Calcineurin preferentially synergizes with PKC-θ to activate JNK and IL-2 promoter in T lymphocytes

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Costimulation of the T cell receptor (TCR) and CD28 is required for optimal interleukin-2 (IL-2) induction. These signals, which can be replaced by the pharmacological agents phorbol ester (PMA) and Ca$^{2+}$ ionophore, synergistically activate the mitogen-activated protein kinase (MAPK) JNK. Cyclosporin A, an inhibitor of the Ca$^{2+}$-dependent phosphatase calcineurin which blocks IL-2 induction, abrogates Ca$^{2+}$-triggered synergistic JNK activation. As protein kinase C (PKC) downregulation inhibits PMA + ionophore-induced JNK activation, we examined whether a particular PKC isosform is preferentially involved in this response. We found that PKC-θ but neither PKC-α nor PKC-ε participates in JNK activation, whereas all three PKCs lead to ERK MAPK activation. PKC-θ specifically cooperates with calcineurin, and together their signals converge on (or upstream of) Rac leading to potent JNK activation. Similarly, calcineurin and PKC-θ specifically synergize to induce transcription of reporters driven by the c-jun and IL-2 promoters. PKC-θ and calcineurin are also partially responsible for the synergistic activation of JNK following TCR and CD28 ligation. Preferential cooperation between PKC-θ and calcineurin is observed in Jurkat T cells but not in HeLa cells. These results indicate that PKC isozymes have distinct biological functions and suggest that synergistic JNK activation is an important function for PKC-θ in T-cell activation.

Keywords: calcineurin/interleukin-2/JNK/PKC-θ/T-lymphocyte activation

Introduction

Engagement of the T cell antigen receptor (TCR) during an immune response is insufficient for T-lymphocyte activation (Schwartz, 1992; Crabtree and Clipstone, 1994). Additional signals are provided by occupancy of auxiliary receptors, such as CD28 (Schwartz, 1992). Several signal amplification cascades composed of protein kinases and phosphatases transduce these signals to the nucleus to induce the interleukin-2 (IL-2) gene, whose product is a major T cell growth factor (Crabtree and Clipstone, 1994; Cantrell, 1996). The mechanisms by which signals generated by TCR and CD28 occupancy are integrated are poorly understood.

Similar to receptor tyrosine kinases which activate phospholipase C (PLC) (Nishizuka, 1992; Berridge, 1993), TCR occupancy leads to recruitment and activation of cytoplasmic protein tyrosine kinases (PTKs), which subsequently associate with and activate PLCγ (Cantrell, 1996). PLCγ translocates to the membrane and cleaves phosphatidylinositol (4,5)-bisphosphate, yielding diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP3). DAG activates PKCs, while IP3 mobilizes Ca$^{2+}$ from intracellular stores (Berridge, 1993; Clapham, 1995). Elevation of intracellular Ca$^{2+}$ is further enhanced by influx from the extracellular milieu. It is believed that Ca$^{2+}$ promotes early events in T-cell activation by stimulating the activity of calcineurin, a Ca$^{2+}$/calmodulin-dependent serine/threonine protein phosphatase (Clipstone and Crabtree, 1993; Crabtree and Clipstone, 1994). Calcineurin is a heterodimer consisting of calcineurin A (CnA), a 61 kDa catalytic subunit, and calcineurin B (CnB), a 19 kDa regulatory subunit with marked homology to other Ca$^{2+}$-binding proteins (Crabtree and Clipstone, 1994). Deletion of the calmodulin-binding site and the adjacent auto-inhibitory domains confers Ca$^{2+}$-independent catalytic activity to CnA (O’Keefe et al., 1992; Clipstone and Crabtree, 1993). A truncated CnAΔCaM-AI mutant has Ca$^{2+}$-independent constitutive phosphatase activity in vitro and synergizes with PMA to induce IL-2 transcription (O’Keefe et al., 1992; Clipstone and Crabtree, 1993). A similar effect is exerted on the nuclear factor of activated T cells (NF-AT) (Clipstone and Crabtree, 1993). NF-AT is a complex of a nuclear component composed of AP-1 proteins and a cytoplasmic component composed of NF-ATc proteins, which enter the nucleus in response to elevated Ca$^{2+}$ and bind cooperatively with AP-1 to NF-AT recognition sites (Jain et al., 1993; Northrop et al., 1993). Together with AP-1 binding to other sites and NF-κB, OBP and CD28RC, NF-AT activates the IL-2 promoter (Crabtree and Clipstone, 1994; Rao, 1994). Calcineurin regulates the translocation of NF-ATc proteins to the nucleus in a Ca$^{2+}$-dependent way (Timmerman et al., 1996). The immunosuppressant cyclosporin A (CsA), a calcineurin antagonist, blocks NF-ATc activation and nuclear entry (Crabtree and Clipstone, 1994; Rao, 1994). CsA forms a complex with a specific cyclophilin which binds and inhibits the catalytic subunit of calcineurin (Schreiber, 1992; Clipstone and Crabtree, 1993; Milan et al., 1994; Cardenas et al., 1995). CsA abrogates ionomycin and PMA-induced IL-2 transcription (Crabtree and Clipstone, 1994), suggesting that calcineurin-regulated and phorbol ester-triggered signaling pathways converge to induce T-cell activation.

