T Cell Activation Up-Regulates the Expression of the Focal Adhesion Kinase Pyk2: Opposing Roles for the Activation of Protein Kinase C and the Increase in Intracellular Ca²⁺

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T Cell Activation Up-Regulates the Expression of the Focal Adhesion Kinase Pyk2: Opposing Roles for the Activation of Protein Kinase C and the Increase in Intracellular Ca^{2+}

Masahiro Tsuchida, Eric R. Manthei, Tausif Alam, Stuart J. Knechtle, and Majed M. Hamawy

T cell activation initiates signals that control gene expression of molecules important for T cell function. The focal adhesion kinase Pyk2 has been implicated in T cell signaling. To further analyze the involvement of Pyk2 in T cell processes, we examined the effect of T cell stimulation on the expression of Pyk2. We found that TCR ligation or PMA increased Pyk2 expression in Jurkat T cells and in normal T cells. In contrast, TCR ligation and PMA failed to induce any detectable increase in the expression of the other member of the focal adhesion kinase family, Fak, in Jurkat T cells and induced only a weak increase in Fak expression in normal T cells. The serine/threonine kinases, protein kinase C and mitogen-activated protein extracellular signal-related kinase kinase (MEK), regulated Pyk2 expression, as inhibitors of these kinases blocked stimulus-induced Pyk2 expression. Cyclosporin A, FK506, and KN-62 did not block Pyk2 expression; thus, calcineurin and Ca^{2+}/calmodulin-activated kinases are not critical for augmenting Pyk2 expression. TCR ligation increased Pyk2 mRNA, and the transcriptional inhibitor actinomycin D blocked Pyk2 expression. Strikingly, Ca^{2+} ionophores, at concentrations that in combination with other stimuli induced IL-2 expression, blocked TCR- and PMA-induced up-regulation of Pyk2 expression. Thus, the increase in Ca^{2+} has opposing effects on IL-2 and Pyk2 expression. Cyclosporin A and FK506, but not KN-62, blocked Ca^{2+} ionophore-mediated inhibition of Pyk2 expression, implicating calcineurin in down-regulating Pyk2 expression. These results show that TCR-triggered intracellular signals increase Pyk2 expression and shed light on the molecular mechanisms that regulate Pyk2 expression in T cells.

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sites within the cell suggest that these PTKs have different cellular functions.

Receptor-mediated activation of T cells has been shown to induce tyrosine phosphorylation of the focal adhesion PTKs (26–31). Notably, although both Pyk2 and Fak become tyrosine phosphorylated following TCR ligation, only Pyk2 appears to become tyrosine phosphorylated upon CD28 ligation (26, 27, 29, 30). To further examine the involvement of Pyk2 and Fak in T cell processes, we examined the effect of T cell stimulation on the expression of Pyk2 and Fak in T cells. We found that the activation of Jurkat T cells and human normal T cells by ligation TCR or by treatment with PMA increased Pyk2 expression. In contrast, TCR ligation or PMA did not increase Fak expression in Jurkat T cells and only weakly increased Fak expression in normal T cells. We also examined the molecular mechanisms that regulate TCR-induced Pyk2 expression and found that the PKC-ERK cascade is involved in up-regulating Pyk2 expression in T cells. Although the Ca^{2+} ionophore did not increase the expression of Pyk2, it completely blocked TCR- and PMA-induced up-regulation of Pyk2 expression. CsA and FK506 at concentrations that inhibited IL-2 completely blocked TCR- and PMA-induced up-regulation of Pyk2 expression. KN-62, an inhibitor of CaMKs, did not block TCR- or PMA-induced up-regulation of Pyk2 expression and did not block Ca^{2+} ionophore-mediated inhibition of Pyk2 expression. Thus, Pyk2 expression in T cells involves the activation of the PKC-ERK cascade and is negatively regulated by the sustained increase in intracellular Ca^{2+}.

Materials and Methods

Reagents

RPMI 1640 was purchased from Mediatech Cellgro (Herndon, VA). The FCS was purchased from HyClone Laboratories (Logan, UT). Antibiotic-antimycotic mixture and glutamine were obtained from Life Technologies (Grand Island, NY). PD98059 and Ro-31-8220 were obtained from Calbiochem (San Diego, CA). Aprotinin, PMSF, H-7, sodium orthovanadate, PMA, Ca^{2+} ionophore A23187, Ca^{2+} ionophore ionomycin, protease-free BSA, and protein A–agarose beads were purchased from Sigma (St. Louis, MO). Cyclosporin A (CsA) was obtained from Sandoz (East Hanover, NJ). FK506 was purchased from Fujisawa USA (Deerfield, IL). KN-62 was obtained from Biomol (Plymouth Meeting, PA). The Lumiglo chemiluminescent substrate kit and TMB peroxidase substrate were obtained from Kirkegaard & Perry Laboratories (Gaithersburg, MD). The RNeasy minikit was obtained from Qiagen (Santa Clarita, CA). Recombinant human IL-2 was obtained from Collaborative Biomedical Products (Bedford, MA).

