Brief Definitive Report

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 (γ_B*CaM-K and ΔCaM-AI, respectively) to explore their relative role in cytokine gene transcription and their interactions with PKC-dependent signaling systems. γ_B*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 γ_B*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, γ_B*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. γ_B*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, α_{33}, β, β', γ_A, γ_B, γ_C, δ_A, δ_B or δ_3, δ_C or δ_2, δ_D or δ_4) 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.

Work from several laboratories has identified calcineurin as a key enzyme needed for IL-2 and IL-4 promoter activity (7–9). In addition to its effects on the intact IL-2 promoter, calcineurin can separately lead to the transactivation of nuclear factors NF-AT and Oct-1 (NF-IL2A, OAP/Oct-1). More recent data have also suggested that calcineurin may act in synergy with PMA to inactivate IκB/MAD3, an inhibitor of NF-κB, thereby resulting in net activation of the NF-κB (10).

Whereas the role of CaM kinase II has been well defined in neuronal tissues, its role in T cell activation remains largely unknown. In human lymphocytes, stimulation via the TCR or with calcium ionophores increases CaM kinase II activity (5, 11). Using constitutively active mutants of CaM kinase II, calcineurin, and PKC, we asked whether CaM kinase II is involved in the regulation of IL-2 and IL-4 promoter transcription and its influence on the effects of calcineurin- and PKC-dependent signaling systems. Our data indicate that CaM kinase downregulates the transcription of these cytokines and provides preliminary evidence for cross-talk among these pathways.

Materials and Methods

Cell Culture. Human Jurkat T cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS, 25 μg/ml gentamicin, and 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO).

Plasmid Constructs. The reporter plasmid IL-2 CAT contains the human IL-2 promoter (base pairs −448 to +43) directing the transcription of the chloramphenicol acetyltransferase (CAT) gene. This plasmid also includes an SV40 polyadenylation site between vector sequences and −448 of the IL-2 promoter to prevent vector-dependent transcription from contributing to the observed CAT activity (7). Plasmid AP-1 CAT (TRE-tk CAT) (a gift from Dr. M. Karin, University of California at San Diego, La Jolla, CA) contains a synthetic oligonucleotide from −73 to −65 of the human collagenase gene cloned into the HindIII/BamHI cut of pBLCAT 2, which contains the Herpes simplex virus thymidine kinase promoter from position −109 to +51 in front of the CAT structural gene (12). Plasmid NF-AT CAT (a gift from Dr. G. R. Crabtree, Stanford University, Stanford, CA) has three copies of NF-AT-binding site containing 5′ IL-2 sequences -255 to −285 linked to the IL-2 promoter sequences from −72 to +47 linked to the CAT gene (8). Plasmid IL-4 CAT (a gift from Dr. G. Thyphronitis, Institute Pasteur, Lille, France) contains the human IL-4 promoter (base pairs −418 to +50) cloned into the Not I site of $pUMSP_L$, which is on a pSV0CAT-based vector that contains the upstream sequences of the *c-mos* gene to stop read-through transcription, and the polylinker of the pBluescript. Plasmid γ_B*CaM-K (a gift from Dr. H. Schulman, Stanford University, Stanford, CA) is a constitutive mutant of the human T lymphocyte γ_BCaM kinase (5). EcoRI linkers were added to the ClaI-NotI fragment containing the full-length γ_BCaM kinase. This fragment was then inserted into pSRαBKS, forming a complete γ_BCaM kinase-SRα expression construct. Subsequently, Thr^{287} was replaced with Asp using site-directed mutagenesis to make mutant γ_B*CaM-K. This mutation mimics the effect of autophosphorylation on disrupting the autoinhibitory domain and generates a CaM kinase which is substantially Ca^{2+}-independent. γ_B*CaM-K was nearly 40% active in the absence of Ca^{2+}/calmodulin (5). In cells stimulated with ionomycin and PMA, and transfected with 10 μg of γ_B*CaM-K, the activity (determined in cytoplasmic extracts using the synthetic peptide substrate syntide that is recognized by this kinase) was two- to threefold higher than in cells transfected with its vector (data not shown). Plasmid double mutant (inactive/constitutive) γ_BCaM-K (also a gift from Dr. H. Schulman) is the catalytically inactive mutant of γ_B*CaM-K. Double mutant γ_B*CaM-K was constructed by replacing Lys^{43} (which is a conserved residue essential for catalytic activity) with Met in the ATP binding (catalytic) domain. Plasmid ΔCaM-AI (a gift from Dr. R. L. Kincaid, Human Genome Sciences, Inc., Rockville, MD) is a deletion mutant of the catalytic subunit of the wild-type murine calcineurin, the sequence of which has 99% homology to the corresponding human subunit. ΔCaM-AI lacks functional CaM-binding and autoinhibitory domains. This truncation was designed to mimic proteolyzed forms of the phosphatase known to have Ca^{2+}-independent, constitutive phosphatase activity in vitro (7). Plasmid ΔPKCβ (a gift from Dr. M.-A. Muramatsu, University of Tokyo, Tokyo, Japan) is the constitutively active mutant of PKCβ which lacks the regulatory domain (phorbol ester binding domain). ΔPKCβ lacks the coding region for amino acids at positions 6–159 of PKCβ (13). In control experiments, the expression vector alone was used.