Phorbol esters, such as PMA, activate members of the PKC family by binding a cysteine-rich region that is physiologically recognized by DAG (Nishizuka, 1992; Newton, 1997). So far 11 different PKCs have been cloned...
and divided into subgroups according to primary structure and binding of Ca$^{2+}$ or phorbol ester (Nishizuka, 1992; Dekker et al., 1995). While both conventional PKCs (cPKCs), like α, βI, βII and γ, and novel PKCs (nPKCs), such as δ, ε, θ and η are activated by PMA, only cPKCs bind Ca$^{2+}$. Atypical PKCs (aPKC), such as ζ and τ(α), as well as PKC-μ/PKD are not activated either by Ca$^{2+}$ or by phorbol esters, although they bind DAG (Dekker et al., 1995; Newton, 1997). T-lymphocyte activation requires PKC (Valge et al., 1988). While most studies investigating the role of PKC in T-cell activation relied on pharmacological agonists and antagonists (Thorpe et al., 1996) or PKC downregulation (Valge et al., 1988; Isakov et al., 1990), others used constitutively active or dominant inhibitory ‘kinase-dead’ mutants (Genot et al., 1995; Baier-Bitterlich et al., 1996). The possibility of examining specific effects of a particular PKC isoform independently of phorbol ester or synthetic DAG treatment underlies the advantage of using such mutants. By using constitutively active mutants of PKCα, -ε and -θ, it was demonstrated that PKC-θ preferentially induces AP-1 transcriptional activity in a murine thymoma cell line (Baier-Bitterlich et al., 1996). While most of these PKCs are present in both T lymphocytes and other cell types, PKC-θ is predominantly expressed in hematopoietic cells (Baier et al., 1993). PKC-θ, but not other isotypes, localizes to the region of contact between the TCR and the antigen-presenting cell (APC) during an immune response (Monks et al., 1997). PKC-θ may thus preferentially propagate signals generated at the TCR. It is, however, unknown whether PKC-θ or any other PKC isoform may specifically synergize with the Ca$^{2+}$-triggered pathway to induce a downstream event important for T-cell activation. That PKCs in general can cooperate with calcineurin is suggested by the synergistic effects of PMA and Ca$^{2+}$ ionophore on both JNK activation and NF-AT activity (Crabtree and Clipstone, 1994; Rao et al., 1994; Su et al., 1994).

JNK or stress-activated protein kinase (SAPK) is a subgroup of MAPKs that bind to the AP-1 component, c-Jun and phosphorylate Ser63 and Ser73 in its N-terminal activation domain (Hibi et al., 1993; Dérijard et al., 1994). Phosphorylation of these serines increases c-Jun transcriptional activity (Su and Karin, 1996). c-Jun can homo- or heterodimerize with other leucine zipper proteins such as Fos to form AP-1 complexes (Su and Karin, 1996). JNK also phosphorylates JunD, another potential component of AP-1 (Kallunki et al., 1996). In T cells, JNK activation correlates with increased AP-1 transcriptional activity and induction of c-jun and IL-2 gene expression (Su et al., 1994). The JNK signal transduction pathway thus appears to play an important role in T-cell activation.

Although MAPK subgroups have different substrate specificities and are activated by distinct extracellular stimuli, they share a common regulatory scheme. MAPKs are activated by a MAPK kinase (MAPKK or MEK), which in turn is activated by a MAPKK or MEK kinase (MAPKKK or MEKKK). In various cell types MEKK1 or a closely related family member is thought to function as the MAPKKK coupling the small GTP-binding proteins Ras and Rac or Cdc42 to JNK activation (Cosso et al., 1995; Minden et al., 1995).

To define the components involved in signal integration leading to JNK activation in T cells, we examined the role of calcineurin and various PKC isoforms in this process. Our results indicate that calcineurin exclusively synergizes with PKC-θ to activate JNK but not ERK. Other PKC isozymes make only minor contributions to JNK activation. The integration of these parallel pathways seems to occur upstream to or at the level of Rac. The cooperation between calcineurin and PKC-θ is probably restricted to lymphocytes as it was not observed in HeLa cells. Similar to their effect on JNK activation, calcineurin preferentially synergizes with PKC-θ to activate the IL-2 promoter. Expression of activated PKC-α or PKC-ε has no effect on either JNK or the IL-2 promoter.

**Results**

**Different Ca$^{2+}$-dependent pathways induce JNK activation in Jurkat and HeLa cells**

Strong activation of JNK in T cells requires costimulation with either PMA and Ca$^{2+}$ ionophore or antibodies to TCR/CD3 and CD28 (Su et al., 1994). Each stimulus by itself results in little or no JNK activation. To investigate whether PKC is required for JNK activation in T cells, we used PKC downregulation. Prolonged PMA exposure downregulates PMA-responsive PKC isoforms resulting in loss of biological responses, such as IL-2 synthesis (Isakov et al., 1990) or T-cell proliferation (Valge et al., 1988). Jurkat and HeLa cells were pre-incubated with or without 100 nM PMA for 24 h, followed by stimulation with ionomycin or PMA, either alone or in combination. PKC downregulation in Jurkat cells totally blocked JNK activation by PMA alone or in combination with ionomycin, suggesting the involvement of a phorbol ester-sensitive PKC (Figure 1). Consistent with these results, we found that low doses of PKC inhibitors, such as staurosporine (25 nM) or Go68505 (250 nM), totally abrogated JNK activation by PMA or PMA+ionomycin (data not shown, but see Figure 3). JNK activation by the translational inhibitor anisomycin was insensitive to PKC downregulation.

