Redox Regulation of the Calcium/Calmodulin-dependent Protein Kinases*

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Reactive oxygen intermediates (ROI) have been viewed traditionally as damaging to the cell. However, a predominance of evidence has shown that ROI can also function as important activators of key cellular processes, and ROI have been shown to play a vital role in cell signaling networks. The calcium/calmodulin-dependent protein kinases (CaM kinases) are a family of related kinases that are activated in response to increased intracellular calcium concentrations. In this report we demonstrate that hydrogen peroxide treatment results in the activation of both CaM kinase II and IV in Jurkat T lymphocytes. Surprisingly, this activation occurs in the absence of any detectable calcium flux, suggesting a novel means for the activation of these kinases. Treatment of Jurkat cells with phorbol 12-myristate 13-acetate (PMA), which does not cause a calcium flux, also activated the CaM kinases. The addition of catalase to the cultures inhibited PMA-induced activation of the CaM kinases, suggesting that similar to hydrogen peroxide, PMA also activates the CaM kinases via the production of ROI. One mechanism by which this likely occurs is through oxidation and consequent inactivation of cellular phosphatases. In support of this concept, okadaic acid and microcystin-LR, which are inhibitors of protein phosphatase 2A (PP2A), induced CaM kinase II and IV activity in these cells. Overall, these results demonstrate a novel mechanism by which ROI can induce CaM kinase activation in T lymphocytes.

The CaM kinases1 are a family of related kinases activated in response to increased calcium levels (for review, see Refs. 1–3). The primary members of this family that demonstrate broad substrate specificity are CaM kinase I, II, and IV. Other family members are dedicated to the phosphorylation of a specific substrate (i.e. myosin light chain kinase, phosphorylase kinase, and elongation factor 2 kinase/CaM kinase III). All members of the CaM kinase family are activated in response to the binding of calcium/calmodulin, which causes a conformational change revealing the substrate binding domain of the kinase (1). However, there are differences in the way each kinase is regulated.

CaM-KII is a multimeric enzyme composed of 10–12 catalytic subunits (4–6). Upon calcium/calmodulin binding, CaM-KII undergoes autophosphorylation in a region of the protein that prevents the autoinhibitory domain from interacting with the kinase domain. Once phosphorylated in this manner, CaM-KII activity becomes independent of calcium (7–9). To return to an inactive state, dephosphorylation of CaM-KII must occur; both protein phosphatases 1 and 2A (PP1 and PP2A) appear to play important physiological roles in the dephosphorylation of CaM-KII (10).

CaM-KIV is phosphorylated by the kinase CaM-KK (11–13). CaM-KK is activated by calcium/calmodulin complexes as well, but the potential for other means of regulation has also been suggested (13–15). Phosphorylation of CaM-KIV by CaM-KK occurs in the pseudosubstrate domain that interacts with the catalytic domain of the kinase. The phosphatase PP2A has been shown to form a complex with CaM-KIV and at least in vitro can cleave the phosphate group off CaM-KIV (16, 17). This interaction is also believed to be important in vivo because transfection of cells with the PP2A inhibitor, SV40 small T antigen, is able to potentiate CaM-KIV induction of cAMP-response element-binding protein (CREB)-dependent transcription (17). Specific cell-permeable inhibitors of the serine phosphatases such as okadaic acid and microcystin-LR can inhibit both PP2A and PPI through binding to the catalytic domain (18, 19). Additionally, hydrogen peroxide has also been shown to inhibit these phosphatases; this is believed to occur via the oxidation of a reactive cysteine residue in the catalytic domain of these phosphatases (20, 21). Inhibition of this phosphatase could conceivably result in the activation of the CaM kinases in the absence of an apparent increase in intracellular calcium.