Antibodies

Anti-human CD28 mAb (Leu28, clone L293) was purchased from Becton Dickinson (San Jose, CA). Anti-human CD3 mAb was obtained from Ancell (Bayport, MN). Anti-phosphotyrosine mAb PY20, anti-Pyk2 mAb, and anti-Fak mAb were obtained from Transduction Laboratories (Lexington, KY). Anti-Csk rabbit polyclonal Ab and anti-Fak (H-1) mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human IL-2 polyclonal Ab and anti-human IL-2 mAb (biotin labeled) were obtained from Endogen (Woburn, MA). Rabbit anti-mouse IgG Ab, HER-conjugated goat anti-mouse Ig Ab, and HRP-conjugated goat anti-rabbit Ig Ab were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cells

T cells were isolated by the blood of healthy humans on Ficoll-His-topaque and washed three times in RPMI containing 10% FCS. The cells were then incubated with goat anti-human IgG-coated Immunul beads according to the manufacturer’s (Becton, Houston, TX) recommendations. Unbound cells (T cells) were collected, and adherent cells were removed by incubating the cells in a tissue culture flask for 3 min at 37 °C. T cell purity was at least 98% as determined by flow cytometry. Acute human T cell leukemia (Jurkat) cells, clone E6-1, were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in suspension in RPMI (RPMI supplemented with 10% heat-inac-

tivated FCS, 4 mM L-glutamine, and antibiotic-antimycotic mixture) at 37°C in 5% CO_{2} and were subcultured three times per week.

Cell activation and preparation of cell lysates

T cell activation was performed as reported previously (27, 32–34). For stimulating T cells in suspension, Jurkat T cells or peripheral blood T cells were washed with RPMI-5% FCS and suspended in the same medium in polystyrene tubes (8 × 10^5 cells in 400 µl of medium). The cells were then incubated with 100 µl of RPMI-5% FCS containing the indicated concentrations of the stimuli with or without inhibitors for 16 h at 37°C. After incubation, the cells were washed once with RPMI 1640 containing 0.001% BSA and immediately solubilized with boiling SDS-PAGE sample buffer. For stimulating T cells with immobilized mAb, T cells were washed with RPMI 1640 containing 5% FCS (RPMI-5% FCS) and then resuspended in the same medium. Meanwhile, wells of 96-well tissue culture plates were coated with the indicated concentrations of the mAb for 2 h at 37°C. After washing the plates, 50 µl of the cell suspension (2 × 10^5 Jurkat T cells or 5 × 10^5 purified normal T cells) was added to each well, and the plates were incubated for the indicated time at 37°C in a water-jacketed incubator. After incubation, the plates were lysed with boiling 2× SDS-PAGE sample buffer. For analysis of total cellular proteins, cell lysates were subjected to SDS-PAGE, electrottransferred to polyvinylidene difluoride membranes, and blotted with the indicated Ab as described below. For immunoprecipitation studies, Jurkat T cells (5 × 10^6 cells/ml in RPMI-5% FCS were treated with the indicated stimulus at 37°C and then immediately solubilized with 2× ice-cold lysis buffer as described previously (27, 33, 34).

Immunoprecipitation and immunoblotting

These were performed as described previously with some modification (27, 32–34). Briefly, 10 µg of rabbit anti-mouse Ig were incubated for 2 h with 50 µl of protein A–agarose beads. After incubation, the beads were pelleted by centrifugation and washed with ice-cold solubilization buffer. The primary Ab was then added to the beads, followed by cell lysates from 5 × 10^5 cells. The mixture was gently rotated for 2 h at 4°C. After incubation, the beads were pelleted by centrifugation and washed five times with ice-cold solubilization buffer. After the final centrifugation, the beads were resuspended in 2× SDS-PAGE sample buffer and boiled for 5 min. For immunoblotting, aliquots from whole cell lysates (WCL) or immunoprecipitates were separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. Immunoblotting with HRP-conjugated anti-phosphotyrosine antibody was performed as previously described (27, 32–34). To confirm similar loading of samples, Abs were stripped from the membranes as previously reported (27, 32–34), and the proteins were re-probed with specific Abs followed by HRP-coupled goat anti-mouse Ig Ab or HRP-coupled goat anti-rabbit Ig Ab (1/50,000 dilution). The signals were visualized using the Lumiglo kit according to the manufacturer’s (Kirkegaard & Perry) recommendations.

The IL-2 assay

The IL-2 level in the supernatants of overnight cultures was determined with a sandwich ELISA technique using combinations of unlabeled and biotin-labeled Abs according to the manufacturer’s recommendations (Endogen). Briefly, flat-bottom 96-well microtiter plates (Corning Costar, Cambridge, MA) were coated with 100 µl of anti-human IL-2 polyclonal Ab (2 µg/ml) overnight at room temperature. After incubation, nonspecific binding sites were blocked with 4% BSA for 1 h at room temperature. Recombinant human IL-2 standard or cultured culture supernatants (50 µl) were added in triplicate to the wells, and the plates were then incubated for 1 h at room temperature. After incubation, 50 µl of biotinylated anti-human IL-2 mAb were added to each well, and the incubation was resumed for additional 1 h at room temperature. After incubation, the wells were washed; 50 µl of HRP-conjugated streptavidin was added to each well, and the plates were incubated for 30 min at room temperature. Following incubation, the wells were washed, and then 50 µl of the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate was added to each well, and the plates were incubated for 30 min at room temperature. The reaction was then stopped with 50 µl of 0.18 M H_{2}SO_{4}. Plates were read at 450 nm in an automated microplate reader (ELX800, Bio-Tek Instruments, Winooski, VT).