By contrast, we found that ionomycin or PMA alone markedly activated JNK in HeLa cells and that the combination of both agonists was additive rather than synergistic (Figure 1). While PKC downregulation completely blocked JNK activation by PMA alone and reduced the response to PMA+ionomycin, it did not affect Ca$^{2+}$-triggered JNK activation. Furthermore CsA, which inhibits the potentiation of JNK activity by Ca$^{2+}$ in Jurkat cells (Su et al., 1994), had no such effect in HeLa cells (Figure 1), suggesting that different Ca$^{2+}$-dependent pathways modulate JNK activation in Jurkat and HeLa cells.

**Role of calcineurin in JNK activation in T cells**

Calcineurin, whose activity is inhibited by CsA, plays a major role in signal transduction leading to T-cell activation (Crabtree and Clipstone, 1994). Calcineurin dephosphorylates the NF-ATc proteins (Jain et al., 1993; Timmerman et al., 1996) and induces IL-2 transcription in cooperation with PKC (O’Keefe et al., 1992; Clipstone and Crabtree, 1993). Since CsA blocks the effect of Ca$^{2+}$ on JNK activation in T lymphocytes but not in HeLa cells we first investigated the role of calcineurin in Jurkat cells. We coexpressed hemagglutinin (HA) epitope-tagged
catalytic (HA-CnA) and regulatory (HA-CnB) subunits of wild-type (wt) calcineurin together with an M2-tagged JNK1 (M2-JNK1). The cells were stimulated with PMA or ionomycin either alone or in combination and JNK activity determined by immunocomplex kinase assay. Overexpression of the two calcineurin subunits further increased JNK activation in response to PMA + ionomycin (Figure 2A). Pretreatment with CsA decreased JNK activation towards its non-stimulated level (data not shown). In the absence of both stimuli, the wt calcineurin subunits had no effect. Indeed, wt calcineurin does not synergize with either PMA or Ca²⁺, ionophore and similar to mock-transfected cells, each stimulus alone resulted in little or no JNK activation. Immunoblot analysis with anti-HA antibody revealed well-expressed full-length HA-CnA and HA-CnB (Figure 2B). Similar results were obtained for non HA-tagged wt calcineurin subunits, verifying that the HA tag located at the N-terminus does not interfere with their function (data not shown).

To assess further the role of calcineurin in JNK activation and to examine whether this phosphatase is the main Ca²⁺-dependent component in this pathway, we constructed an HA-tagged constitutively active form of CnA (ΔCaM-AI). Overexpression of CnAΔCaM-AI in Jurkat cells potentiated JNK activation by PMA (Figure 2A). The constitutively active CnA does not activate JNK by itself and even at high expression levels, its effect is strictly dependent on costimulation with PMA. A marginal increase in JNK activity was observed upon further treatment with ionomycin, suggesting that calcineurin is probably the main Ca²⁺-dependent component involved in JNK activation in T lymphocytes. Consistent with this hypothesis we found that when coexpressed with CnAΔCaM-AI, the Ca²⁺-binding regulatory subunit CnB could increase the response to PMA + ionomycin (data not shown). Furthermore, increasing doses of CsA almost totally abrogated activation of JNK by PMA + CnAΔCaM-AI (Figure 2C), consistent with its inhibition of PMA + ionomycin-induced JNK activation and the known ability of the cyclophilin–CsA complex to bind and inhibit the catalytic domain of calcineurin (Milan et al., 1994; Cardenas et al., 1995). At a high dose of CsA (500 ng/ml), JNK activity was a mere 15% of the maximal level induced by PMA + ionomycin.

In contrast to the effect on JNK, CsA had no effect on ERK activity (Su et al., 1994). In support of these results, CnAΔCaM-AI did not activate ERK by itself and did not potentiate ERK activation by either PMA or constitutively active Ha-Ras(L61) (data not shown). Taken together, these results strongly support the notion that calcineurin is a key component of the Ca²⁺-signaling pathway leading to JNK activation in T cells.

