Calcium/Calmodulin-dependent Protein Kinase II Downregulates Both Calcineurin and Protein Kinase C-mediated Pathways for Cytokine Gene Transcription in Human T Cells

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Summary

Engagement of the T cell receptor for antigen activates phospholipase C resulting in an increase in intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) and activation of protein kinase C (PKC). Increased [Ca\(^{2+}\)]\(_i\) activates Ca\(^{2+}\)/calmodulin-dependent kinases including the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM-K II), as well as calcineurin, a type 2B protein phosphatase. Recent studies have identified calcineurin as a key enzyme for interleukin (IL)-2 and IL-4 promoter activation. However, the role of CaM-K II remains unknown. We have used mutants of these kinases and phosphatases (γB*CaM-K and ΔCaM-AI, respectively) to explore their relative role in cytokine gene transcription and their interactions with PKC-dependent signaling systems. γB*CaM-K and ΔCaM-AI, known to exhibit constitutive Ca\(^{2+}\)-independent activity, were cotransfected (alone or in combination) in Jurkat T cells with a plasmid containing the intact IL-2 promoter driving the expression of the chloramphenicol acetyltransferase reporter gene. Cotransfection of γB*CaM-K with the IL-2 promoter construct downregulated its transcription in response to stimulation with ionomycin and phorbol myristate acetate (PMA). The inhibitory effect of CaM-K II on IL-2 promoter was associated with decreased transcription of its AP-1 and NF-AT transactivating pathways. Under the same conditions, ΔCaM-AI superinduced IL-2 promoter activity (approximately twofold increase). When both mutants were used in combination, γB*CaM-K inhibited the induction of the IL-2 promoter by ΔCaM-AI. Similar results were obtained when a construct containing the IL-4 promoter also was used. γB*CaM-K also downregulated the activation of AP-1 in response to transfection with a constitutively active mutant of PKC or stimulation with PMA. These results suggest that CaM-K II may exert negative influences on cytokine gene transcription in human T cells, and provide preliminary evidence for negative cross-talk with the calcineurin- and PKC-dependent signaling systems.

IL-2 gene transcription is controlled by a promoter region extending ~326 bp upstream of the transcription start site. This promoter region contains binding sites for at least five nuclear proteins including nuclear factors NF-AT, Oct-1, NF-κB, AP-1, and CD28RC (1, 2). Both an increase of cytoplasmic calcium (Ca\(^{2+}\)) and activation of protein kinase C (PKC) are required for its activation in resting T cells.

Recent studies have established that the Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaM kinase II) is the general or multifunctional kinase of Ca\(^{2+}\)-signaling systems (reviewed in 3). CaM kinase II is present in most tissues as an oligomer composed of 6–12 subunits, depending on the isoform and tissue. All 11 isoforms described to date (α, α33, β, β′, γ\(_A\), γ\(_B\), γ\(_C\), δ\(_A\), δ\(_B\) or δ\(_S\), δ\(_C\) or δ\(_S\), δ\(_D\) or δ\(_S\)) share a highly conserved catalytic domain at the NH\(_2\)-terminal portion of the molecule, an autoinhibitory sequence overlapping with a calmodulin binding region and an association domain which is important for the formation of the holoenzyme (3–5). After an increase in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), Ca\(^{2+}\)/calmodulin activates CaM kinase II causing the kinase to phosphorylate itself on the Thr\(^{286}\) (α isoform) or Thr\(^{287}\) (β and γ isoforms) site of the autoinhibitory domain. This phosphorylation event results in disruption of the autoinhibitory domain and Ca\(^{2+}\)-independent activity (~20–80% of maximal Ca\(^{2+}\)-stimulated activity) (3). Mutations at Thr\(^{286}\) (e.g., change to Asp) result in mutants with 20–40% activity in the absence of Ca\(^{2+}\)/calmodulin stimulation (3). Constitutively active mutants of CaM kinase II can be used to investigate its role in the transcriptional regulation and to isolate the potential effects of this enzyme from other Ca\(^{2+}\)-dependent events (6).