Gene expression analysis

Jurkat T cells (2 × 10^6) in RPMI-5% FCS were stimulated with 1 ng/ml of PMA, 3 µg/ml of anti-CD3 mAb, or the combination of 1 ng/ml PMA and 1 µM Ca^{2+} ionophore A23187 for the indicated time at 37°C. After incubation, the cells were washed, and total RNA was extracted using the
FIGURE 1. Jurkat T cell activation by ligating CD3 or CD28 or by PMA treatment up-regulates the expression of the focal adhesion PTK Pyk2 but not Fak. A, Jurkat T cells were incubated in RPMI-5% FCS with the indicated concentrations of anti-CD3 mAb (α-CD3), anti-CD28 mAb (α-CD28), PMA, or the Ca\(^{2+}\) ionophore A23187 for 16 h at 37°C. After incubation, the cells were washed with RPMI 1640 containing 0.001% BSA and immediately lysed with boiling SDS-PAGE sample buffer. Proteins in WCL were then transferred to membranes and immunoblotted with anti-Pyk2 mAb (upper panel), anti-Fak mAb (middle panel), or anti-Csk Ab (lower panel). B, Densitometry of Pyk2 expression obtained from results similar to those shown in the upper panel of A. Results are expressed as the mean ± SD (n = 3) for the percent increase in Pyk2 in stimulated cells over that in untreated Jurkat T cells. C, For stimulation with immobilized anti-CD3 mAb, Jurkat T cells (2 × 10^5 cells in 100 μl of RPMI-5% FCS) were added to uncoated wells (lane 1) or to wells coated with the indicated concentrations of anti-CD3 mAb (lanes 2–4). For stimulation with soluble anti-CD3 mAb, Jurkat T cells (2 × 10^5 cells in 50 μl of RPMI-5% FCS) were added to polyethylene tubes containing 50 μl of RPMI-5% FCS (lane 5) or to tubes containing 50 μl of RPMI-5% FCS and the indicated concentration of anti-CD3 mAb (lanes 6–8). Following incubation for 16 h at 37°C, the cells were lysed with 2× boiling sample buffer. Proteins in WCL were then transferred to membranes and immunoblotted with anti-Pyk2 mAb (upper panel) or anti-Csk Ab (lower panel). This experiment was repeated twice with similar results. D, Densitometry of Pyk2 expression obtained from results shown in the upper panel of C. Results are expressed as the percent increase in Pyk2 in stimulated cells over that in untreated Jurkat T cells. E, Jurkat T cells were stimulated for 16 h at 37°C with immobilized anti-CD3 mAb as described in C. Proteins in WCL were then transferred to membranes and immunoblotted with anti-Pyk2 mAb (upper panel), anti-Fak mAb (middle panel), or anti-Csk Ab (lower panel). This experiment was repeated three times with similar results.

Statistics
For statistical analysis, Student's t test for paired data was used to compare the expression of Pyk2 induced by various stimuli. The results are expressed as the mean ± SD, and p < 0.05 was accepted as significant.

Results
The TCR ligation and PMA increase the expression of Pyk2 in Jurkat T cells and in normal T cells, but increase the expression of Fak only in normal T cells

To examine the effect of T cell stimulation on the expression of Pyk2 and Fak, we incubated Jurkat T cells in suspension with the indicated concentrations of anti-CD3 mAb, anti-CD28 mAb, PMA, or the Ca\(^{2+}\) ionophore A23187 for 16 h at 37°C. Such incubation did not affect the number of the cells, nor did it affect the viability of the cells, as determined by trypan blue exclusion assay (data not shown). After incubation, the cells were washed and immediately lysed. The proteins in WCL were transferred to membranes and blotted with anti-Pyk2 mAb, anti-Fak mAb, or, as a control, anti-Csk Ab (in initial studies we found that the expression of Csk in Jurkat T cells is resistant to the various treatments outlined in the present manuscript). As shown in Fig. 1A, anti-CD3 mAb, anti-CD28 mAb, and PMA increased the level of total cellular Pyk2, but not that of Fak, in a dose-dependent manner. In contrast, the Ca\(^{2+}\) ionophore A23187 failed to increase the level of either PTK (Fig. 1A). Similar results were obtained with the Ca\(^{2+}\) ionophore ionomycin (data not shown). The level of Csk was similar in all lanes, indicating equal loading of cell material (Fig. 1A).

The extent of the increase in the cellular level of Pyk2 varied depending on the stimulus, with PMA inducing the most increase...
in the Pyk2 level. Densitometric and statistical analysis showed that the increases after CD3 ligation, CD28 ligation, and PMA treatment were significant (Fig. 1B).