The CaM kinases have been implicated recently in T cell receptor-, hydrogen peroxide-, and PMA-induced IκB kinase activation and IκB phosphorylation in Jurkat T lymphocytes. Treatment of T lymphocytes with hydrogen peroxide induces calcium fluxes in these cells similar to those caused by antibodies to the T cell receptor (22). PMA treatment of T lymphocytes is not reported to induce a calcium flux. Surprisingly, PMA-induced NF-κB activation is reported to be sensitive to calmodulin antagonists and CaM kinase inhibitors (23, 24). In
addition, we have reported previously that hydrogen peroxide-induced IκB phosphorylation was not blocked by incubation of the cells with BAPTA and EGTA, although CaM kinase inhibitors did block this response (22). These results suggest that calcium-independent activation of the CaM kinases is occurring. In this report we demonstrate the activation of both CaM-KII and CaM-KIV in the absence of increases in intracellular calcium. This activation is induced by ROI as well as by PMA. Additionally, we report that phosphatase inactivation can also lead to the activation of the CaM kinases. We postulate that both ROI and PMA can induce CaM kinase activation through the inactivation of phosphatases by oxidation.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—The human Jurkat T cell line was obtained from ATCC (Manassas, VA) and cultured in RPMI 1640 with 5% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Ionomycin, PMA, and microcystin-LR were purchased from Calbiochem (San Diego, CA) and dissolved in Me2SO. Protein kinase C inhibitor peptide (A.A.19–31), protein A/G beads, and okadaic acid were also purchased from Calbiochem. Mouse immunoglobulin (IgG1), syntide-2, and protein kinase inhibitor peptides were purchased from Sigma. Anti-CaM kinase II, anti-CaM kinase IV, anti-phosphoThr-308 Akt, and anti-PP2A antibodies were purchased from Cell Signaling Technology (Beverly, MA). Fluoro-3 was purchased from Molecular Probes (Eugene, OR) and dissolved in Me2SO.

**Sample Preparation for Kinase Assay**—Cells were washed in phosphate-buffered saline and resuspended in serum-free RPMI 1640. Fifteen ml containing 1.25 × 10⁶ cells/ml were added to 50 ml of cold 1.25 mM CaCl₂ and placed at 37 °C for at least 1 h prior to stimulation. EGTA (2 mM) was added to the cells 30 min prior to stimulation to produce calcium-free conditions. Cells were stimulated with hydrogen peroxide (10 mM), ionomycin (500 nM), or PMA (100 nM) for the time period indicated. Cells were treated with microcystin-LR (500 nM) or okadaic acid (1 μM) for 15 min. In the experiments employing catalase (10,000 units), it was added for 10 min prior to stimulation. Following treatments, cells were centrifuged, and the supernatant was removed. The cell pellets were resuspended in 300 μl of cold lysis buffer (50 mM HEPES, pH 7.5, 2 mM EDTA, 2 mM EGTA, 2 mM benzamidine, 10 mM NaPO₄, 50 mM NaFl, 5 mM dithiothreitol, 0.5% IGEPAL, 1 mM Na₃VO₄, 0.5 mM phenylmethysulfonyl fluoride, and 10 μg/ml each of the following: soybean trypsin inhibitor, aprotinin, antipain, leupeptin, pepstatin A, and N-p-tosyl-L-lysine chloromethyl ketone), transferred to microcentrifuge tubes, and placed on ice for 20 min. Lysates were centrifuged for 15 min at 14,000 rpm in a refrigerated Eppendorf microcentrifuge. The supernatant was transferred to a new microcentrifuge tube and frozen until needed.

**Immunoprecipitation**—To preclarify the lysates, 25 μl of protein A/G bead suspension was added to 300 μl of the supernatant from above, incubated with rocking for 2 h at 4 °C, and spun, and the supernatant was placed in a fresh tube for further analysis. Either the anti-CaM kinase IV or II antibody (2.5 μg) was added to the preclarified lysates, which were then placed on a rocker for 2 h at 4 °C. Protein A/G beads (25 μl) were then added to the lystate antibody mixture and rocked for an additional 1 h at 4 °C. Samples were centrifuged at 14,000 rpm, and the beads were washed twice in the lysis buffer. The beads were then resuspended in 40 μl of CaM kinase dilution buffer (50 mM HEPES, pH 7.5, 1 mg/ml bovine serum albumin, 10% ethylene glycol). A portion of the immunoprecipitate was used to ensure equal amounts of the kinase were immunoprecipitated. To do this, 5 μl were combined with 2 μl of 3.3× sample buffer (200 mM Tris-HCl, pH 6.8, 33% glycerol, 6.6% SDS, 18.6% 2-mercaptoethanol, 0.04% bromphenol blue), boiled for 5 min.