**Characterization of the PKC isoform that synergizes with calcineurin**

Several isoforms of PKCs are present in T cells and may play specific roles in signal transduction (Valge et al., 1988; Isakov et al., 1990; Genot et al., 1995; Baier-Bitterlich et al., 1996). Recently, it was shown that PKC-θ, an nPKC isoform predominantly expressed in hematopoietic cells (Baier et al., 1993), selectively activates the AP-1 transcription factor in murine EL4 thymoma cells (Baier-Bitterlich et al., 1996). However, in contrast to Jurkat or circulating T cells, phorbol esters or activated PKC mutants are sufficient for induction of IL-2 in EL4 cells (Baier-Bitterlich et al., 1996). It is, therefore, unclear if PKC-θ can cooperate with calcineurin in Jurkat or other lymphoid cell types. To address this question we used both ‘kinase-dead’ and constitutively active PKC-θ mutants. The ‘kinase-dead’ PKC-θ (K409R) is mutated in the ATP-binding site, while PKC-θ (A148E) is mutated in the pseudosubstrate site and was shown to be functionally independent of any agonist (Baier-Bitterlich et al., 1996). Jurkat cells were cotransfected with increasing amounts of PKC-θ (K409R) together with M2-JNK1. As shown in Figure 3A, expression of ‘kinase-dead’ PKC significantly abrogated PMA or PMA + ionomycin-induced JNK activation. Similarly PKC-κ (K409R) blocked the synergism between PMA and CnAΔCaM-AI (data not shown) and partially inhibited the response to anti-CD3 + anti-CD28 (Figure 3A). However, the ability of PKC-θ (K409R) to inhibit JNK activation is not isotype-specific because overexpression of a catalytically inactive PKC-α (KR) mutant also resulted in inhibition of JNK activation (data not shown). Therefore, we examined the effect of two selective PKC inhibitors Gö6976 (indolocarbazole) and Gö6850 (bisindolylmaleimide) on JNK activation by either PMA + ionophore or anti-CD3 + anti-CD28. Gö6976 was described to specifically block the activity of PKC isoforms belonging to the conventional class and unable to inhibit nPKC or aPKCs (Martiny-Baron et al., 1993). Gö6850, on the other hand, blocks most known PKC isoforms.
Fig. 2. Effect of calcineurin on JNK activation in Jurkat cells. (A) Jurkat cells were transfected with 0.25 μg of M2-JNK vector and 2 μg of empty vector, 1 μg of each of the wild-type calcineurin subunit expression vectors, HA-CnA and HA-CnB or 2 μg of HA-CnAΔCaM-AI. Thirty hours post-transfection, cells were stimulated for 30 min with 100 nM PMA either alone or in combination with 1 μM ionomycin. JNK activity was measured by immunocomplex kinase assay as described in Figure 1, using anti-M2 to immunoprecipitate M2-JNK1, fold-activation was quantitated by a phosphorimager. These experiments were repeated four times with similar results. (B) Expression of the M2-JNK1 and the HA-tagged calcineurin constructs was analyzed by immunoblotting with anti-M2 or -HA antibodies as described in Materials and methods. The dots indicate a non-specific band crossreactive with anti-HA. These experiments were repeated four times with similar results. (C) Jurkat cells were cotransfected with 0.5 μg of M2-JNK1 and 10 μg of HA-CnAΔCaM-AI or the same amount of pSRα. CsA was added at the indicated concentration (ng/ml) for 15 min and cells were stimulated with PMA as described above. JNK activity was assayed and quantitated by phosphorimaging. The antagonistic effect of CsA was plotted as a function of maximal JNK activation induced by PMA in HA-CnAΔCaM-AI transfected cells in the absence of CsA. Shown are the mean and standard errors (SE) of three separate experiments.

including PKC-θ, with variable efficacies (Toullec et al., 1991; Martiny-Baron et al., 1993). Gö6976 did not inhibit JNK activation, whereas Gö6850 partially inhibited JNK activation by anti-CD3+anti-CD28 and completely inhibited the response to PMA+ionomycin (Figure 3B).

To examine further which PKC isoform can synergize with calcineurin to activate JNK, we used constitutively activated mutants of PKC-θ, PKC-α and PKC-ε. Although PKC-θ (A148E) alone activated JNK even at the lowest amount transfected, ionomycin costimulation was required to reach maximal JNK activation (Figure 4A). Overexpressed wt PKC-θ was unable to activate JNK either alone or in combination with Ca2+-ionophore, although it slightly increased the response to PMA+ionomycin (data not shown). Since PKC-θ lacks the Ca2+-binding C2 region and therefore belongs to the Ca2+-independent class (Baier et al., 1993), our results suggested that a Ca2+-dependent component, possibly calcineurin, synergizes with PKC-θ to activate JNK. Therefore we cotransfected M2-JNK1 with increasing amounts of PKC-θ (A148E) in the presence or absence of HA-CnAΔCaM-AI. Although PKC-θ (A148E) activated JNK by itself, it synergized with the constitutive calcineurin mutant leading to enhanced JNK activation (Figure 4B). At high amounts of transfected PKC-θ this effect was even more pronounced than the synergism induced by PMA or costimulation with
PKC-θ was assessed by cotransfecting CnA alone (data not shown). The specificity of this response was illustrated by using PKC-α (A148E), PKC-ε (A159E), PKC-θ (A148E) expression vectors. The total amount of DNA was kept constant with pSRα. Expression of HA-ERK2 was determined by immunoblotting with anti-HA antibody. HA-ERK2 activity was determined by immunocomplex kinase assay using anti-HA to immunoprecipitate HA-ERK2 and MBP as substrate. Stimulation with PMA (15 min) and cotransfection with RafBXB were used as positive controls. Fold-activation was determined using a phosphorimager. The dot indicates a non-specific band cross-reactive with anti-HA. These experiments were repeated three times.

Calcineurin preferentially synergizes with PKC-θ to induce T-cell activation

Induction of IL-2 gene expression requires cooperative interaction between ubiquitous and lymphocyte-specific transcription factors such as AP-1, NF-κB, OBP, NF-AT, CD28RC (Clipstone and Crabtree, 1994; Rao, 1994; Cantrell, 1996). Costimulation of T cells increases AP-1 transcriptional activity which correlates with and appears to depend on JNK activation (Su et al., 1994). NF-AT transcriptional activity, which is a combinational response to AP-1 and the NF-ATc proteins (Jain et al., 1993; Timmerman et al., 1996), is induced by costimulation by either PMA and CnAΔCaM-AI (Clipstone and Crabtree, 1993) or by ionomycin and activated mutants of PKC-α or PKC-ε but not PKC-ζ (Genot et al., 1995). We investigated whether, similar to JNK activation, calcineurin would synergize with PKC-θ to modulate the transcriptional activities of NF-AT, NF-κB and the c-jun and IL-2 promoters. Among the three PKC isoforms tested, PKC-θ (A148E) preferentially increased the activities of all four reporter genes in synergism with CnAΔCaM-AI (Figure 6A and B and data not shown).