Increase in [Ca\(^{2+}\)]\(_i\) and activation of calmodulin may also activate Ca\(^{2+}\)/calmodulin-dependent phosphatases such as calcineurin, a type 2B serine/threonine protein phosphatase.
Materials and Methods

Results

γB*CaM-K Does Not Substitute for The Ca2+-dependent Signaling Pathway for IL2 Promoter Transactivation. In the Jurkat model system, ΔCaM-AI can substitute for the Ca2+ signal required for IL-2 gene transcription (7, 8, 14). To investigate the role of CaM kinase II in the activation of the IL-2 promoter, we cotransfected a plasmid containing the IL-2 promoter linked to the CAT gene along with plasmids ΔCaM-AI or γB*CaM-K into these cells. Cells were stimulated with ionomycin and/or PMA. As shown in Fig. 1, both ionomycin and PMA were required for IL-2 promoter activation. ΔCaM-AI was able to partially substitute for the Ca2+ ionophore (~50% activity as compared to stimulation with ionomycin and PMA). Furthermore, addition of ionomycin further induced IL-2 promoter activity by approximately two-fold (range 1.8-2.4) suggesting that in addition to calcineurin other Ca2+-dependent pathways are also involved in IL-2 promoter activity. The effect of calcineurin was specific since it did not affect the activity of the unrelated promoter PCMV.Gal (98.9 + 15.3% of activity of cells treated with ionomycin and PMA). As shown in Fig. 1, both ionomycin and PMA were required for IL-2 promoter activation. ΔCaM-AI was able to partially substitute for the Ca2+ ionophore (~50% activity as compared to stimulation with ionomycin and PMA). Furthermore, addition of ionomycin further induced IL-2 promoter activity by approximately two-fold (range 1.8-2.4) suggesting that in addition to calcineurin other Ca2+-dependent pathways are also involved in IL-2 promoter activity. The effect of calcineurin was specific since it did not affect the activity of the unrelated promoter PCMV.Gal (98.9 + 15.3% of activity of cells treated with the vector alone). As shown in the same figure, these pathways most likely do not involve CaM kinase, since in similar experiments γB*CaM-K not only could not substitute for ionomycin but, to the contrary, exerted a negative effect on the transactivation of IL-2 promoter in response to stimulation with ionomycin and PMA (Fig. 1) (see below).

γB*CaM-K Inhibits IL-2 Promoter Activation by Down-regulating Its Transactivating Pathways AP-1 and NF-AT. The transcription factors that bind to the AP-1 and NF-AT sites are critical for IL-2 gene expression (1). To determine whether the inhibitory effect of γB*CaM-K on IL-2 promoter was
Figure 1. ΔCaM-Al but not γ*CaM-K acts in synergy with phorbol esters to mediate the transactivation of the IL-2 promoter. Jurkat cells were transiently transfected with 5 μg of plasmid IL-2 CAT (which contains the IL-2 promoter driving the expression of CAT gene) alone or in combination with plasmids ΔCaM-Al or γ*CaM-K (10 μg). Cells were stimulated with PMA alone or in combination with ionomycin.

mediated by these cis-acting elements, cells were transfected with constructs containing the intact IL-2 promoter or its AP-1 and NF-AT sites linked to the CAT reporter gene. γ*CaM-K decreased the activity of all three constructs (Fig. 2). In four different experiments γ*CaM-K decreased the activity of the IL-2 promoter construct by ∼50% (53.4 ± 9.2), of the AP-1 by ∼70% (68.5 ± 3.9), and NF-AT by ∼35% (34.2 ± 7.4). The downregulatory effect of γ*CaM-K was specific since it did not affect the activity of the unrelated promoter pCMV.Gal (95.8 ± 9.3% of the activity of cells transfected with the vector alone). Furthermore, cotransfection of the double mutant (inactive/constitutive) γ*CaM-K did not downregulate the IL-2 promoter activity (122.2 ± 8.9% of the activity of cells transfected with the vector alone in four independent experiments) (not shown).