**FIG. 1. The CaM kinases are activated by hydrogen peroxide.** Jurkat cells were washed and resuspended in RPMI 1640 at a concentration of 1.25 × 10⁶ cells/ml. For each treatment, 15 ml of cells were warmed to 37 °C. As indicated, cells were pretreated with 2 mM EGTA for 30 min. Cells were then treated with either H₂O₂ (10 mM) or ionomycin (Iono) (500 nM) for 7 min. The bars labeled Control represent untreated cells. Cells were immediately centrifuged, and cells were lysed. Lysates were used to immunoprecipitate CaM kinase II (A) or CaM kinase IV (B). Immunoprecipitates were used in a kinase assay in the absence of calcium to measure the phosphorylation of the synthetic peptide, syntide-2 (A and B). In C and D calcium and calmodulin were added to the kinase assays to measure total kinase activity obtainable in the samples. Counts were measured and are shown as the average of two separate assays. Data are expressed as cpm (CPM) ± S.D. cpm values shown are the average of two separate assays. In E, CaM-KII or CaM-KIV was immunoprecipitated following stimulation as indicated above. These immunoprecipitates were then immunoblotted with the immunoprecipitating antibody. Binding of the primary antibody was detected using an AP-labeled goat anti-mouse immunoglobulin and an AP-BCIP/NBT developing system.
and frozen until utilized in immunoblot analysis. The remaining immunoprecipitate was used in a kinase assay immediately. In some cases immunoprecipitates were performed using 2.5 μg H9262 of mouse immunoglobulin to ensure the specificity of immunoprecipitations.

**Kinase Assay**—Kinase assays were performed as described previously by Park et al. (16). Five μl of the immunoprecipitates were assayed for either CaM kinase II or IV activity in 25 μl of a reaction mixture containing 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 2 mM dithiothreitol, 5 μM protein kinase inhibitor, 2 μM protein kinase C inhibitor peptide, 1 μM microcystin-LR, 200 μM ATP (4 μCi of [γ-32P]ATP), and 40 μM peptide substrate syntide-2. Total kinase activity was determined using 800 μM Ca2+ and 1 μM calmodulin. Autonomous activity was determined in the presence of 5 mM EGTA. Duplicates were performed for each reaction, as well as kinase reactions lacking the syntide-2 peptide to account for autoprophosphorylation (background activity). The background activity was subtracted from the samples containing syntide-2 peptide to determine the kinase activity. The reactions were carried out for 12 min at 30 °C and terminated by the addition of 5 μl of 200 mM EDTA. Twenty-five μl of the kinase reaction mix were spotted on Whatman P81 filter paper. The filter papers were washed three times in 0.5% phosphoric acid, air-dried, and counted on a liquid scintillation counter. In one series of experiments hydrogen peroxide (10 mM) was added directly to the CaM-KII or CaM-KIV immunoprecipitates to determine whether the effects of hydrogen peroxide were mediated directly on the CaM kinases.

**Measurement of Intracellular Calcium**—Intracellular calcium was measured by loading the cells with Fluo-3 as described previously (22). Briefly, cells were washed in phosphate-buffered saline and resuspended at 5 x 10^6/ml in RPMI containing 5% serum and 10 μM Fluo-3. Cells were incubated at room temperature for 30 min. Cells were then washed and resuspended at 1 x 10^6 cells/ml in RPMI 1640 (serum- and phenol red-free) in a black opaque 96-well plate. Cells were stimulated as indicated in the figures, and the fluorescence was monitored over time on a Bio-Tek FL600 fluorescence/absorbance spectrophotometer (Winooski, VT). The wavelength for excitation was 485 nm, and emission was measured at 530 nm.

**FIG. 2.** PMA does not produce a detectable calcium flux in Jurkat cells. Jurkat cells were washed and resuspended in RPMI 1640 and loaded with Fluo-3 (10 μM) for 30 min at room temperature. Cells were washed and resuspended in phenol red-free RPMI 1640 in a black opaque 96-well plate and treated with PMA (10 nM) or hydrogen peroxide (1 mM). Fluo-3 fluorescence was measured using a Bio-Tek fluorescent microtiter plate reader (excitation, 495 nm, and emission, 520 nm) over the indicated time period (in s).