It is noteworthy, however, that while the activated PKC-α and PKC-ε mutants could not synergize with calcineurin to activate JNK or increase c-jun (data not shown) or IL-2 promoter activities (Figure 6B), both were capable of stimulating the NF-AT-dependent reporter, although not as efficiently as activated PKC-θ (Figure 6A). These results are consistent with those of Genot et al. (1995),...
who found that activated PKC-α and PKC-ε can stimulate the NF-AT reporter. As the c-jun promoter is responsive to AP-1 (Angel et al., 1988) but not to NF-ATc, it appears that the more relaxed PKC-responsiveness is due to the ability of NF-ATc to respond to all three PKC isozymes. By using the selective PKC inhibitors, G66976 and
Go6850, we found that activation of the NF-AT-dependent reporter by PMA+calcineurin mostly involves PKC-θ rather than PKC-α (Figure 6C). To confirm the selectivity of these inhibitors, we coexpressed CnAΔCaM-AI with NF-AT-LUC with or without PKC-α (A25E) or PKC-θ (A148E). While Go6850 inhibited induction of the NF-AT reporter by all three agonists, Go6976 only blocked the response to PKC-α (A25E) but not the response to PMA or PKC-θ (A148E) (Figure 6C). These results suggest that among the different PKC isoforms present in Jurkat cells, PKC-θ or a PKC-θ-like isozyme is most probably involved in activation of NF-AT in cooperation with calcineurin. Taken together, our findings strongly suggest that calcineurin preferentially cooperates with PKC-θ (or PKC-θ-like isozymes) to transduce mitogenic signals involved in IL-2 gene induction and T-cell activation.

**Role of the small GTP-binding protein, Rac**

In various cell lines Ras and Rac or Cdc42 couple extracellular mitogenic signals to the JNK pathway (Coso et al., 1995; Minden et al., 1995). In T cells, Ras is involved in coupling TCR occupancy to ERK activation (Cantrell, 1996). Rac is more specifically involved in JNK activation and mediates signals from PKC, CD3 and CD28 (Jacinto et al., 1998). We examined whether signals generated by PKC-θ and calcineurin that lead to JNK activation are integrated at the level of these GTPases. Jurkat cells were cotransfected with HA-CnAΔCaM-AI and M2-JNK1 expression vectors and with dominant inhibitory mutants of either Ras [Ha-(RasN17)] or Rac [Rac1(N17)]. While 10 µg of the Rac1(N17) vector decreased PMA-induced JNK activation by >80% (Figure 7A), twice that amount of Ha-Ras(N17) had no effect (Figure 7B). Similar results were obtained when PKC-θ

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**Fig. 7.** The calcineurin and PMA signals leading to JNK activation are integrated at the level of Rac1 or upstream of it. (A) and (B) Jurkat cells were transfected with 0.1 µg of M2-JNK1 in the presence or absence of 5 µg HA-CnAΔCaM-AI plus the indicated amount (µg) of (A) Rac1(N17) or (B) Ha-Ras(N17). Total transfected DNA was kept constant using pSRα. Where indicated, cells were stimulated with PMA alone or in combination with ionomycin. Expression of M2-JNK1, HA-CnA and Ha-Ras(N17) was determined by immunoblotting. M2-JNK1 activity was measured as described above by immunocomplex kinase assay. After quantitation with a phosphorimager, the highest level of JNK1 activity was given an arbitrary value of 100% and the other values were expressed relative to that. The dots indicate non-specific bands cross-reactive with anti-HA. These experiments were repeated three times. (C) Jurkat cells were transfected with 2.5 µg of HA-ERK2 in the absence or presence of 20 µg Ha-Ras(N17) expression vector. Where indicated, cells were stimulated with PMA (15 min). Expression of each protein was measured by immunoblotting. HA-ERK2 activity was measured by immunocomplex kinase assay. After quantitation with a phosphorimager, the highest level of ERK2 activity was given an arbitrary value of 100% and the other values were expressed relative to that. Similar results were obtained in two other experiments. (D) Jurkat cells were transfected with 0.1 µg of M2-JNK1 together with 5 µg of HA-CnAΔCaM-AI or 20 µg of Ha-Ras(L61) expression vectors either alone or in combination. As in (A) and (B) total DNA was kept constant with pSRα. Where indicated, cells were stimulated with PMA or ionomycin either alone or in combination. Expression of each protein was measured by immunoblotting. M2-JNK1 activity was measured by immunocomplex kinase assay and fold-activation determined using a phosphorimager. The dot indicates a non-specific band cross-reactive with anti-HA. These experiments were repeated five times. (E) Cells were transfected with 0.1 µg of M2-JNK1 in the absence or presence of 2 µg Rac1(V12) expression vector. The total amount of DNA was kept constant with pSRα. Where indicated, cells were stimulated with PMA or ionomycin either alone or in combination. JNK activity was measured by immunocomplex kinase assay. Fold-activation was quantitated by phosphorimager after normalization for the amount of recovered M2-JNK1 and one representative experiment out of three is shown.
The synergy between calcineurin and PKC-θ is T cell-specific