Figure 2. γ*CaM-K inhibits IL-2 promoter activation by downregulating its transactivating pathways AP-1 and NF-AT. Cells were cotransfected with plasmids IL-2 CAT, AP-1 CAT or NF-AT CAT (5 μg) and γ*CaM-K (10 μg) or its vector, and stimulated with ionomycin and PMA. Results (expressed as percentage of activity observed when cells were stimulated in the presence of vector) were normalized to the activity of pCMV.Gal which was used to control for transfection efficiency (mean ± SE).

γ*CaM-K Downregulates the Transcription of Other Ca2+-dependent Promoters. In addition to IL-2 promoter the Ca2+ pathway is also involved in the regulation of other cytokine promoters such as IL-4, a Th2 promoter. Even though the signal transduction pathway may be different in IL-2– and IL-4–producing Th1 and Th2 clones, both pathways share the [Ca2+]i elevation (15). Recent data have suggested that calcineurin alone may upregulate the transcriptional activity of IL-4 (reference 9 and Paliogianni, F., N. Hama, G. J. Mavrothalassitis, G. Thyphronitis, and D. T. Boumpas, manuscript in preparation). To determine the effects of γ*CaM-K on this promoter, Jurkat cells were transfected with a construct of IL-4 promoter linked to the CAT reporter gene. Similar to IL-2, transfection with γ*CaM-K resulted in a small (∼40%) but reproducible inhibition of the transcriptional activity of the IL-4 promoter in response to ionomycin or ΔCaM-Al (Fig. 3). These data suggest that γ*CaM-K may counteract the effects of calcineurin (and probably other Ca2+-pathways).

γ*CaM-K Counteracts the Effects of ACaM-AI on IL-2 Promoter. To further explore the interaction between calcineurin and CaM kinase, we cotransfected Jurkat cells with the IL-2 promoter and ΔCaM-Al alone or in combination with γ*CaM-K. In cells stimulated with ionomycin and PMA cotransfection with γ*CaM-K completely abolished the superinduction of IL-2 promoter in the presence of ΔCaM-Al (Fig. 4). Similar to its effects on the IL-2 promoter, γ*CaM-K partially downregulated the increase in the activity of the NF-AT construct in response to stimulation with ionomycin and PMA alone or in combination with ΔCaM-Al (not shown). These results provide further evidence that γ*CaM-K may counteract the effects of calcineurin dependent-pathways on T cell activation.

Figure 3. γ*CaM-K decreases the activity of IL-4 promoter in response to activation with ionomycin or ΔCaM-Al. Cells cotransfected with 15 μg of IL-4 CAT (which contains IL-4 promoter linked to the CAT gene) in combination with γ*CaM-K and/or ΔCaM-Al (7.5 μg).
II with PKC-dependent pathways during the activation of T cells, Jurkat cells were cotransfected with an AP-1 construct linked to the CAT reporter gene alone or in combination with constitutive active mutants of CaM kinase II, calcineurin, and PKC (γCaM-K, DCaM-Al, and ΔPKCβ, respectively). As shown in Fig. 5, cotransfection with ΔPKCβ increased the baseline activity of AP-1 by approximately sixfold. Stimulation with PMA further induced the activity of AP-1. Under both conditions cotransfection with γCaM-K downregulated the AP-1 activity by ∼60%. In addition to γCaM-K, DCaM-Al also downregulated the AP-1 activity in response to stimulation with PMA (∼50% inhibition, Fig. 6). As shown in the same figure, the downregulatory effects of DCaM-Al and γCaM-K were not synergistic. These data suggest that under certain conditions both calcineurin and CaM kinase II may downregulate PKC-dependent transactivation pathways.