**FIG. 3.** PMA activates the CaM kinases by a redox-regulated mechanism in the absence of a calcium flux. Jurkat cells were washed and resuspended in RPMI 1640 at a concentration of 1.25 x 10^6 cells/ml. For each treatment 15 ml of cells were warmed to 37 °C. As indicated, cells were pretreated with either 2 mM EGTA for 30 min (A and B) or 10,000 units of catalase (cat) for 10 min (C and D). Cells were then treated with either PMA (10 nM) or ionomycin (Iono) (500 nM) for 7 min. CaM kinase II (A and C) or CaM kinase IV (B and D) was then immunoprecipitated from cell lysates and used in a kinase assay containing EGTA to measure the autonomous phosphorylation of the synthetic peptide, syntide-2. The cpm of 32P incorporated into the peptide was measured on a scintillation counter. Data are expressed as cpm (CPM) ± S.D. cpm values shown are the average of two separate assays.
Sample Preparation for Immunoblot Analysis—Cells were washed and resuspended in serum-free RPMI 1640. One ml containing 1.25 × 10^6 cells was added to microcentrifuge tubes and warmed at 37 °C for at least 1 h prior to the start of the experiment. Cells were stimulated with indicated treatments. The tubes were then centrifuged in a microcentrifuge for 30 s, and the supernatants were removed. Cell pellets were then resuspended in 110 μl of cold lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 2% IGEPAL, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μM leupeptin, 0.5 mM Na3VO4) and placed on ice for 15 min with intermittent shaking. Lysates were then centrifuged for 10 min at 14,000 rpm in a refrigerated Eppendorf microcentrifuge, and the supernatants (98 μl) were removed and mixed with 42 μl of 3.3× sample buffer. Samples were boiled (5 min) and frozen (−20 °C) until used in the immunoblot analysis.

Immunoblot Analysis—Samples were electrophoresed through 10% SDS-PAGE gels prior to electrophoretically transferring proteins to polyvinylidene fluoride membranes. Membranes were then incubated with TBST and developed with the colorogenic substrates BCIP and NBT (AP-conjugated goat anti-mouse immunoglobulin (Promega, 1:10,000 dilution). Membranes were incubated with alkaline phosphatase (AP)-conjugated streptavidin diluted in blocking buffer for 1 h at room temperature. The blots were washed twice in TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.025% Tween 20). Membranes were blocked overnight at 4 °C in blocking buffer (25 mM Tris-HCl, pH 8.0, 1% bovine serum albumin, 0.1% sodium azide). Following blocking, the membranes were incubated for 2 h with the primary antibody diluted in blocking buffer (anti-CaM kinase II, 1:1000; anti-CaM kinase IV, 1:2000; anti-Akt, 1:1000; antiphospho-Akt (Thr-308), 1:1000; and anti-PP2A, 1:1000). Membranes were then washed in TBST (25 mM Tris-HCl, pH 8.0, 125 mM NaCl, 0.025% Tween 20). Membranes were incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse immunoglobulin (Promega, 1:10,000 in TBST) for 1 h at room temperature. The blots were washed twice in TBST and developed with the colorogenic substrates BCIP and NBT (Promega proteoblot AP system).

Phosphatase Assay—Cells were washed in phosphate-buffered saline and resuspended in serum-free RPMI 1640. Fifteen ml containing 1.25 × 10^6 cells/ml were added to 15-ml conical tubes and placed at 37 °C for at least 1 h prior to stimulation. Cells were treated with hydrogen peroxide (1 μM) or mcostatin-LR (4 μM) for 15 min. Cells were lysed in 300 μl of cold lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 1% IGEPAL, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μM leupeptin, 0.5 mM Na3VO4). Phosphatase activity was determined using the serine/threonine phosphatase assay system from Promega (Madison, WI). This system measures the amount of free phosphate generated in a reaction by measuring the absorbance of a molybdate-malachite green-phosphate complex (25, 26). Briefly, free phosphate was removed from the samples (250 μl) using a Sephadex® G-25 spin column (600 μl) were removed and mixed with 42 μl of 3.3× sample buffer. Samples were boiled (5 min) and frozen (−20 °C) until used in the immunoblot analysis.