The T cell response to stimulation by immobilized anti-CD3 mAb has been shown to be stronger than the response to the mAb in suspension, a phenomenon attributed to the exaggerated CD3 ligation by the immobilized Ab. Thus, we examined whether stimulating T cells with immobilized anti-CD3 mAb would further enhance Pyk2 expression and increase Fak expression. Stimulating T cells with immobilized anti-CD3 mAb induced only a modest increase in Pyk2 expression over that induced by stimulating T cells with anti-CD3 mAb in suspension (Fig. 1, C and D). Interestingly, stimulating T cells with immobilized anti-CD3 mAb failed to induce any detectable increase in Fak or Csk expression (Fig. 1E).

Time-course studies showed that the increase in the expression of Pyk2 after T cell activation was apparent within 4 h and reached a maximum by 6–8 h (Fig. 2). No stimulus-induced increase in the level of Fak or Csk was seen at any time point (Fig. 2).

To examine the effect of normal T cell activation on Pyk2 and Fak expression, T cells were purified from the blood of healthy volunteers and were then incubated with immobilized anti-CD3 mAb or PMA for 16 h at 37°C. Stimulating normal T cells with immobilized anti-CD3 mAb or PMA increased Pyk2 level in a dose-dependent manner (Fig. 3, A and B). Interestingly, stimulating T cells with immobilized anti-CD3 mAb failed to induce any detectable increase in Fak or Csk expression (Fig. 3).

Time-course studies showed that the increase in the expression of Pyk2 after T cell activation was apparent within 4 h and reached a maximum by 6–8 h (Fig. 2). No stimulus-induced increase in the level of Fak or Csk was seen at any time point (Fig. 2).

To examine the effect of normal T cell activation on Pyk2 and Fak expression, T cells were purified from the blood of healthy volunteers and were then incubated with immobilized anti-CD3 mAb or PMA for 16 h at 37°C. Stimulating normal T cells with immobilized anti-CD3 mAb or PMA increased Pyk2 level in a dose-dependent manner (Fig. 3, A and B). Interestingly, stimulating T cells with immobilized anti-CD3 mAb induced a slight increase in Pyk2 expression (Fig. 3). However, Fak expression in normal T cells appears to be very low, as Fak detection (using two different commercially available mAb) in immunoblotts prepared from the lysates of TCR- or PMA-activated 2.5 × 10^5 normal T cells required extended exposure of the membranes to x-ray films. Furthermore, we found that Fak expression in normal T cells was markedly lower than that in Jurkat T cells, in accordance with previously published reports (28). This is in contrast to Pyk2, which was readily detected in the lysates from Jurkat T cells and normal T cells.

Because the increase in the expression of Pyk2 was readily detected in both Jurkat T cells and normal T cells, we chose to focus on examining the molecular mechanisms that regulate TCR-induced Pyk2 expression in T cells.

**T cell activation up-regulates Pyk2 gene transcription**

To determine whether T cell activation increases the level of cellular Pyk2 by up-regulating Pyk2 gene expression, we stimulated Jurkat T cells for 8 h at 37°C with anti-CD3 mAb or PMA and then examined the cells for the expression of the mRNA for Pyk2 by the RT-PCR technique. As shown in Fig. 4A, both anti-CD3 mAb and PMA increased the mRNA for Pyk2. As a control, we measured the level of the mRNA for GAPDH. No increase in the level of mRNA for this gene was seen in cells treated with either anti-CD3 mAb or PMA (Fig. 4A). To determine whether the increase in the mRNA for Pyk2 is due to the increase in Pyk2 gene transcription, we pretreated the cells with different concentrations of the transcriptional inhibitor actinomycin D (Act-D) for 5 min, and then stimulated the cells, in the continuous presence of Act-D, with PMA for 8 or 16 h at 37°C. As shown in Fig. 4, B and C, this pretreatment blocked the PMA-induced increase in Pyk2 in a dose-dependent manner, suggesting that the stimulus-induced increase in cellular Pyk2 is due to the increase in Pyk2 gene transcription.

**Inhibitors of PKC block stimulus-induced up-regulation of Pyk2 expression**

T cell activation through CD3 has been shown to activate the serine/threonine kinase PKC (35, 36). Furthermore, the increase in Pyk2 expression after PMA treatment suggests a role for PKC in regulating Pyk2 expression, as PKC is the intracellular receptor for PMA (37). Therefore, we examined the involvement of PKC in regulating Pyk2 expression by investigating the effect of the PKC inhibitors H-7 and Ro-31–8220 on stimulus-induced increase in Pyk2 expression. As shown in Fig. 5, both PKC inhibitors blocked the PMA- and CD3-induced increase in Pyk2 expression in a dose-dependent manner. These blockers had no effect on the viability of
the cells, as determined by trypan blue exclusion assay (data not shown). Blotting the membranes with anti-Csk Ab confirmed that a similar amount of WCL was loaded in each lane (Fig. 5). These results strongly implicate PKC in the signaling pathways that up-regulate Pyk2 expression.