DNA Transfections. Transfections of Jurkat T cells were carried out by the DEAE-dextran method as previously described (14). To monitor and control for transfection efficiency, cells were cotransfected with pCMV.Gal which contains the CMV-IE promoter directing transcription of the bacterial β-galactosidase (β-gal) gene (7).

CAT Assay. The CAT assay was carried out as described previously (14). The CAT activity was then normalized to the β-gal activity.

Results

*γ_B*CaM-K Does Not Substitute for The Ca^{2+}-dependent Signaling Pathway for IL-2 Promoter Transactivation.* In the Jurkat model system, ΔCaM-AI can substitute for the Ca^{2+} 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 γ_B*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 Ca^{2+} ionophore (~50% activity as compared to stimulation with ionomycin and PMA). Furthermore, addition of ionomycin further induced IL-2 promoter activity by approximately twofold (range 1.8–2.4) suggesting that in addition to calcineurin other Ca^{2+}-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 γ_B*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).

*γ_B*CaM-K Inhibits IL-2 Promoter Activation by Downregulating 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 γ_B*CaM-K on IL-2 promoter was

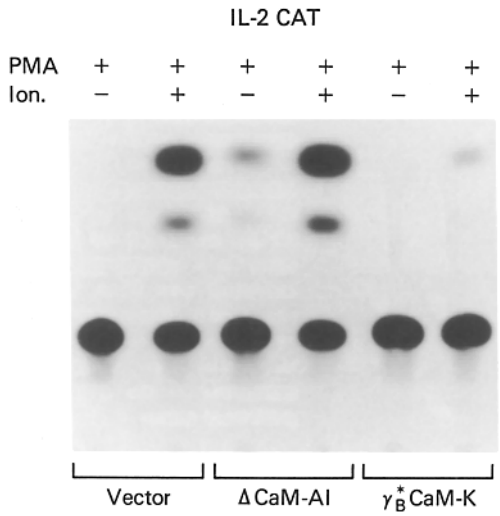

Figure 1. ΔCaM-AI but not γ_B*CaM-K acts in synergy with phorbol esters to mediate the transactivation of the IL-2 promoter. Jurkat cells were transiently transfected with 5 μg of plasmid IL-2 CAT (which contains the IL-2 promoter driving the expression of CAT gene) alone or in combination with plasmids ΔCaM-AI or γ_B*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_B*CaM-K decreased the activity of all three constructs (Fig. 2). In four different experiments γ_B*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_B*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_BCaM-K did not downregulate the IL-2 promoter

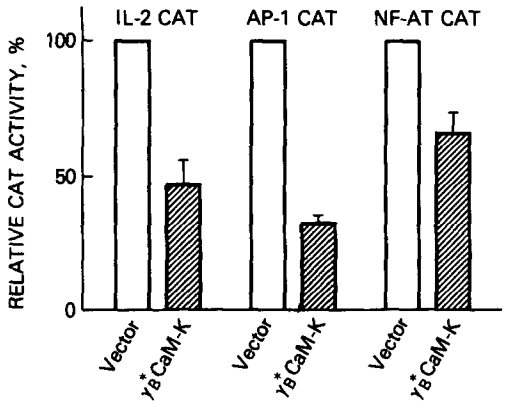

Figure 2. γ_B*CaM-K inhibits IL-2 promoter activation by downregulating its transactivating pathways AP-1 and NF-AT. Cells were cotransfected with plasmids IL-2 CAT , AP-1 CAT or NF-AT CAT (5 μg) and γ_B*CaM-K (10 μg) or its vector, and stimulated with ionomycin and PMA. Results (expressed as percentage 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).

activity (122.2 ± 8.9% of the activity of cells transfected with the vector alone in four independent experiments) (not shown).

*γ_B*CaM-K Downregulates the Transcription of Other Ca^{2+}-dependent Promoters.* In addition to IL-2 promoter the Ca^{2+} 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 $[Ca^{2+}]_i$ 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_B*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_B*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-AI (Fig. 3). These data suggest that γ_B*CaM-K may counteract the effects of calcineurin (and probably other Ca^{2+}-pathways).

*γ_B*CaM-K Counteracts the Effects of ΔCaM-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-AI alone or in combination with γ_B*CaM-K. In cells stimulated with ionomycin and PMA cotransfection with γ_B*CaM-K completely abolished the superinduction of IL-2 promoter in the presence of ΔCaM-AI (Fig. 4). Similar to its effects on the IL-2 promoter, γ_B*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-AI (not shown). These results provide further evidence that γ_B*CaM-K may counteract the effects of calcineurin dependent-pathways on T cell activation.