Thiol Labeling—Reduced thiols groups were labeled using Nε-(3-maleimidylpropionyl)biocytin (MPB) (27, 28). Cells were washed in phosphate-buffered saline and resuspended in serum-free RPMI 1640. Fifteen ml containing 1.25 × 10^6 cells/ml were added to 15-ml conical tubes and placed at 37 °C for at least 1 h prior to stimulation. Cells were treated with hydrogen peroxide (1 μM) or PMA (100 nM) for 15 min. Cells were lysed in 300 μl of cold lysis buffer (25 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5% sodium deoxycholate, 1% IGEPAL, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μM leupeptin, 0.5 mM Na3VO4). Phosphatase activity was determined using the serine/threonine phosphatase assay system from Promega (Madison, WI). This system measures the amount of free phosphate generated in a reaction by measuring the absorbance of a molybdate-malachite green-phosphate complex (25, 26). Briefly, free phosphate was removed from the samples (250 μl) using a Sephadex® G-25 spin column (600 μl) were removed and mixed with 42 μl of 3.3× sample buffer. Samples were boiled (5 min) and frozen (−20 °C) until used in the immunoblot analysis.

RESULTS

We and others have suggested the possibility for the activation of the CaM kinases in the absence of a calcium flux (22, 23). To test this possibility, we stimulated Jurkat cells in the presence and absence of 2 mM EGTA. We have shown previously that this amount of EGTA is sufficient to block any calcium flux into the cells and also strips calcium from the cells, preventing the release of internal calcium stores (22). We then measured the ability of immunoprecipitates of CaM kinase II and IV from these cells to phosphorylate the CaM kinase-specific substrate syntide-2. This kinase assay was performed in the presence of calcium and calmodulin to measure total activity but also in the presence of EGTA to measure autonomous activity. The presence of EGTA in the cultures prior to stimulation elucidates the role of calcium in the activation process of the kinases. The presence of EGTA in the kinase reaction determines the autonomous activity of the kinase; the kinase has already been activated and is not free of any calcium requirement. We found that CaM-KII and CaM-KIV activity was increased following stimulation with hydrogen peroxide both in the presence and absence of EGTA during stimulation (Fig. 1, A and B). These increases in CaM kinase activity were similar to the levels induced by ionomycin, a calcium ionophore. However, unlike hydrogen peroxide-induced activity, ionomycin-induced CaM kinase activity was blocked by the addition of EGTA to the cultures. Thus, hydrogen peroxide could induce CaM kinase activity both in the presence or absence of a calcium flux, whereas ionomycin only induced CaM kinase activity in the presence of a calcium flux (Fig. 1, A and

FIG. 4. Glucose oxidase activates the CaM kinases. Jurkat cells were washed and resuspended in RPMI 1640 at a concentration of 1.25 × 10^6 cells/ml. For each treatment 15 ml of cells were warmed to 37 °C. Cells were treated with glucose oxidase (Glucose Ox.) (10 units/ml) or ionomycin (500 nM) for 7 min. CaM kinase II (A) or CaM kinase IV (B) was then immunoprecipitated from cell lysates and used in a kinase assay containing EGTA to measure the autonomous phosphorylation of the synthetic peptide, syntide-2. The cpm of 32P incorporated into the peptide was measured on a scintillation counter. Data are expressed as cpm (CPM) ± S.D. cpm values shown are the average of two separate assays.