The synergistic activation of JNK by PKC and elevation of intracellular Ca²⁺ are involved in a variety of signaling responses (Nishizuka, 1992; Berridge, 1993; Clapham, 1995). Generally, these signaling pathways act synergistically although the molecular basis for synergy is obscure. While the rise in intracellular Ca²⁺ is only transient upon cell stimulation, PKC activation is more prolonged and may mediate in part some of the more sustained signaling responses. In T cells, PKC activation and calcium mobilization are regulated by PLCγ, which is activated following TCR stimulation (Berridge, 1993; Cantrell, 1996). T cells that are defective in Ca²⁺ or PKC signaling display decreased responsiveness to mitogenic stimulation (Goldsmith and Weiss, 1988; Valge et al., 1988; Straus and Weiss, 1992).

Among the Ca²⁺-dependent kinases and phosphatases, calcineurin mediates a critical positive signal necessary for IL-2 induction (O’Keefe et al., 1992; Clipstone and Crabtree, 1993; Crabtree and Clipstone, 1994), while Ca²⁺/calmodulin-dependent protein kinase II (CaM-K II) negatively regulates this response (Nghiem et al., 1994; Hama et al., 1995). The important role of calcineurin in T-cell activation is underscored by the effects of immunosuppressive drugs, such as CsA and FK506, which inhibit calcineurin activity. Moreover, targeted disruption of the calcineurin-α gene in mice results in defective antigen-specific T-cell responsiveness (Zhang et al., 1996).

While calcineurin mainly transduces the Ca²⁺ signal, a pathway triggered by PKC (or DAG), which cooperates with calcineurin, is required in T cells for full activation. However, it was heretofore not clear whether all PKC-responsive PKCs or only a particular isoform can synergize with calcineurin. How these two signaling pathways are integrated to induce IL-2 gene transcription also remains to be addressed. We investigated the mechanisms involved in integration of these pathways by focusing on their effect on JNK, a class of MAPKs that are synergistically activated by phorbol esters and Ca²⁺ in T cells (Su et al., 1994). JNK activation is believed to play an important role in T-cell activation (Su and Karin, 1996), an assertion that is supported by the phenotype of T cells whose JNK kinase 1 (JNKK1 or SEK1) gene was disrupted (Nishina et al., 1997).

Calcineurin is the major Ca²⁺-dependent component that mediates the effect of that ion on the JNK cascade (Figure 2). Costimulation with PMA is needed to reveal the effect of activated calcineurin on JNK activity. As expected, the PMA signal is mediated by PKC, but the surprising result is that only a particular PKC isoform can synergize with calcineurin to cause JNK activation. Only PKC-θ, but neither PKC-α nor PKC-ε, synergizes with calcineurin to activate JNK in T cells (Figure 4). A PKC
inhibitor that targets both conventional and novel PKC isotypes almost totally abrogated the response of JNK to PMA+ionomycin and partially inhibited its activation by anti-CD3+anti-CD28 (Figure 3). In contrast, a PKC inhibitor which targets only conventional PKCs has no effect on JNK activation. The latter inhibitor, Gö6976, has no effect on NF-AT activation by PKC-θ but inhibits its response to PKC-α (Figure 6C). These results support a role for PKC-θ in JNK activation by either the pharmacological activators PMA+ionomycin or the more physiological activators anti-CD3+anti-CD28. The cause for the partial sensitivity of the latter agonists to PKC inhibition is related to their ability to trigger the activation of multiple signaling pathways leading to JNK activation (Jacinto et al., 1998). Likewise, CsA which completely blocks synergistic JNK activation by PMA+ionomycin only partially inhibits its activation by anti-CD3+anti-CD28 (Su et al., 1994). In contrast to JNK, ERK activation can be triggered non-selectively by the three PKCs that were tested, suggesting that several PKC-dependent pathways may converge to cause activation of this MAPK cascade. Indeed, ERK activation induced by PMA is markedly higher than the effect of each individual PKC. This functional distinction between PKC isozymes may increase the diversity of various downstream responses, such as activation of transcription factors. Similar to the effect on JNK, calcineurin selectively synergizes with PKC-θ to activate the AP-1-responsive c-Jun and IL-2 promoters. Surprisingly, very little c-Jun or IL-2 promoter activation was achieved in response to coexpression of activated calcineurin with activated PKC-α or PKC-ε. The NF-AT-dependent reporter, on the other hand, was not as strictly dependent on PKC-θ although PKC-θ was also most effective in activating this reporter, considerable activation was observed in response to PKC-α or PKC-ε. As NF-AT activity requires interaction of AP-1 with the nuclear translocated NF-ATc proteins (Jain et al., 1993; Northrop et al., 1993; Timmerman et al., 1996), the latter factors may be responsible for the broader sensitivity of this reporter. However, as the IL-2 promoter is completely dependent on coordinated activation of several transcription factors including AP-1 (Crabtree and Clipstone, 1994), it shows strict dependence on PKC-θ. Further evidence for a specific role of PKC-θ in T-cell activation is provided by the finding that this PKC isoform specifically translocates and clusters with the TCR at the APC docking region, suggesting that PKC-θ but not -α, -βI, -βII, -δ, -η nor -ζ transduces signals generated at the TCR during an immune response (Monks et al., 1997). It should be noted, however, that in T-cell clones the co-custering of PKC-θ and the TCR was linked to mitogenesis rather than to cytokine induction. PKC-θ does not translocate to the APC docking region when TCR/CD3, CD4, LFA-1 or CD28 are separately capped with specific monoclonal antibodies, suggesting that its translocation and possibly activation, requires costimulatory signals.

