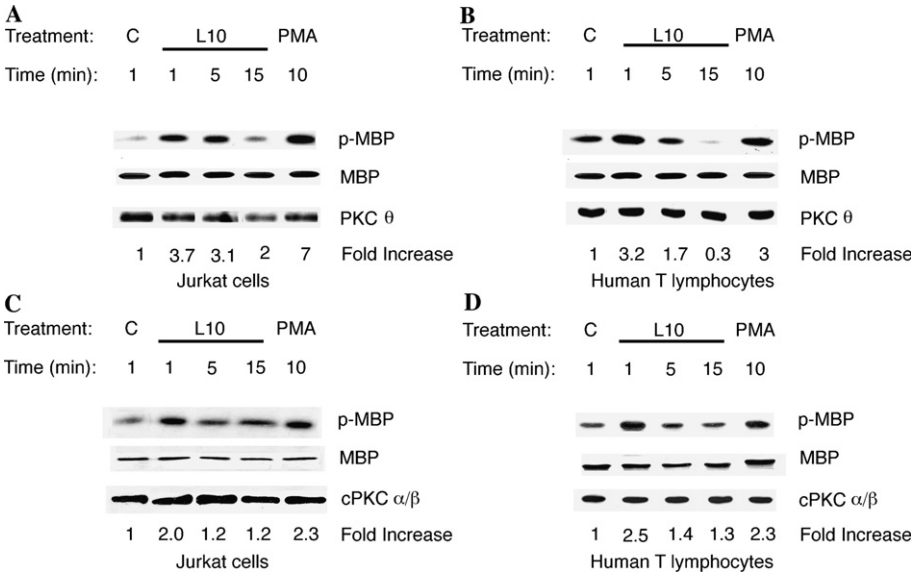

Fig. 2. CD43 engagement promotes PKCθ and PKCα/β catalytic activity. Jurkat cells (A,C) or human T lymphocytes (B,D) were stimulated as described under Materials and methods with RαMIg only (“C”), the anti-CD43 L10 mAb or with PMA. PKCθ (A,B) or PKCα/β (C,D) were immunoprecipitated from precleared cell lysates and enzymatic activities were evaluated by an in vitro kinase reaction using MBP as a substrate. Phosphorylated MBP (p-MBP) was resolved by SDS–PAGE and detected by autoradiography (top panel). Total MBP was visualized by Coomassie blue staining (middle panel). The actual amount of PKC present in each lane was determined by Western blot analysis with anti-PKCθ or PKCα/β antibodies (bottom panel). “Fold Increase” represent the phosphorylation levels of MBP corrected for the amount of MBP present, and PKCθ or cPKC immunoprecipitated in each experimental condition was compared with the value obtained in control cells. A representative experiment of three is shown.

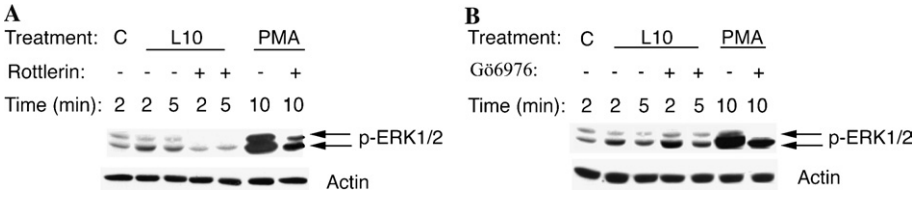

Fig. 3. Activation of the MAP kinase pathway induced in response to CD43-mediated signals is PKCθ-dependent. Jurkat cells were activated with RαMIg (“C”), with the anti-CD43 L10 mAb or with PMA. Before activation, cells were incubated with rottlerin (A) or Gö6976 (B). Whole cell extracts were prepared and the levels of phosphorylated ERK (p-ERK) were determined by Western blot analysis. The levels of actin were determined as a loading control.

that in human T cells, CD43 signaling enhances AP-1, NF-κB, and NFAT DNA binding activity [13], we investigated whether the positive effect on the DNA binding activity of these transcription factors in response to CD43 ligation was mediated through a PKCθ-ERK pathway. Blocking PKCθ or MEK activation with rottlerin or PD98059, respectively, reduced AP-1 DNA binding activity in response to CD43 engagement (Fig. 4A), while consistent with data presented above, the cPKC inhibitor, Gö6976, had a minor effect on AP-1 binding. These results suggest that PKCθ and ERK activation is required to promote AP-1 DNA binding activity in response to CD43 crosslinking.