Specific substrate syntide-2. This kinase assay was performed in the presence of calcium and calmodulin to measure total activity but also in the presence of EGTA to measure autonomous activity. The presence of EGTA in the cultures prior to stimulation elucidates the role of calcium in the activation process of the kinases. The presence of EGTA in the kinase reaction determines the autonomous activity of the kinase; the kinase has already been activated and is not free of any calcium requirement. We found that CaM-KII and CaM-KIV activity was increased following stimulation with hydrogen peroxide both in the presence and absence of EGTA during stimulation (Fig. 1, A and B). These increases in CaM kinase activity were similar to the levels induced by ionomycin, a calcium ionophore. However, unlike hydrogen peroxide-induced activity, ionomycin-induced CaM kinase activity was blocked by the addition of EGTA to the cultures. Thus, hydrogen peroxide could induce CaM kinase activity both in the presence or absence of a calcium flux, whereas ionomycin only induced CaM kinase activity in the presence of a calcium flux (Fig. 1, A and
We also examined the CaM kinase activity in these samples (Fig. 1, C and D) following the addition of calcium and calmodulin to the kinase reactions. The levels of activity of both CaM kinase II and CaM kinase IV were increased by the addition of exogenous Ca\textsuperscript{2+} and calmodulin confirming that the kinase activity found in the immunoprecipitates can be attributed to the CaM kinases. The addition of Ca\textsuperscript{2+}/CaM leads to the full activation of CaM kinase II because of autophosphorylation (8, 29). As can be seen in Fig. 1C the level of activity does not change with treatment. These data indicate two things; 1) equal amounts of CaM-KII are present in the immunoprecipitates, and 2) the activity is because of CaM-KII. If the activity were because of a calcium-independent protein following stimulation with hydrogen peroxide then the differences between the treatment groups would still exist on top of the total CaM-KII activity. These results are similar to the results that many other investigators report when calcium and calmodulin are added to CaM-KII kinase assays (29, 30). Immunoblots of CaM-KII and CaM-KIV immunoprecipitates indicate that the treatments did not influence the amount of kinase present in the immunoprecipitates (Fig. 1E).

When an irrelevant antibody (mouse immunoglobulin) was used to perform similar immunoprecipitates, no changes in kinase activity could be noted following hydrogen peroxide treatment (control, 14,144 ± 5577, versus hydrogen peroxide, 15,803 ± 8269). Additionally, no changes in kinase activity could be noted when assayed in the presence of calcium and calmodulin. It should be noted that the same antibody serves as an irrelevant control for both CaM-KII and CaM-KIV.

Hughes et al. (23) demonstrated that PMA was capable of inducing NF-\kappaB activation in these cells and that this response was inhibited by the addition of CaM kinase inhibitors. We have reported previously that similar to their findings, PMA can induce I\kappaB degradation and that this degradation can be blocked by the addition of a CaM kinase inhibitor (22). Additionally, it has been suggested that PMA can cause the production of oxygen radicals in Jurkat cells and that binding of NF-\kappaB to a probe is inhibited by both catalase and antioxidants.
Jurkat cells were washed and resuspended at 1.25x10^6 cells/ml in RPMI 1640 containing 5% fetal calf serum. Cells were treated with either (A) okadaic acid (1 μM) or (B) microcystin-LR (500 nM) for the indicated time. Cells were lysed and subjected to immunoblot analysis using antibodies against phospho-Akt (Thr-308) or total Akt. Bands were visualized using an AP-BCIP/NBT developing system.

PMA does not cause a calcium flux in these cells as measured using Fluor-3 (Fig. 2). Stimulation of the cells with PMA results in the activation of both CaM kinase II and IV (Fig. 3, A and B). Because PMA does not cause a calcium flux, this activation must also be occurring in a manner independent of increases in intracellular calcium. Additionally, PMA was also able to activate the CaM kinases in the presence of EGTA, and as before, ionomycin-induced CaM kinase activity was blocked by the addition of EGTA to the cultures (Fig. 3, A and B) suggesting that PMA-induced activation of the CaM kinases also occurs in the absence of increases in intracellular calcium. To determine whether this activity was dependent on the induction of oxygen radicals we stimulated Jurkat cells with PMA in the presence of catalase. Catalase was shown to inhibit the activation of both CaM kinase II and IV by PMA (Fig. 3, C and D). Catalase has no effect on ionomycin-stimulated cells demonstrating that catalase is specific in its actions. These results suggest that PMA is acting via the production of oxygen radicals. In support of this, glucose oxidase, another compound demonstrating that catalase is specific in its actions. These results suggest that PMA is acting via the production of oxygen radicals. In support of this, glucose oxidase, another compound that causes the production of peroxide, is also able to activate both CaM kinase II and IV (Fig. 4). These results, taken together, suggest that both CaM kinase II and IV can be activated in a calcium-independent manner in response to ROI.

