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Phosphatidylinositol-3-kinase regulates PKCθ activity in cytotoxic T cells

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Abstract

Protein kinase C (PKC) θ plays a crucial role in T cell activation. We, therefore, examined the regulation of PKCθ activity in cytotoxic T lymphocytes (CTL). We demonstrated that PMA did not stimulate PKCθ activation and phospholipase C inhibition did not block anti-CD3-stimulated PKCθ activation in a CTL clone. This suggests that diacylglycerol is neither sufficient nor required for PKCθ activation. Furthermore, PKCθ was only activated in a CTL clone stimulated with plate-bound anti-CD3 but not soluble anti-CD3. However, PMA or cross-linked anti-CD3 stimulated phosphorylation of PKCθ as measured by a migratory shift, suggesting that phosphorylation was not sufficient for activity. Phosphatidylinositol 3-kinase activity was required for anti-CD3, but not PMA, stimulated phosphorylation and for immobilized anti-CD3-triggered PKCθ activity. A substantial fraction of PKCθ was constitutively membrane associated and PMA or CD3 stimulation did not significantly increase membrane association. Our data indicate that phosphorylation of PKCθ is not a suitable surrogate measurement for PKCθ activity and that additional, yet to be defined steps, are required for the regulation of PKCθ enzymatic activity in CTL.

Keywords: T lymphocytes; Protein kinases; Phosphorylation; Cellular activation; Signal transduction; Protein kinase C; Phosphatidylinositol-3-kinase

1. Introduction

Cytotoxic T lymphocytes (CTL) are $CD8^+$ T cells essential for the clearance of most viral infections. One effector mechanism involves the release of cytolytic granules upon engagement of the T cell receptor (TCR). We have found that in contrast to many T cell hybridomas, non-transformed CTL do not mediate a functional response to anti-TCR or CD3 crosslinking in suspension (Berg et al., 1998). Degranulation in response to antibodies against the TCR complex occurs only if the antibody is presented on a solid matrix such as a plastic surface or a cell size bead, even though cross-linked antibodies trigger all of the membrane proximal signals examined to date (Berg et al., 1998). One possible explanation for this requirement for adherence is that the immobilized antibodies allow for the dramatic cytoskeletal changes associated with T cell activation (Bunnell et al., 2001; Parsey and Lewis, 1993), which in turn may allow for the assembly of signaling complexes (Bunnell et al., 2002). Alternatively, matrix-bound antibodies may allow for the polarization of signaling which may be essential for CTL degranulation, as has been suggested by the observed proximity of signaling proteins such as protein kinase C (PKC) θ to the cytolytic granules (Stinchcombe et al., 2001). Stimulation with immobilized antibodies may, therefore, more closely mimic the cytoskeletal changes and polarization induced by target cell contact.

The novel PKCθ isozyme is most highly expressed in lymphocytes (Baier et al., 1993) and is thought to play an important role in their activation (Arendt et al., 2002). For example, PKCθ has been shown to regulate the transcription factors NF-κB (Dienz et al., 2000; Khoshnan et al.,

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2000) and AP-1 (Baier-Bitterlich et al., 1996), which play critical roles in T cell activation. T cells from PKCθ deficient mice fail to fully activate AP-1 and NF-κB and exhibit reduced cytokine production and proliferation in response to stimulation (Pfeifhofer et al., 2003; Sun et al., 2000). PKCθ is unique among PKC isozymes in that only PKCθ is relocalized to the central region of the contact site between a T cell and its cognate antigen-presenting cell (Monks et al., 1998,1997; Potter et al., 2001). The mechanism for this selective movement is unknown. Transfection studies in Jurkat T leukemia cells suggest that PKCθ and the guanine nucleotide exchange factor Vav participate in a common pathway (Dienz et al., 2000; Villalba et al., 2000). It has been proposed that Rac-mediated cytoskeletal reorganization downstream of Vav results in recruitment of PKCθ to the cell membrane, where it becomes activated (Villalba et al., 2000). Vav contains a plekstrin homology domain, which binds 3-phosphatidylinositol lipids generated by phosphatidylinositol-3-kinase (PI3-kinase) (Han et al., 1998), and a PI3-kinase inhibitor was found to block membrane recruitment of PKCθ (Villalba et al., 2002).