The MAPK MEK is involved in regulating Pyk2 expression in T cells

As described in the introduction, TCR-initiated signals in T cells have been shown to activate the MAPK MEK-ERK cascade, leading to the phosphorylation and activation of transcription factors. Similarly, PMA-activated PKC has been shown to activate the Ras-Raf pathway, which, in turn, links PKC to the MEK-ERK pathway. To gain insight into the pathways that regulate Pyk2 expression in T cells, we examined the effect of the selective MEK blocker PD98059 on stimulus-induced increase in Pyk2 level. As shown in Fig. 6, PD98059 blocked the CD3- and PMA-induced increase in Pyk2 expression in a dose-dependent manner, suggesting that the MEK-ERK cascade is involved in regulating Pyk2 expression. The inhibition of Pyk2 expression by PD98059 correlated with the inhibition of MEK activation by PD98059, as determined by the decrease in the tyrosine phosphorylation of ERK (Fig. 6B).
allografts (38). Both agents form complexes with intracellular proteins, which, in turn, bind calcineurin, leading to the inhibition of its phosphatase activity (39). We examined the effects of CsA and FK506 on Pyk2 expression in T cells by treating the cells for 1 h with CsA and then stimulating the cells, in the continuous presence of CsA or FK506, with anti-CD3 mAb or PMA. As shown in Fig. 7, A and B, CsA and FK506 only slightly (if at all) blocked the CD3- and PMA-induced increase in Pyk2 expression. Yet, these concentrations of CsA and FK506 effectively blocked stimulus-induced IL-2 production (Fig. 7C). Neither CsA nor FK506 affected the viability of the cells, as determined by trypan blue exclusion assay (data not shown). Blotting the membranes with anti-Csk Ab confirmed that a similar amount of WCL was loaded in each lane (Fig. 7). These data strongly suggest that calcineurin is not critical for up-regulating Pyk2 expression in T cells.

**KN-62, a CaMK inhibitor, fails to block stimulus-induced increase in Pyk2 expression**

T cell activation has been shown to stimulate CaMKs, including CaMKII and CaMKIV (8, 9). These kinases are implicated in regulating the activity of several transcription factors and, in turn, in regulating gene expression. To examine the role of CaMKs in stimulus-induced up-regulation of Pyk2 expression, we pretreated the cells with KN-62 and then stimulated the cells with PMA. As shown in Fig. 7D, concentrations as high as 10 μM KN-62 failed to block the PMA-induced increase in Pyk2 expression. These results suggest that CaMKs are not critical for up-regulating Pyk2 expression in T cells.

**Ca2⁺ ionophores block stimulus-induced up-regulation of Pyk2 expression**

Optimal T cell activation and cytokine gene expression require the integration of intracellular signal transduction pathways (40–47). For example, TCR ligation is a weak stimulator of IL-2 production from T cells, whereas TCR-induced IL-2 release is markedly increased if TCR is ligated in the presence of anti-CD28 mAb or PMA. Similarly, treating T cells with PMA or Ca2⁺ ionophores alone is not sufficient to induce optimal T cell activation. Yet, the combination of PMA and Ca2⁺ ionophores strongly initiates gene expression (48). To examine whether Pyk2 expression is also dependent on the integration of various intracellular signals, we examined the effect of T cell costimulation on Pyk2 expression. As shown in Fig. 8A, coligating TCR and CD28 with optimal concentrations of anti-CD3 and anti-CD28 mAb did not appear to significantly increase the expression of Pyk2 above that seen with anti-CD3 mAb alone. Also, combining anti-CD3 mAb or anti-CD28 mAb with PMA did not increase the expression of Pyk2 beyond that achieved by PMA alone (Fig. 8A). Strikingly, however, the Ca2⁺ ionophore A23187 completely blocked PMA-, CD28-, and CD3-triggered up-regulation of Pyk2 expression (Fig. 8A). This compound was very effective in blocking stimulus-induced up-regulation of Pyk2 expression, as 1 μM markedly blocked Pyk2 expression (Fig. 8, B and C). The vehicle DMSO at a concentration of 1% (20 times more than that found in the 1 μM ionophore A23187 solution) did not block stimulus-induced up-regulation of Pyk2 expression (data not shown). These results were surprising, as the combination of Ca2⁺ ionophores and PMA has been shown to increase the gene expression of several cytokines. Thus, we examined whether the combination of Ca2⁺ ionophores and PMA induces IL-2 expression under the same conditions that lead to the inhibition of Pyk2 expression. As previously reported, Ca2⁺ ionophore A23187 treatment of T cells did not by itself induce IL-2 production (data not shown). However, the Ca2⁺ ionophore A23187 in combination with PMA induced IL-2 release in a dose-dependent manner (Fig. 8B, graph). As shown in Fig. 8B, there was an inverse relationship between the IL-2 release and the increase in Pyk2 level, suggesting that the Ca2⁺ influx has contrasting effects on the expression of IL-2 and Pyk2 in T cells. Similar results were obtained with the Ca2⁺ ionophore ionomycin (Fig. 8D). Notably, the Ca2⁺ ionophore A23187 also completely blocked stimulus-induced up-regulation of Pyk2 expression in normal T cells (Fig. 8E).