Because of their sensitivity to oxidation, one potential mechanism for the increase in CaM kinase activity is the inactivation of regulatory phosphatases. When the PP1/PP2A inhibitors okadaic acid or microcystin-LR were added to the cultures, activation of both CaM kinase II and IV occurred (Fig. 5, A and B). Both of these inhibitors induce a transient calcium flux in these cells that can be blocked by EGTA pretreatment (Fig. 5C). OKT3 is an antibody directed toward the T cell receptor and serves as a positive control for inducing an intracellular calcium flux in these cells. Similar to the results obtained with hydrogen peroxide, EGTA has no effect on the ability of these inhibitors to induce kinase activity suggesting that activation of the CaM kinases by phosphatase inhibitors also occurs in a calcium-independent manner (Fig. 5, A and B).

We have shown previously that hydrogen peroxide can cause the phosphorylation of the antiapoptotic kinase Akt (22). This phosphorylation was inhibited by CaM kinase inhibitors but was insensitive to calcium chelators EGTA and BAPTA-AM. We can also show that the phosphatase inhibitors okadaic acid and microcystin-LR induce the phosphorylation of Akt on Thr-308 (Fig. 6). These results show that inhibition of phosphatase activity is sufficient in itself to induce Akt phosphorylation, mimicking the effects of hydrogen peroxide and PMA. Taken with the above results, we have shown that phosphatase inhibition can activate the CaM kinases in a manner similar to treatment with hydrogen peroxide or PMA. Further, downstream CaM kinase targets are also phosphorylated after phosphatase inhibition.

Although it has been shown that hydrogen peroxide can inhibit phosphatase activity in other cell types, we determined whether this occurs in the Jurkat cell line. After treatment with hydrogen peroxide, we tested the ability of whole lysates to cleave the phosphopeptide from a synthetic peptide in PP2A-specific phosphatase buffer. Free phosphopeptide could be detected by the use of a molybdate dye and measured in a spectrophotometric assay. We found that following treatment with hydrogen peroxide, the phosphatase activity in these lysates decreased (Fig. 7). Furthermore, this activity was similar to the activity seen after treatment with the phosphatase inhibitor microcystin-LR at a level consistent with inhibition of PP2A (Fig. 7). Others have shown that PP2A will form a complex with CaM-KIV and can be co-immunoprecipitated with CaM-KIV (17). It is believed that in vivo PP2A is integral in the inactivation of CaM-KIV (16, 17).

We attempted to determine the phosphatase activity in immunoprecipitates of CaM-KIV, but we found that PP2A is inactivated because of oxidation during the immunoprecipitation process. As an alternative method, we used N^-(3-maleimidylpropionyl)biotin, which will only react with reduced cysteine residues (27, 28). Oxidation of the active cysteine on PP2A results in its inactivation. We immunoprecipitated CaM-KIV and performed an immunoblot to demonstrate that PP2A
does indeed co-immunoprecipitate with CaM-KIV (Fig. 8A). These data also indicate that an equal amount of PP2A immunoprecipitates with CaM-KIV regardless of the treatment group. It should be noted that the PP2A seen in the immunoblots in Fig. 8, A and B, represents only the PP2A associated with CaM-KIV and likely represents only a small portion of the total PP2A in the cell. Control immunoprecipitates using an irrelevant antibody (mouse immunoglobulin) failed to immunoprecipitate PP2A (Fig. 8C). Cysteine labeling shows that following hydrogen peroxide treatment, PP2A is indeed oxidized (Fig. 8B). Additionally, PMA treatment also results in the oxidation of PP2A (Fig. 8B), supporting our earlier results showing catalase inhibition of PMA-induced CaM kinase activity. This oxidation of PP2A that associates with CaM-KIV (Fig. 8B) as well as the total cellular PP2A (Fig. 8D). Cysteine labeling demonstrates that CaM-KIV is also oxidized under these conditions (Fig. 8C). To determine whether this change in cysteine oxidation could directly activate CaM-KIV we added hydrogen peroxide directly to CaM-KIV or CaM-KII immunoprecipitates. When kinase assays were performed on these samples, a reduction in kinase activity could be noted indicating that the direct oxidation of CaM-KIV or CaM-KII was not mediating the increase in activity (Fig. 9). Considering both the phosphatase assay as well as cysteine labeling, we can demonstrate that hydrogen peroxide causes the oxidation and subsequent inactivation of the phosphatase PP2A. Inactivation of this phosphatase is a potential mechanism for CaM kinase activation by ROI.