In T cells, TcR activation up-regulates c-Fos expression, resulting in AP-1 binding activity and transactivation of target genes such as the IL-2 gene [52]. c-Fos expression depends, at least in part, on activation of the transcription factor Elk1 through the ERK pathway [53–55]. Since our data suggested that ERK activation was downstream of PKCθ in the CD43-mediated signaling cascade leading to AP-1 DNA binding, we tested whether PKCθ activation was required to induce c-Fos expression in response to CD43 engagement in T cells. The levels of the c-Fos protein detected in nuclear extracts from CD43-activated Jurkat cells were higher than those found in control cells. This was prevented by the PKCθ inhibitor rottlerin, while blocking MEK or cPKCα/β catalytic activities had no effect on the levels of c-Fos found in the nucleus of CD43-stimulated cells (Fig. 4B). CD43 engagement did not alter STAT-1 protein levels (Fig. 4B), indicating that the increase in c-Fos levels is specific and that it does not reflect a generalized effect on gene expression. This also shows that at the concentrations used the PKC inhibitors did

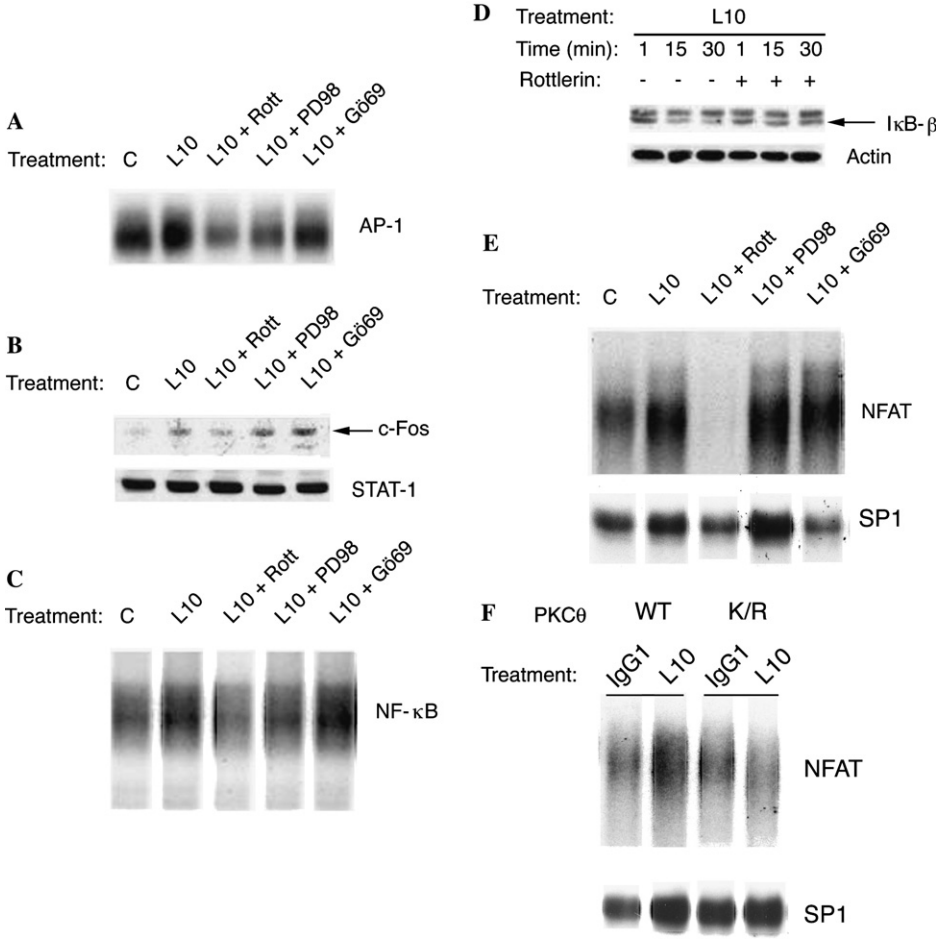

Fig. 4. PKCθ activation is required to induce AP-1, NF-κB, and NFAT DNA binding in response to CD43 engagement. (A) Jurkat cells were stimulated as described under Materials and methods with RαMIg only ("C") or the anti-CD43 mAb L10 for 8 h. Where indicated, cells were stimulated in the presence of rottlerin (Rott), PD98059 (PD98), or Gö6976 (Gö69). Nuclear extracts were prepared and the AP-1 binding activity was determined by EMSA analysis. (B) Nuclear extracts were prepared from cells stimulated as described in (A) and the levels of c-Fos and STAT-1 were determined by Western blot analysis. (C) Cells were treated as in (A) and nuclear extracts were used to determine NF-κB binding activity by EMSA analysis. (D) Jurkat cells were stimulated with the anti-CD43 L10 mAb in the presence or absence of rottlerin. Total cell extracts were prepared and the levels of IκB-β and actin were determined by Western blot analysis. (E) Nuclear extracts from Jurkat cells stimulated as in (A) and were used to determine NFAT DNA binding activity by EMSA analysis. (F) Jurkat cells transfected with wild type PKCθ (WT) or with a dominant negative PKCθ mutant (K/R) were stimulated with isotype control antibodies (IgG1) or with the anti-CD43 mAb L10 for 8 h and nuclear extracts were used to determine NFAT and SP1 binding activity. Similar results were obtained in two independent experiments.

not have a pleiotropic effect on the cell metabolism. These results indicate that PKCθ regulates AP-1 DNA binding activity by inducing c-Fos expression in response to CD43-specific signals.