To examine whether the Ca2⁺ ionophore A23187 blocks the increase in cellular Pyk2 by inhibiting stimulus-induced Pyk2 gene expression, cells were treated for 8 h at 37°C with PMA with or without the Ca2⁺ ionophore A23187 and then examined for the
level of Pyk2 mRNA. As shown in Fig. 9A, the Ca^{2+} ionophore A23187 blocked the PMA-induced increase in the mRNA for Pyk2, but did not affect the mRNA for GAPDH. Notably, the Pyk2 level in cells pretreated for 16 h with PMA did not decrease when the cells were subsequently incubated for up to 8 h with the Ca^{2+} ionophore A23187, suggesting that Ca^{2+} ionophore-triggered signals do not down-regulate Pyk2 post-translationally (Fig. 9B, compare lanes 2 and 5). Thus, Ca^{2+} ionophores appear to trigger signals that interfere with the transcription of the gene for Pyk2 and/or with the stability of Pyk2 mRNA. This issue is subject to further investigation.

Cyclosporin A and FK506, but not KN-62, block Ca^{2+} ionophore-mediated inhibition of activation-induced up-regulation of Pyk2

Ca^{2+} ionophores induce a sustained Ca^{2+} influx in T cells, leading to the sustained activation of various enzymes, such as CaMKs and calcineurin (1, 2). To examine whether CaMKs and/or calcineurin are involved in the inhibition of Pyk2 expression by the Ca^{2+} ionophore A23187, T cells were treated with PMA and the Ca^{2+} ionophore A23187 in the presence or the absence of KN-62, CsA, or FK506. As shown in Fig. 10A, KN-62 at concentrations as high as 10 μM failed to block Ca^{2+} ionophore A23187-induced inhibition of Pyk2 expression. In contrast, both CsA and FK506 partially blocked Ca^{2+} ionophore A23187-induced inhibition of Pyk2 expression in a dose-dependent manner (Fig. 10B and C). These results suggest that calcineurin, but not CaMK, activation negatively regulates Pyk2 expression in T cells.

The newly expressed Pyk2 becomes involved in TCR and CD28 signaling

To examine whether the overexpressed Pyk2 becomes involved in receptor signaling, we performed the following studies. In the first set of experiments, T cells were stimulated with anti-CD3 mAb for the indicated time at 37°C. After cell solubilization, Pyk2 was immunoprecipitated and analyzed by immunoblotting with anti-

FIGURE 8. Ca^{2+} ionophores completely block activation-induced up-regulation of Pyk2 expression. A, Jurkat T cells were incubated with 3 μg/ml of anti-CD3 mAb (α-CD3), 1 μg/ml of anti-CD28 mAb (α-CD28), 1 ng/ml PMA, 1 μM Ca^{2+} ionophore A23187 (Iono), or combinations of these stimuli for 16 h at 37°C. After incubation, the cells were washed and immediately lysed with boiling SDS-PAGE sample buffer. Proteins in WCL were then transferred to membranes and immunoblotted with anti-Pyk2 mAb. This experiment was repeated three times with similar results. B, Jurkat T cells were incubated with PMA in the presence of the indicated concentrations of the Ca^{2+} ionophore A23187 for 16 h at 37°C. C, Jurkat T cells were incubated with 3 μg/ml of anti-CD3 mAb (α-CD3) in the presence of the indicated concentrations of the Ca^{2+} ionophore A23187 for 16 h at 37°C. D, Jurkat T cells were incubated with 1 ng/ml of PMA in the presence of the indicated concentrations of the Ca^{2+} ionophore ionomycin for 16 h at 37°C. B–D, After incubation, the tubes were centrifuged, the supernatants were collected and analyzed for IL-2 (B–D; graphs) as described in Materials and Methods, and the cells were washed and immediately lysed with boiling SDS-PAGE sample buffer. Proteins in WCL were then transferred to membranes and immunoblotted with anti-Pyk2 mAb or anti-Csk Ab. This experiment was repeated three times with similar results. E, The Ca^{2+} ionophore A23187 blocks stimulus-induced up-regulation of Pyk2 expression in normal T cells. T cells purified from the blood of healthy volunteer as described in Materials and Methods were incubated in RPMI-5% FCS (lane 1), RPMI-5% FCS containing 1 ng/ml PMA (lane 2), or RPMI-5% FCS containing the combinations of 1 ng/ml of PMA and the indicated concentration of Ca^{2+} ionophore A23187 (lanes 3–5) for 16 h at 37°C. After incubation, the cells were washed and lysed with boiling SDS-PAGE sample buffer. Proteins in WCL were then transferred to membranes and immunoblotted with anti-Pyk2 mAb or anti-Csk Ab. This experiment was repeated twice with similar results.
phosphotyrosine mAb. As shown in Fig. 11A, more tyrosine-phosphorylated Pyk2 was precipitated from cells activated with anti-CD3 mAb for 16 h than from cells activated for 8 or 4 h. In the second set of experiments, T cells were stimulated for 16 h with PMA at 37°C. After incubation, the cells were washed and stimulated for 5 min at 37°C with anti-CD3 mAb or anti-CD28 mAb. After cell solubilization, Pyk2 was immunoprecipitated and analyzed by blotting with anti-phosphotyrosine mAb. As shown in Fig. 11B, the basal level of tyrosine phosphorylation of Pyk2 was substantially increased in cells pretreated with PMA (compare lanes 1 and 4). Furthermore, stimulating PMA-treated T cells with anti-CD3 mAb or anti-CD28 mAb increased Pyk2 tyrosine phosphorylation, indicating that at least some of the newly expressed Pyk2 becomes involved in TCR and CD28 signaling. Interestingly, PMA has been shown to modulate TCR and CD28 function; thus, it is tempting to speculate that the substantial increase in cellular Pyk2 and in its tyrosine phosphorylation in PMA-treated cells may play a role in the effects of PMA on TCR and CD28 function.