We have shown previously that cross-linked antibodies differentially regulate the requirement of PKC for ERK activation in T cells compared to immobilized antibody (Puente et al., 2000) suggesting that PKC activation may be differentially regulated downstream of the TCR. Given that PKCθ may be particularly sensitive to directional signals since it localizes to the point where a CTL contacts a target cell (Potter et al., 2001; Stinchcombe et al., 2001), we set out to examine the regulation of this enzyme in CTL. Herein, we demonstrate that PKCθ activity appears to be regulated independently of the phospholipase C (PLC) pathway but is regulated by a PI3-K-dependent pathway in CTL. Although PKCθ undergoes a PMA and CD3-induced phosphorylation, this phosphorylation is not sufficient for activation of PKCθ. Our studies suggest that PKCθ may be activated only when a polarized signal is received by the CTL.

2. Materials and methods

2.1. Cell culture

The murine $CD8^+$ CTL clone AB.1 was described previously (Kane et al., 1989). Clones were maintained by weekly stimulation with irradiated splenocytes from C57BL/6J mice and IL-2 and used 4–7 days later. The L1210 lymphoma cell line expressing chimeric class I MHC (L1210-K^b) was a generous gift from Dr. Kevin Kane (University of Alberta) (Durairaj et al., 2003). L1210 cells were grown in DMEM supplemented with 8% heat-inactivated defined calf serum.

2.2. Antibodies and reagents

The hybridomas producing 145-2C11 (anti-CD3ε) and MB4B4 (anti-CD45) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Anti-PKCθ (C-18) and anti-goat Ig^{HRP} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Dr. Dan Littman (HHMI, Skirball Institute of Biomolecular Medicine, NY) generously provided anti-PKCθ polyclonal antiserum. Anti phospho-Akt (Ser473) and anti-phospho-PKCθ (Thr538) were obtained from cell signaling (Beverly, MA). Anti-paxillin was acquired from BD BioSciences (Mississauga, ON). Anti-phospho-PKCθ Ser676 and 695 were obtained from BioSource International (Camarillo, CA). Anti-ERK (ERK1 + ERK2) monoclonal antibody (mAb) was purchased from Zymed (San Francisco, CA). Rabbit anti-hamster Ig and anti-$mouse^{HRP}$ antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). PMA and myelin basic protein (MBP) were purchased from Sigma (Mississauga, Ont.). Wortmannin, Ly-294002, U73122 and U73343 were purchased from Calbiochem (San Diego, CA).

2.3. T cell stimulation

AB.1 cells were stimulated by one of the three methods. For immobilized anti-CD3 mAb stimulation, 96-well flat bottom Falcon 3912 microtitre plates (Becton Dickinson, Oxnard, CA) or 60 mm non-treated polystyrene dishes (Fisher, Nepean, ON) were coated with 10 μg/ml 145-2C11 overnight at 4 °C. Wells were washed twice with phosphate-buffered saline (PBS), blocked with 2% BSA in PBS for 30–60 min at 37 °C, then washed twice with PBS before addition of cells. Cells were incubated at 37 °C for the indicated time before lysis. For soluble, cross-linked anti-CD3 mAb stimulation, cells in PBS were incubated with 10 μg/ml 145-2C11 on ice for 15 min, then washed and resuspended in fresh PBS. Rabbit anti-hamster Ig was added at 5 μg/ml and cells were incubated at 37 °C for 10 min or the indicated time. For PMA stimulation, cells were treated with 100 ng/ml of PMA and incubated in plates or dishes as described above for the indicated time. CTL were preincubated with inhibitory compounds or DMSO carrier control for 30 min prior to stimulation. Cell viability was confirmed by trypan blue exclusion. None of the pharmacological agents used had a significant impact on cell viability at the concentrations used for these experiments.

2.4. PKCθ immunoprecipitation

As described above, $1–2 \times 10^7$ cells were collected and stimulated. Cells were resuspended in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NP-40, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4) and nuclei removed by centrifugation. PKCθ was recovered by immunoprecipitation with antibody and protein A-Sepharose 4B, washed in lysis buffer and separated by SDS-PAGE on a 7.5% gel. In some cases, the PKCθ was subjected to phosphatase treatment by overnight incubation with alkaline phosphatase (Promega, Madison, WI) at room temperature.