

## **Prostaglandin E<sub>2</sub> and Other Cyclic AMP Elevating Agents Inhibit Interleukin 2 Gene Transcription by Counteracting Calcineurin-dependent Pathways**

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### **Summary**

We have previously shown that prostaglandin E<sub>2</sub> and other cAMP elevating agents inhibit the nuclear transcription of the human IL-2 gene by interfering with a Ca<sup>2+</sup>-sensitive T cell signal transduction pathway. Calcineurin, a Ca<sup>2+</sup>/calmodulin-dependent 2B protein phosphatase, is an essential component of the T cell receptor signal transduction pathway leading to IL-2 gene expression. We have therefore tested the hypothesis that this phosphatase may be a target for the inhibitory effects of cAMP on IL-2 gene transcription. We report here that PGE<sub>2</sub> markedly reduces the IL-2 promoter activity that is induced by a constitutively active form of calcineurin. In contrast to the complete inhibition of promoter activity produced by the immunosuppressants cyclosporin A and FK-506, this partial block suggests that PGE<sub>2</sub> modulates downstream events needed for lymphokine gene activation. Overexpression of calcineurin in Jurkat cells decreases their apparent sensitivity to the inhibitory effects of PGE<sub>2</sub> consistent with the fact that this enzyme plays a physiological role in dephosphorylating substrates of cAMP-dependent kinases in several tissues. These results provide evidence that cAMP-dependent pathways may antagonize calcineurin-regulated cascades for T cell activation in vivo, and suggest crosstalk between the Ca<sup>2+</sup> and the cAMP signaling pathways during T cell activation.

Prostaglandins (PG) of the E series have been shown to modulate immune responses by inhibiting T cell activation events including IL-2 gene expression (1–4). PGE<sub>2</sub> release from macrophages is induced by diverse stimuli such as phagocytosis, immune complexes, and complement products (5, 6). The effects of PGE<sub>2</sub> are mediated by the intracellular second messenger, cAMP, through binding to its intracellular receptor protein kinase A (PKA). As with other agents that increase cAMP (histamine, adenosine, forskolin, and cholera toxin), the mechanism by which PGE<sub>2</sub> activates PKA involves the binding of cAMP to the inactive tetrameric holoenzyme dissociating it into two regulatory and two catalytic subunits (7). This kinase-mediated activation leads to inhibition of IL-2R expression, IL-2 production (mediated by both a decrease in IL-2 nuclear transcription and IL-2 mRNA stability) (8), and T cell proliferation (1–4).

IL-2 gene transcription is controlled by a promoter region extending ~326 bp upstream of the transcription start site. Both an increase of cytoplasmic-free calcium and activation of protein kinase C (PKC) are required for its transcription in resting T cells (9, 10). Recent findings have identified cal-

calcineurin, a Ca<sup>2+</sup>/calmodulin-dependent (type 2B) serine/threonine protein phosphatase, as a key enzyme needed for IL-2 promoter activity (11, 12). A deletion mutant ( $\Delta$ CaM-AI) of the calcineurin catalytic subunit (11) has been shown to mimic proteolyzed forms of the phosphatase known to have Ca<sup>2+</sup>-independent, constitutive phosphatase activity in vitro (13, 14); this has allowed demonstration of IL-2 promoter activation in the presence of phorbol ester alone (11).

We have previously shown that cAMP elevating agents blocked IL-2 gene transcription by inhibiting Ca<sup>2+</sup>-dependent pathways of T cell activation. In vitro data have shown that calcineurin exhibits a relatively narrow substrate specificity, preferring substrates of cAMP-dependent kinases (including the regulatory subunit of the kinase itself) (15–18). More importantly, it appears that this phosphatase plays a physiological role in counteracting cAMP-dependent signal pathways in skeletal muscle (19) and in intact kidney cells (20). We therefore hypothesized that a different type of reciprocal relationship may exist in T cells, i.e., that cAMP-mediated inhibition of IL-2 gene transcription may downregulate calcineurin-dependent pathways of lymphokine gene activation.

To this end, we have used transient DNA transfection assays with plasmids carrying the mutant calcineurin ( $\Delta$ CaM-AI) and the IL-2 promoter in Jurkat cells.