PKCθ has also been shown to be required for NF-κB activation [2,23,26]. To test whether PKCθ played a role in the NF-κB activation mediated by CD43 signals, the NF-κB DNA binding activity of nuclear extracts obtained from Jurkat cells stimulated with the mAb L10 was evaluated in the presence of PKCθ or MEK inhibitors. The induction in NF-κB DNA binding activity observed after CD43 engagement was partially abrogated by rottlerin or PD98059 (Fig. 4C). In contrast, preventing cPKC activation with the inhibitor Gö6976 had no effect on the CD43-induced NF-κB DNA binding. Activation of NF-κB requires the degradation of its inhibitor IκB, which maintains NF-κB in an inactive form in the cytoplasm [56,57]. Consistent with NF-κB activation following CD43 engagement, CD43 ligation resulted also in IκB degradation, in a PKCθ-dependent fashion (Fig. 4D). These results suggest that the CD43-induced NF-κB DNA binding activity is dependent on PKCθ and ERK activation.

It was recently shown that following TcR engagement, PKCθ regulates the DNA binding activity of NFAT by modulating IP_3 and Ca^{2+} levels [24]. Since CD43 engagement results also in activation of this transcription factor [13], we assessed whether the CD43-induced NFAT DNA binding activity was mediated by PKCθ activation. Nuclear extracts obtained from Jurkat cells stimulated through CD43 were tested for NFAT DNA binding activity. Interestingly, the CD43-medi-

ated NFAT binding activity was completely blocked by rottlerin, while Gö6976 had no effect (Fig. 4E). Contrary to AP-1 and NF-κB, inhibition of ERK did not affect NFAT binding activity (Fig. 4E). The fact that similar levels of SP-1 DNA binding activity were observed in extracts from all experimental conditions (Fig. 4E) indicates that the differences in NFAT DNA binding activity observed were not due to differences in the quality of the nuclear extracts. To confirm that the effect of rottlerin on the NFAT binding activity was due to the specific inhibition of PKCθ, Jurkat cells were transfected with expression vectors encoding wild type or a dominant negative PKCθ mutant and the DNA binding activity of NFAT was determined after CD43 engagement. In contrast to wild type PKCθ, expression of the dominant negative PKCθ mutant abolished the NFAT binding activity resulting from CD43 ligation (Fig. 4F). The effect of the dominant negative PKCθ mutant on NFAT binding was specific since the binding of the SP1 transcription factor was not affected (Fig. 4F). Together, these results suggest that in response to CD43 engagement, PKCθ but not PKCα/β regulates activation of AP-1 and NF-κB by an ERK-dependent pathway. Furthermore, these data also suggest that PKCθ regulates NFAT activation, independently of the ERK pathway.