Since TCR engagement generates the second messengers Ca²⁺ and DAG which activate calcineurin and PKC, respectively, it is puzzling why this process is insufficient for full PKC-θ activation. One explanation may be that PKC-θ requires a second activating signal. For instance, PKC-δ, another nPKC, is phosphorylated on tyrosine upon activation (Li et al., 1994). This mechanism may hypothetically be involved in PKC-θ activation upon phosphorylation by a CD28-recruited PTK (Cantrell, 1996). Such a mechanism may augment the ability of PKC-θ to synergize with calcineurin. Consistent with this possibility, it was observed that CD28 engagement further enhances PMA+Ca²⁺ ionophore-induced JNK activation (Su et al., 1994). Costimulation could also modulate the duration and amplitude of the Ca²⁺ signal. These two variables were proposed as a means by which Ca²⁺ could achieve specificity in signaling (Werlen et al., 1993; Clapham, 1995; Dolmetsch et al., 1997). In B lymphocytes, for example, a large transient Ca²⁺ rise activates JNK, whereas NF-ATc translocation to the nucleus is induced by a low sustained Ca²⁺ plateau (Dolmetsch et al., 1997). In other cell lines, such as HL-60, induction of the early response genes c-fos and c-jun also requires fine-tuned Ca²⁺ transients (Werlen et al., 1993). Similarly, we found that JNK activation in T cells is likely to depend on the pattern of the Ca²⁺ transient as suggested by reduced duration and fold-activation by PMA+ionomycin in the absence of extracellular Ca²⁺ (data not shown).

In the absence of costimulation, TCR engagement may not provide a sufficient Ca²⁺ transient to cause sustained calcineurin activation. As calcineurin is regulated by two different Ca²⁺-binding proteins (CnB and calmodulin), a low Ca²⁺ transient may jump-start the enzyme but a larger sustained Ca²⁺ plateau may be required for full activation. Sustained high intracellular Ca²⁺ is required to maintain NF-ATc in the nucleus of activated T lymphocytes (Timmerman et al., 1996). A large Ca²⁺ transient may also enhance PKC-θ activation. This could account for the higher level of JNK activation produced by PMA+ionomycin as compared with TCR and CD28 costimulation (Su et al., 1994). Ionomycin also induces saturating intracellular Ca²⁺ which does not reflect Ca²⁺ transients during physiological cell stimulation, (Werlen et al., 1993; Dolmetsch et al., 1997). As a consequence of the sustained Ca²⁺ signal, calcineurin may directly increase PKC-θ activity. However, we found that CnΔ-Acalpha-1 potentiates the effect of PKC-θ (A148E) on JNK activity, but does not directly affect PKC-θ kinase activity (data not shown). Therefore, the site for integration of the Ca²⁺ signal is not PKC-θ itself. This assertion is supported by the fact that PKC-θ belongs to the Ca²⁺-independent nPKC class.

It appears that the PKC and calcineurin signals are integrated upstream or at the level of the small G protein, Rac. Rac, but not Ras, can also couple the CD28-generated signal leading to JNK activation (Jacinto et al., 1998), supporting the idea that the CD28 signal enhances or is coupled to the PKC and calcineurin pathways. While Rac mediates JNK activation, Ras is believed to couple TCR-generated signals to the ERK cascade (Cantrell, 1996). Upstream regulators of JNK such as JNKK1 (MKK4) or JNKK2 (MKK7), the MAPKKs that activate the JNKs, and the MEKKs, the MAPKKKs responsible for JNKK activation (Lin et al., 1995; Wu et al., 1997), may also integrate costimulatory signals. Both JNKK1 and JNKK2 are synergistically activated by either CD3+CD28 coligation or PMA+Ca²⁺ ionophore (Wu et al., 1997). Expression of catalytically inactive MEKK1 totally abrogates synergistic JNK activation by PMA+Ca²⁺ ionophore or CD3+CD28 coligation (data not shown). Hence, the
Ca\(^{2+}\) and PKC signals are integrated upstream to the JNKKs, possibly at the level of Rac or a Rac exchange factor. The proteins that regulate Rac activity will provide important clues as to the possible integration mechanism. GTP exchange factors such as Vav, which may regulate Rac seem to play a crucial role in T-cell development and signaling (Fischer et al., 1995; Zhang et al., 1995). In fibroblasts, Vav can activate JNK in a Rac-dependent manner (Crespo et al., 1996). It remains to be established whether Vav plays a critical role in JNK activation in T cells.