**DISCUSSION**

We and others have suggested the possibility of a mechanism that activates the CaM kinases in the absence of a calcium flux (22, 23). In this paper we elucidate this novel form of regulation of the CaM kinases. Using in vitro kinase assays, we were able to demonstrate that hydrogen peroxide can activate both CaM kinase II and IV within Jurkat T cells (Fig. 1). To the best of our knowledge, no one has demonstrated that the CaM kinases are sensitive to redox changes in the cell. Although H₂O₂ can cause a calcium flux in these cells, we found that blocking this calcium flux with EGTA has no effect on the activation of the CaM kinases (Fig. 1). These results are novel as an increase in intracellular calcium has always been thought to be necessary for the activation of these kinases.

Hughes et al. (23) demonstrated that PMA was capable of inducing NF-κB activation in Jurkat cells and that this response was inhibited by the addition of CaM kinase inhibitors. In this work we demonstrate that PMA treatment activates both CaM kinase II and IV (Fig. 3). It should be noted that PMA treatment of human T cells resulted in an increase in intracellular hydrogen peroxide as measured using a fluorescent indicator (35). In addition, treatment of cells with either N-acetylcysteine, an antioxidant (33), or catalase (34) could block PMA-induced NF-κB activity. The addition of catalase to the cells before PMA stimulation was able to prevent the activation of both CaM kinase II and IV (Fig. 3). It has been shown previously that PMA treatment of human T cells resulted in an increase in intracellular hydrogen peroxide as measured using a fluorescent indicator (35). In addition, treatment of cells with either N-acetylcysteine, an antioxidant (33), or catalase (34) could block PMA-induced NF-κB activity. The addition of catalase to the cells before PMA stimulation was able to prevent the activation of both CaM kinase II and IV (Fig. 3). Because catalase is specific for hydrogen peroxide, we have shown that the PMA-induced activation of the CaM kinases is directly related to hydrogen peroxide production in the cell. Because catalase can block PMA-induced activation of the CaM kinases, we postulate that both PMA and hydrogen peroxide are activating the kinases in a similar manner.

Although calcium is not required for activation of the CaM kinases, it was shown previously that calmodulin antagonists
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Figure 9. Direct oxidation of the CaM kinases does not result in increases in their activity. Jurkat cells were washed and resuspended in RPMI 1640 at a concentration of 1.25 × 10⁶ cells/ml. For each treatment, 15 ml of cells were warmed to 37 °C. Cells were then either treated with H₂O₂ (10 mM, in situ) or left untreated for 7 min. The bars labeled Control represent untreated cells. Cells were immediately centrifuged, and cells were lysed. Lysates were used to immunoprecipitate CaM kinase II or CaM kinase IV. Immunoprecipitates of either CaM kinase II or IV from untreated cells were incubated with 10 mM hydrogen peroxide in cold lysis buffer for 7 min (in vitro). Immunoprecipitates were used in a kinase assay in the absence of calcium to measure the phosphorylation of the synthetic peptide, syntide-2.

In summary, we have demonstrated that hydrogen peroxide can cause the activation of both CaM kinase II and IV. Most significantly, this activation occurred in the absence of any detectable calcium flux into the cell. In addition, PMA activation of the CaM kinases is also dependent on peroxide production and also occurs in the absence of a calcium flux. This activation of the CaM kinases is likely because of the oxidation and inactivation of phosphatases within the cell. These results demonstrate a novel mechanism by which the CaM kinases can be activated. These results may be significant for the activation of T lymphocytes in the inflammatory environment. Additionally, any cellular process resulting in the production of oxygen radicals can potentially activate the CaM kinases even in the absence of any calcium flux.

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