The CD43-dependent CD69 expression requires PKCθ activation

In T cells, PKCθ has been implicated in regulating the expression of CD69 following CD3, CD3/CD28 or PMA activation, through activation of AP-1, NF-κB, and NFAT [23,26,58,59]. We have shown that CD43 ligation induces CD69 expression in Jurkat cells [13]. Since, CD43 engagement resulted in activation of these transcription factors in a PKCθ-dependent manner, we evaluated the participation of PKCθ in the CD43-dependent CD69 expression. As previously reported, ligation of CD43 with the L10 mAb for 24 h induced CD69 expression in Jurkat cells (Fig. 5A) and human T lymphocytes (Fig. 5B). In the presence of rottlerin, CD69 expression was inhibited by 86% in Jurkat cells (Fig. 5C), whereas in human peripheral blood T cells a 50% inhibition was observed (Fig. 5D). In the presence of the MEK selective inhibitor PD98059, the CD43-dependent CD69 expression was reduced by 50% in Jurkat cells and 35% in human T lymphocytes (Figs. 5C and D, respectively). In contrast, impairing the PKCα/β catalytic activity with the inhibitor Gö6976 in Jurkat cells or human T lymphocytes had no effect on the CD43-mediated CD69 expression. The increase in CD69 expression was specific for CD43 signals, since only 1% of the total cells were $CD69^+$ when cells were incubated with an isotype control antibody (IgG1), in the absence or presence of

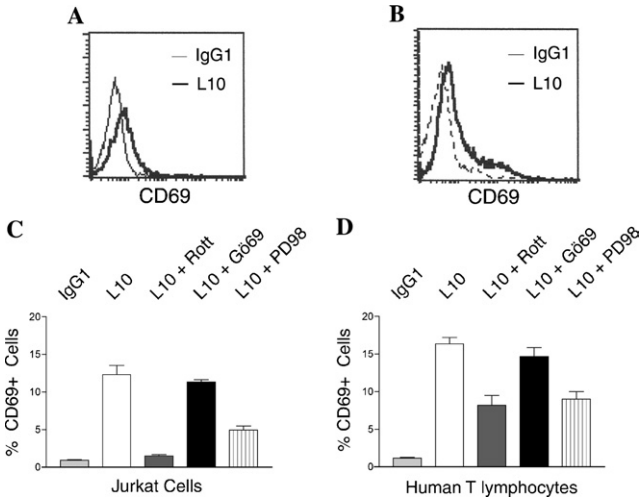

Fig. 5. CD43-dependent CD69 expression requires PKCθ activation. (A) Jurkat cells (1×10^6) or (B) human T lymphocytes (1×10^6) were stimulated for 24 h as described under Materials and methods with isotype control antibody (IgG1) or with the anti-CD43 L10 mAb, then cells were stained and CD69 expression was evaluated by flow cytometry. (C) Jurkat cells or (D) human T lymphocytes were incubated with rottlerin, Gö6976, or PD98059 for 15 min before activation and the expression of CD69 was determined 24 h later by flow cytometry, data represent the average of three independent experiments.

rottlerin (Figs. 5C and D, and data not shown). These results suggest that activation of PKCθ and ERK is necessary to induce CD69 expression in response to CD43 engagement.