Discussion

An important step toward understanding the role of signaling molecules in receptor function involves determining whether receptor ligation modulates the expression of such molecules. In this report we showed that TCR ligation induces intracellular signals that increase the expression of the focal adhesion PTK Pyk2 in Jurkat T cells and human normal T cells. We also showed that Pyk2 expression in T cells involves the activation of the PKC-ERK cascade and is negatively regulated by the sustained increase in intracellular Ca²⁺.

The TCR ligation stimulates PTKs that lead to the activation of phospholipase C followed by the metabolism of inositol lipids to diacylglycerol and inositol triphosphate, triggering both the activation of PKC and the increase in intracellular Ca²⁺ (1, 2). A number of studies have shown that the activation of cellular PKC in T cells induces the expression of several immediate response genes, including c-fos and Elk-1 (43, 49). The specific signaling pathways involved in this process are not known with certainty. Activated PKC has been shown to stimulate the small GTPase Ras (50), which, in turn, activates the serine/threonine kinase Raf (3, 4, 51). Activated Raf phosphorylates and activates the MAPK MEK, which, in turn, phosphorylates and activates the MAPK ERK (4). Phosphorylated ERK translocates to the nucleus, where it phosphorylates and activates transcription factors such as c-Fos and Elk-1, leading to the initiation of gene transcription (3, 4). Our results implicate PKC in the signaling pathways that up-regulate Pyk2 expression in T cells: 1) PMA, a potent activator of PKC, strongly up-regulated Pyk2 expression; and 2) the PKC inhibitors H-7 and Ro-31-8220 blocked the CD3- and PMA-induced increases in Pyk2 expression. The inhibition of the CD3-induced increase in Pyk2 expression by the specific MEK inhibitor PD98059 strongly suggests that PKC is linking CD3-initiated signals to the MEK-ERK cascade to up-regulate Pyk2 expression. Because in our studies PD98059 did not completely block TCR-induced up-regulation of Pyk2 expression, we cannot at this time rule out the involvement of signaling pathways other than the PKC-MEK-ERK pathway in up-regulating Pyk2 expression.

Experimental evidence suggests that the increase in intracellular Ca²⁺ following TCR ligation (52) leads to the activation of calmodulin; binding of calmodulin to regions on CaMKs and calcineurins displaces the autoinhibitory domains of the enzymes and exposes their active sites (5, 12, 53). CaMKs and calcineurins control gene expression by regulating the activation of various transcription regulators (5, 12, 53). Our data, however, strongly suggest that the Ca²⁺/calmodulin-CaMK and Ca²⁺/calmodulin-

![FIGURE 9](https://www.jimmunol.org/)

![FIGURE 10](https://www.jimmunol.org/)
calcineurin cascades are not critical for TCR-induced up-regulation of Pyk2 expression: 1) Ca\(^{2+}\) ionophores failed to increase Pyk2 expression; 2) both CsA and FK506, at concentrations well above those that completely inhibited IL-2 production, failed to block CD3- and PMA-induced up-regulation of Pyk2 expression; 3) KN-62 failed to block stimulus-induced increase in Pyk2 expression; and 4) PMA, which does not increase intracellular Ca\(^{2+}\) and is not an activator of CaMKs or calcineurin, strongly up-regulated Pyk2 expression. Our findings that PMA by itself can up-regulate Pyk2 expression and that PKC and MEK inhibitors block CD3-induced up-regulation of Pyk2 indicate that the activation of the PKC-ERK cascade is sufficient to link CD3-initiated signals to Pyk2 expression in T cells.