*γ_B*CaM-K Downregulates the Effects of PKC on Transactivation of AP-1.* In contrast to NF-AT whereby Ca^{2+} is essential for its activation, stimulation with phorbol esters alone is sufficient and more potent than ionomycin to induce AP-1 activity (16, 17). To explore the interaction of CaM kinase

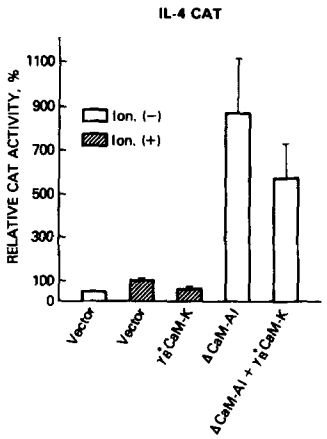

Figure 3. γ_B*CaM-K decreases the activity of IL-4 promoter in response to activation with ionomycin or ΔCaM-AI. Cells cotransfected with 15 μg of IL-4 CAT (which contains IL-4 promoter linked to the CAT gene) in combination with γ_B*CaM-K and/or ΔCaM-AI (7.5 μg).

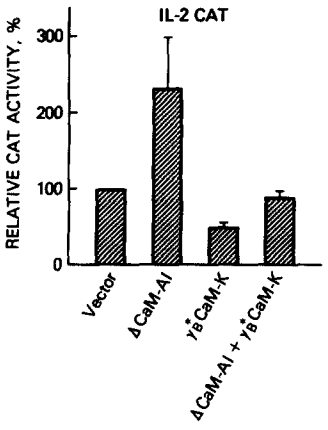

Figure 4. γ_B*CaM-K counteracts the effects of ΔCaM-AI on IL-2 promoter. Cells were cotransfected with IL-2 CAT (5 μg) in combination with ΔCaM-AI and/or γ_B*CaM-K (10 μg) and stimulated with ionomycin and PMA. Results (expressed as percentage activity observed in cells transfected with vector alone) were normalized to the activity of pCMV.Gal which was used to monitor for transfection efficiency (mean ± SE).

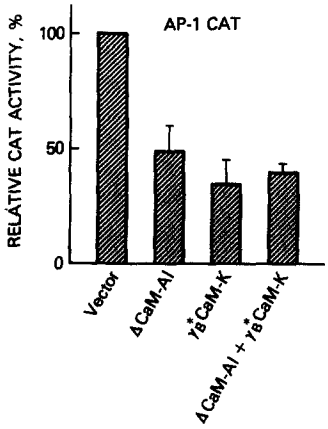

Figure 6. Both γ_B*CaM-K and ΔCaM-AI downregulate the transactivation of AP-1 in response to stimulation with PMA. Cells were cotransfected with plasmids AP-1 CAT (5 μg), γ_B*CaM-K (10 μg) and ΔCaM-AI (10 μg) (alone or in combination), and stimulated with PMA. Results (expressed as a percentage activity observed in cells transfected with the pSRα vector alone) were normalized to the activity of pCMV.Gal (mean ± SE).

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 (γ_B*CaM-K, ΔCaM-AI, 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 γ_B*CaM-K downregulated the AP-1 activity by ~60%. In addition to γ_B*CaM-K, ΔCaM-AI 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 ΔCaM-AI and γ_B*CaM-K were not synergistic. These data suggest that under certain conditions both calcineurin and CaM kinase II may downregulate PKC-dependent transactivation pathways.

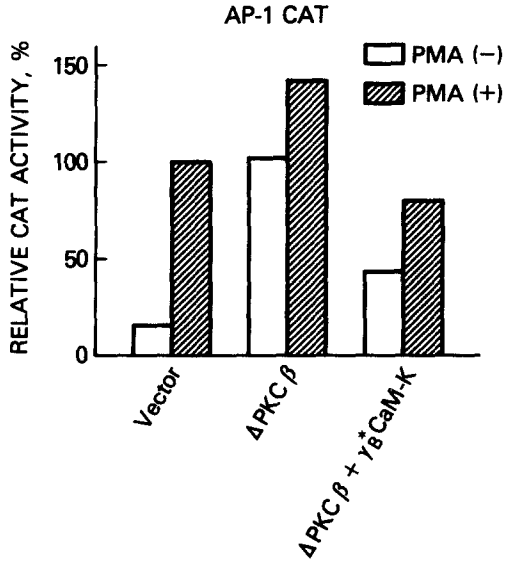

Figure 5. γ_B*CaM-K downregulates the transactivation of AP-1 in response to cotransfection with ΔPKCβ and/or stimulation with PMA. Cells were cotransfected with plasmids AP-1 CAT (5 μg) in combination with γ_B*CaM-K (10 μg) and/or ΔPKCβ (20 μg). Data are representative of results in five independent experiments.

Discussion

In this study we have presented evidence that CaM kinase II may downregulate Ca^{2+}- 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 pCMV.Gal. Furthermore, the double mutant (inactive/constitutive) γ_BCaM-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 Ca^{2+}-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 $[Ca^{2+}]_i$ 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^{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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