Discussion

CD43 has been implicated in T cell activation, IL-2 production, and proliferation [5,6,10,13]. Although, a role for members of the PKC family has been suggested in the CD43-dependent activation of T lymphocytes [1,38,60], the direct participation of members of this family of kinases remains to be demonstrated. Here we show that in human T cells, CD43 ligation boosted the levels of membrane-bound PKC, particularly that of the cPKCα/β, the nPKCε and θ, the aPKCζ isoforms as well as PKCμ. Activation of cPKC (α/β), nPKC (ε and θ) by CD43 is consistent with their requirement for DAG and Ca^{2+} for being activated and with the fact that CD43 engagement results in DAG generation and Ca^{2+} mobilization [1]. Activation of PLCγ plays a central role for DAG production and increase in Ca^{2+} levels through IP_3-induced release, ultimately leading to PKC activation. We have shown that PLCγ-2 is activated in response to CD43 engagement [61] and that activation of PKCα/β as well as of PKCθ can be prevented with the PLCγ inhibitor U73122 (data not shown). Overall, these data suggest that in human T cells, CD43 ligation induces the enzymatic activity of PLCγ, increasing the

levels of DAG and Ca^{2+}, ultimately leading to activation of cPKCα/β and nPKC (ε and θ).

The activation of PKCθ has been shown to activate PKCμ in T cells [62], in agreement with this the activation of PKCθ precedes the recruitment to the membrane of PKCμ in response to CD43 engagement, thus it is possible that the activation of PKCμ resulting from CD43 ligation is mediated by PKCθ. Although, there is experimental evidence suggesting that the activation of PKCζ may involve a PI3K-dependent pathway [63] and CD43 ligation promotes PI3K activity ([38], Oceguera-Yañez unpublished data), the molecular mechanism by which CD43 ligation leads to PKCζ activation remains to be elucidated.

CD43 engagement resulted not only in the membrane localization of cPKCα/β and PKCθ molecules but it induced also their kinase activity in Jurkat cells as well as in human peripheral T lymphocytes. We have previously published that PKC activation was necessary to promote ERK activation in response to CD43 engagement [12]. Data presented here point that PKCθ but not PKCα/β activity is required to activate ERK in response to CD43. Interestingly, in γδ human T lymphocytes stimulated with a isopentenyl pyrophosphate from mycobacterium, the cPKCα/β isoforms have been found to induce ERK activation [64]. Altogether, this suggests that the specific PKC isoform activating the ERK pathway in a given cell type may be determined by the receptor engaged. Moreover, the fact that PKCθ-deficient mice show no defect in ERK activation in response to TcR/CD28 ligation [23,24] suggests that the activation of ERK by PKCθ may be a species- or cell-specific mechanism.

In human T cells, CD43 engagement promotes the expression of several genes involved in innate and adaptive immunity [13,65,66], and whose expression depends on the activation of transcription factors such as AP-1, NF-κB, and NFAT. We had previously shown that ligation of CD43 on human T cells promotes the DNA binding activity of AP-1, NF-κB, and NFAT [13]. The data we present here suggest that, PKCθ participates in the CD43-dependent signaling leading to activation of these transcription factors, since the PKCθ catalytic activity was required to enhance c-Fos expression and AP-1 DNA binding activity. Although the ERK pathway has been shown to be required for AP-1 recruitment as well as for c-Fos expression by inducing binding of Elk-1 to the SRE [53,54], preventing ERK activation with the MEK inhibitor PD98059 had no effect on c-Fos expression in response to CD43 engagement. This result suggests that the effect of PKCθ on c-Fos expression is independent of ERK activation, and that PKCθ may use an alternative pathway to regulate SRF or Elk1 transcriptional activities to promote c-Fos expression. In agreement with this, PKCα and PKCε have been shown to induce the transcriptional activity of SRF independent of ERK activation [67].

Induction of NF-κB by PKCθ involves the participation of the IκB kinases (IKKα or β) [26]. Active IKKs phosphorylate IκB at specific amino-terminal serine residues, allowing its ubiquitination and degradation by the 26S proteasome [68]. The fact that the IκB-β degradation and the NF-κB DNA binding activity we found were dependent on the catalytic activity of PKCθ suggests that the PKCθ molecules recruited in response to CD43 signals lead to NF-κB activation through an IKK-dependent mechanism. Moreover, consistent with the activation of both IκB kinases through the MAP kinase pathway [69,70], the CD43-mediated activation of NF-κB was also dependent on the ERK kinase pathway. The activation of IKK in response to CD43 engagement in human T cells remains to be documented. The fact that the NF-κB DNA binding activity was not completely prevented by rottlerin suggests that additional PKC isoforms such as PKCε [71] and PKCζ [72] or PKC independent mechanisms may also play a role in NF-κB activation in response to CD43 engagement.