The inhibition of CD3- and PMA-induced up-regulation of Pyk2 expression by Ca\(^{2+}\) ionophore is intriguing, as we are not aware of Ca\(^{2+}\) ionophores inhibiting the expression of other signaling PTKs. Interestingly, Ca\(^{2+}\) ionophore-mediated inhibition of Pyk2 expression was apparent at concentrations that synergized with PMA in inducing IL-2 expression; thus, Ca\(^{2+}\) ionophore-triggered signals appear to have opposing effects on IL-2 and Pyk2 expressions. Our results implicate calcineurin as having a role in Ca\(^{2+}\) ionophore-mediated inhibition of CD3- and PMA-induced up-regulation of Pyk2 expression, as CsA and FK506 partially blocked the effects of Ca\(^{2+}\) ionophore on Pyk2 expression. The fact that two structurally different compounds (CsA and FK506) blocked the effects of Ca\(^{2+}\) ionophore suggests that this inhibition is not due to nonspecific effects of the drugs. Calcineurin has been shown to up-regulate the transcriptional activity of several transcription factors. Among these transcription factors, calcineurin has been shown to dephosphorylate c-NF-AT, allowing it to translocate into the nucleus to initiate gene transcription of several cytokines. Calcineurin has also been shown to down-regulate gene transcription and expression. For example, calcineurin has been implicated in down-regulating the expression of TGF-\(\beta\) in human T cells (54); of c-Myb, Egr-1, and c-Fos in the murine erythroleukemia cell line ELM-1-1 (55), and of c-fos in a T cell lymphoma cell line (56) and in MCT, a murine renal cell line (57). However, the mechanisms by which calcineurin down-regulated protein expression in these studies are not clear. Notably, in contrast to the up-regulation of NF-AT activity as a result of dephosphorylation by calcineurin, recent studies have shown that calcineurin-induced dephosphorylation of the transcription factor Elk-1 down-regulates the transcriptional activity of Elk-1 (13). Elk-1 has been shown to be phosphorylated by PMA through the MEK-ERK pathway and to associate with ERK through the so-called D domain of Elk-1 (58). Notably, treating COS cells with CsA significantly enhanced epidermal growth factor-induced Elk-1 phosphorylation, whereas ionomycin inhibited the Elk-1 phosphorylation (13). Thus, Elk-1 is potentially a transcription factor that could play a role in Pyk2 gene transcription in T cells. Calcineurin has also been shown to associate with cellular proteins such as protein kinase A anchoring protein (59) and the inositol triphosphate (IP-3) and ryanodine receptors (60) and to induce the dephosphorylation of the Bcl-2 family protein BAD (61). Thus, further studies are necessary to identify the mechanisms by which calcineurin down-regulates Pyk2 expression.

As described above, the calcineurin inhibitors CsA and FK506 only partially blocked Ca\(^{2+}\) ionophore-mediated down-regulation of Pyk2 expression. Thus, mechanisms initiated by Ca\(^{2+}\) ionophores other than calcineurin activation are also important for down-regulating Pyk2 expression. Our data suggest that the Ca\(^{2+}\)/calmodulin-activated CaMKII and CaMKIV are not involved in the Ca\(^{2+}\) ionophore’s effect, as the CaMK inhibitor KN-62 did not appear to block Ca\(^{2+}\) ionophore-mediated inhibition of the stimulus-induced up-regulation of Pyk2 expression. Ca\(^{2+}\) ionophores mobilize Ca\(^{2+}\) from intracellular stores and induce Ca\(^{2+}\) influx, leading to a prolonged and sustained increase in intracellular Ca\(^{2+}\). The fact that two structurally distinct Ca\(^{2+}\) ionophore compounds (A23187 and ionomycin) blocked Pyk2 expression suggests that this inhibition is most likely the result of the increase in intracellular Ca\(^{2+}\) and is not due to nonspecific effects of the drugs. Although we do not know why TCR ligation, which also increases intracellular Ca\(^{2+}\), increased Pyk2 expression, we can speculate that down-regulating Pyk2 expression may require sustained activation of calcineurin and other enzymes, a process that might be achieved with the prolonged and sustained increase in Ca\(^{2+}\) by Ca\(^{2+}\) ionophore, but not by TCR ligation, as the TCR-induced increase in Ca\(^{2+}\) appears to subside with time (7, 10). There is now experimental evidence that the magnitude and the duration of the Ca\(^{2+}\) signal can elicit differential gene transcription and distinct cell responses. For example, studies with T cell clones showed that only the sustained influx of extracellular Ca\(^{2+}\) was required for Fas ligand induction and killing; yet both the release of Ca\(^{2+}\) from intracellular stores and the sustained influx of extracellular Ca\(^{2+}\) were required for perforin/granule exocytosis (47). Similarly, a transient increase in intracellular Ca\(^{2+}\) after TCR ligation in J.CaM1, a mutant of Jurkat T cells, failed to induce IL-2 production (62). Remarkably, in B cells the early rise in Ca\(^{2+}\) was sufficient to provoke prolonged activation of the transcription regulators c-Jun terminal kinase and NF-kB, whereas prolonged...
activation and nuclear localization of NF-AT required sustained increase in intracellular Ca\(^{2+}\) (63). A similar requirement for sustained Ca\(^{2+}\) for prolonged NF-AT nuclear localization was evident in cells other than B cells (12). Thus, additional studies are required to further clarify the mechanisms by which Ca\(^{2+}\)-ionophores down-regulate stimulus-induced up-regulation of Pyk2 expression.

Interestingly, although the expression of the other member of the focal adhesion PTK family Fak increased, albeit at a low level, after TCR ligation in normal T cells, its expression did not increase following receptor ligation in Jurkat T cells. Because the Jurkat T cells are transformed cells, it is plausible that a defect/alteration in TCR signaling in Jurkat T cells led in one way or another to the blockage of the signaling pathways that regulate Fak expression. However, because such a defect/alteration affects Fak expression but not Pyk2 expression, it is likely that at least some of the signaling pathways that lead to Fak and Pyk2 expression in T cells are different. This issue requires further investigation.

Acknowledgments

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10. Nghiem, P., T. Ollick, P. Gardner, and H. Schulman. 1994. Interleukin-2 transduction in cells other than B cells (12). Thus, additional studies are required to further clarify the mechanisms by which Ca\(^{2+}\)-ionophores down-regulate stimulus-induced up-regulation of Pyk2 expression.

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