Discussion

In this study we have presented evidence that CaM kinase II may downregulate Ca2+- and PKC-dependent transactivating pathways for cytokine gene transcription in human T cells. The effect of CaM kinase II seems to be specific since CaM kinase II does not affect the activity of the unrelated promoter pCMVGal. Furthermore, the double mutant (inactive/constitutive) γCaM-K does not affect the IL-2 promoter activity, suggesting that inhibition of gene transcription requires the presence of a catalytically active domain of CaM kinase II and it is not due to the competing effects of the vectors used in these experiments. These results provide a molecular basis for the previously reported negative effects of the Ca2+-pathway on T cells, such as negative selection and anergy (18–20).

IL-2 is one of the major growth factors for the cells of the immune system. Inadequate production of IL-2 has significant consequences for the T cells and may result in anergy and tolerance. Exogenous IL-2 or costimulation via the CD28 pathway may reverse these states suggesting that the total amount of IL-2 produced in an immune response plays a critical role in determining the extent of the anergy (20, 21). In addition to playing a major role in the physiological immune responses against foreign antigens IL-2 may also be involved in autoimmune phenomena. A stringent control of the production of IL-2 is therefore required to avoid excess production which may lead to breaking of tolerance and autoimmune phenomena.

Work from several laboratories has associated the lack of IL-2 production in anergic states to defects in the transactivation of nuclear factors, AP-1 (22), NF-AT, and NF-κB (23). These results suggest that activation of CaM kinase II may provide an additional regulatory mechanism for the fine tuning of IL-2 production during T cell activation. It is possible that the downregulation of these transactivating pathways may be due, at least in part, to activation of CaM kinase II. Although in these experiments CaM kinase II only partially inhibited IL-2 transcription, this may be sufficient to result in autocrine rather than paracrine levels of IL-2 production (20). Furthermore, the degree of inhibition may differ among different types or clones of T cells.
In the murine system of T cell anergy, costimulation of T cells through the CD28 pathway augments IL-2 production from autocrine to paracrine levels and prevents induction of anergy (21). Although increases in [Ca\textsuperscript{2+}] during activation through TCR will activate both calcineurin (which augments IL-2 production) and CaM kinase II (which decreases it), it is conceivable that additional accessory stimuli (e.g., stimulation via CD2, CD5, or CD28) may shift the balance towards one or the other of these enzymes.

Downregulation of IL-4 production of CaM kinase II is also of interest. IL-4 is the other major T cell growth factor and is essential for the regulation of humoral immune responses. Anergic T cells lose their responsiveness to IL-4 (20). Decreased production of IL-4 by CaM kinase II may be an additional proximal mechanism to prevent proliferative response by these cells.

This study has presented preliminary data on cross-talk between the CaM kinase II and calcineurin signaling systems. During T cell activation the partial inhibition of IL-2 or IL-4 promoter activity by CaM kinase II suggests that their effects may be exerted at more distal sites in the calcineurin regulated pathways. Calcineurin is known to activate several discrete transactivating pathways including NF-AT, Oct-1/OAP, and NF-κB and to downregulate the activity of AP-1 (7–10). Hashimoto et al. (24) have shown that calcineurin is phosphorylated by the autophosphorylated form of CaM kinase II. Whether these in vitro data have any relevance to explaining the attenuating effects of CaM kinase II on calcineurin in vivo can not be addressed by these studies.

Whereas it is well established that PKC may exhibit both negative and positive cross-talk with CaM kinase II (25), the effects of CaM kinase on PKC are less well characterized. Our data provide for the first time evidence that CaM kinase II may downregulate PKC-dependent pathways for T cell activation. Once more the partial inactivation of the PKC mediated AP-1 induction suggests that CaM kinase II more likely affects distal sites in the PKC mediated pathway.

In summary, our data have identified CaM kinase II as a potential candidate for some of the negative effects of the Ca\textsuperscript{2+}-pathway on T cell activation. Furthermore, these data provide indirect evidence for cross-talk between the calcium and the PKC pathways during T cell activation. Additional studies should further delineate this interaction and determine its importance for the activation of T cells.

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Note added in proof: Work published by Ngheim et al. (26) after the original submission of this manuscript corroborates our findings for attenuation of IL-2 gene transcription by CaM kinase II.

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