The first genetic analysis of PKCθ indicated no physiological role for this isoenzyme in the TcR/CD28-induced NFAT activation, yet it confirmed its role in AP-1 and NF-κB activation [23]. However, in a second independent study, PKCθ deficiency abrogated the TcR/CD28-dependent NFAT activation, suggesting that PKCθ participates in NFAT activation [24]. Consistent with this, we show that NFAT activation in response to CD43 ligation on human T cells requires PKCθ kinase activity, since the addition of rottlerin abrogated the CD43-induced NFAT binding activity. The possibility that the effect of rottlerin on NFAT binding was due to the inhibition of another atypical PKC was ruled out by the fact that a dominant negative PKCθ mutant prevented NFAT binding activity. This, together with the fact that CD43 engagement did not induce the membrane localization of PKCδ and failed to induce the phosphorylation of PKCδ on Thr^{505} (data not shown), which has been shown to correlate with PKCδ activation [73], strongly suggests that the effect of rottlerin on NFAT binding resulted from the specific inhibition of PKCθ. The data obtained with T lymphocytes from the PKCθ-deficient mice indicate that PKCθ is necessary for the generation of IP_3 and for the sustained Ca^{2+} mobilization needed to maintain NFAT in the nucleus in response to TcR engagement [24]. Although in murine T cells it is possible that PKCθ is an upstream regulator of PLCγ, in human cells however, PLCγ is required for PKCθ activation in response to TcR/CD28 engagement [74]. In the CD43 signaling pathway, PKCθ is most likely located downstream of PLCγ, since preventing PLCγ activity with the inhibitor U73122 blocked PKCθ activation (data not shown).

Therefore, we propose that activation of PLCγ by CD43 promotes on one hand, the rise in Ca^{2+} concentration induced by IP3 and on the other hand the activation of PKCθ by DAG. PKCθ may in turn lead to further rise in Ca^{2+} levels by opening membrane Ca^{2+} channels [75] in order to reach the critical Ca^{2+} concentration required for NFAT activation.

$PKC\theta^{-/-}$ T lymphocytes show defects in CD69 expression and proliferation as a result of impaired TcR-induced IL-2 production [23]. In Jurkat cells, recent experimental evidence indicates that PKCθ is important for the TcR/CD28-dependent NFAT activation and CD69 expression [2,30,76]. We had previously reported that ligation of the CD43 molecule induced CD69 expression [13]. Here we provide evidence that CD69 up-regulation in response to CD43 engagement is dependent on PKCθ activity in Jurkat cells and partially dependent in human peripheral T lymphocytes. Activation of PKCθ and the subsequent recruitment of NF-κB and NFAT resulting of CD43 engagement is probably not exclusive of the CD69 or IL-2 genes [13] but constitutes rather a general mechanism through which CD43 signaling modulates gene expression during immune response. Consistent with this, the CD43-dependent co-stimulatory signals were found to enhance the TcR-induced HIV transcription and virus production through a mechanism involving the participation of NF-κB and NFAT [8].

The fact that almost all PKC isoforms are expressed in T cells suggests overlapping functions for these kinases. However, the results from the PKCθ-deficient mice show that this isoform plays an important role in the TcR/CD28-mediated activation. Data we report here indicate that following CD43 engagement on human T lymphocytes, PKCθ promotes activation of the AP-1, NF-κB, and NFAT transcription factors, regulating specific biological responses such as IL-2 production and CD69 expression. Although the precise role of the additional PKC isoforms recruited in response to CD43 ligation (α/β, ε, ζ, and μ) remains to be investigated, it is tempting to think that they may be involved in distinct CD43-induced biological functions such as T cell adhesion, polarization, and migration.

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