In contrast to Jurkat cells, ionomycin activates JNK by itself in HeLa cells, but does not synergize with PMA. CnAΔCaM-Al had no effect on JNK activity in HeLa cells either alone or in combination with activated PKCs (Figure 8). Since all three activated PKCs can stimulate JNK activity in HeLa cells one might speculate that the lack of synergy or any response to CnA is due to the absence of a T cell-specific factor, which integrates the PKC and CnA signals. Unlike the Ca\(^{2+}\) response in T cells, the Ca\(^{2+}\)-signaling pathway leading to the JNK module in HeLa cells is not well understood. Nevertheless, various Ca\(^{2+}\)-dependent components such as CaM-KII or CaM-KIV may be involved. In PC12 cells, CaM-KIV and its upstream kinase CaM-KK activate MAPK cascades (Enslen et al., 1996), while CaM-KII mediates Ca\(^{2+}\)-induced early gene expression (Bading et al., 1993). CaM-KII also negatively affects IL-2 transcription in T cells (Nghiem et al., 1994; Hama et al., 1995), consistent with our unpublished observations that CaM-KII does not mediate the Ca\(^{2+}\) signal to JNK in T cells. Whether CaM-KII or CaM-KIV mediate JNK activation in HeLa cells remains to be determined. Another possible mediator of the Ca\(^{2+}\) signal in HeLa cells is a tyrosine kinase related to Pyk2 (Tokiwa et al., 1996).

In conclusion, we have demonstrated that PKC-θ, but not α- or ε significantly activates JNK in T cells, while all three PKCs stimulate ERK activity non-selectively. These results clearly demonstrate that different PKC isoforms have distinct biological roles, most likely due to either differential subcellular distribution or to phosphorylation of unique substrates. Calcineurin, a major mediator of Ca\(^{2+}\) signaling in T cells, markedly and exclusively potentiates the effect of PKC-θ on the JNK MAPK module. In addition, calcineurin and PKC-θ, but not other PKC isoforms, lead to induction of IL-2 and c-jun promoter activity, an effect that correlates with their effect on JNK activity. In HeLa cells by contrast, no difference was detected in the effect of the different PKCs on JNK activity and none of them synergizes with calcineurin. Unraveling the molecular mechanism by which the synergistic signals mediated by PKC-θ and calcineurin are specifically integrated in T cells should further our understanding of T-cell activation and hopefully provide the means to design new and highly specific immunosuppressive drugs, as this cooperation is cell type-specific.

Materials and methods

Cell culture, stimulation and transfection

Jurkat cells and Tag-Jurkat stably expressing the SV40 large T antigen (gift from G.Crabtree) were grown in complete RPMI 1640 medium containing 10% fetal calf serum (FCS), 1 mM glutamate, 100 U/ml penicillin and 100 µg/ml streptomycin. HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS, 1 mM glutamate, 100 U/ml penicillin and 100 µg/ml streptomycin. When indicated, cells were pretreated for 24 h with 100 nM phorbol myristate acetate (PMA). The drugs CsA (a generous gift from Sandoz), G06976 or G06850 (purchased from Alexis) were added at the indicated concentrations 15 min prior to stimulation with 1 µM ionomycin (Calbiochem) or 100 nM PMA (Sigma) either alone or in combination. The usual stimulation was for 30 min. Jurkat cells were lysed in 200 µl and HeLa cells in 800 µl of whole-cell extract (WCE) lysis buffer (Hibi et al., 1993), respectively. The protein content of each WCE was determined by Lowry assay (Bio-Rad) and the quantity of lysate used for JNK assay was normalized according to its JNK content determined by immunoblotting.

Transient transfection and kinase assays were done as described (Iacinto et al., 1998). For reporter gene assays cells were stimulated 16–24 h post-transfection with the indicated drugs and harvested 10 h later. Cells were lysed in 100 µl of buffer II (100 mM Tris–acetae pH 7.8; 10 mM Mg acetate; 1 mM EDTA) containing 1% Triton X-100 and 1 mM DTT. Luciferase activities were determined as described (Su et al., 1994) using a Microtum luminometer (EG&G Berthold). HeLa cells grown in 35 mm dishes to 40% confluency were transfected using lipofectamine (Gibco-BRL) according to the manufacturer’s protocol, keeping total DNA at 3 µg per plate. Cells were harvested after 30 h, lysed in 300 µl of WCE lysis buffer and assayed for JNK activity.

Expression vectors and reporters

The calcineurin expression vectors (pBJ5-CNa-4 and pBJ5-CNβ1) (gifts from Dr G.Crabtree) were HA-epitope tagged by adding an Ncod site at the N-terminus of the different open reading frames by PCR and subcloned between the Ncod and BglII sites of the pSRα vector (Minden et al., 1995). HA-CnAΔCaM-Al was constructed by PCR using the primer 5’GGCAGAATCTAGTTTCTGA TGACTTC, which introduces a stop codon at position 1194 of the CnA open reading frame. The M2Δ, 1JNK1, HA-RCK2, Rac1(V12), Rac1(N17), Ha-Ras(L61) and Raf(BBX) expression vectors were previously described (Dérijard et al., 1994; Minden et al., 1995). All constructs were subcloned into the pSRα vector (Takebe et al., 1988). The PKC-θ expression vectors were gifts from Dr A.Altsman (Baier-Bitterlich et al., 1996). PKC-α and PKC-ε expression vectors were gifts from Dr P.Parker (Genot et al., 1995). The NF-AT-LUC reporter was provided by Dr G.Crabtree (Northrop et al., 1993). The IL-2-LUC reporter construct was previously described (Su et al., 1994).

Antisera and reagents

Anti-HA and anti-M2 antibodies were described elsewhere (Hibi et al., 1998). Ras monoclonal antibody was kindly provided by Dr B.Sefton. Expression and purification of GST-cJun(1–79) were described before (Hibi et al., 1993). MBP was purchased from Sigma.

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Activation of JNK by PKC-θ and calcineurin


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