## Materials and Methods

**Cell Culture.** Human Jurkat T cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated FCS, 25  $\mu$ g/ml gentamicin, and  $5 \times 10^{-5}$  M 2-ME (Sigma Chemical Co., St. Louis, MO). Cultures were stimulated at the indicated time points and concentrations with the following reagents: cholera toxin (CT; List Biol. Laboratories, Inc., Campbell, CA), ionomycin, forskolin, 8-bromo-cAMP (8 Br-cAMP), or 3-isobutyl-1-methyl-xanthine, (all from Sigma Chemical Co.). cAMP elevating agents were added into cell cultures 30 min before stimulation.

**Plasmids.** Plasmid  $\Delta$ CaM-AI 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 (11). As discussed elsewhere (11),  $\Delta$ CaM-AI was constructed by polymerase chain reaction (PCR) amplification of a CN $\alpha$ -4 template and insertion of a fragment encoding amino acids 1 to 398 into the SmaI site of pUC18. Consequently the 5' and 3' untranslated sequences present in CN $\alpha$ 4 were also deleted. The clone was subsequently inserted into the EcoRI site of pcDL-SR $\alpha$ 296.  $\Delta$ CaM-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 (13, 14). In control experiments, the expression vector alone without calcineurin was used. 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. Plasmid SV40 CAT vector (Promega Corp., Madison, WI) contains the SV40 enhancer and promoter regions linked to the CAT gene.

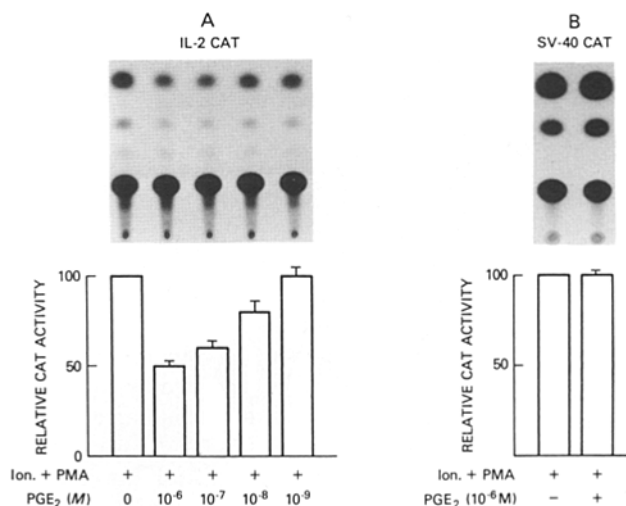
**DNA Transfections.** Transfections of lymphoid cells were carried out by the (DEAE)-dextran method as previously described (21). To decrease variations in transfection efficiency, cells were transfected in single batches, which were then separated into different drug treatment groups. For each treatment,  $1.5-2 \times 10^7$  cells harvested in log phase of growth ( $3-5 \times 10^5$  cells/ml) were incubated with 20  $\mu$ g DNA and 100  $\mu$ g/ml DEAE-dextran in serum-free RPMI-1640 for 70 min at room temperature. After two washes with serum-free RPMI-1640, they were replated in complete medium. 24 h after transfection, cells were treated with various concentrations of cAMP elevating agents for 30 min. Then they were stimulated for 12 h with ionomycin 0.7  $\mu$ g/ml and 20 ng/ml of phorbol myristate acetate (PMA). In titration experiments this dose of ionomycin was found to induce maximal reporter gene activity. After stimulation, cells were harvested and extracts were prepared by rapid freeze-thawing. Protein concentration was determined by the Bradford assay and equivalent amounts of protein extracts were assayed for enzyme activity.

**Chloramphenicol Aminotransferase (CAT) Assay.** The CAT assay was carried out as described previously (21) by the incubation of 50  $\mu$ g of cell lysate with 0.1  $\mu$ Ci of [ $^{14}$ C]chloramphenicol (sp act 57.3 mg/mmol, NEN Research Products, Boston, MA) in the presence of 9 mM acetyl-coenzyme A (Pharmacia Inc., Piscataway, NJ) for 3 h at 37°C. Acetylated and unacetylated chloramphenicol were separated by thin layer chromatography. Conversion of [ $^{14}$ C]chlor-

amphenicol to its acetylated forms was quantified by the use of a Betagen Betascope (Waltham, MA).

## Results

**cAMP Inhibits Protein Kinase C- and Calcium-mediated trans-Activation of the IL-2 Promoter.** In earlier studies, we showed that cAMP partially inhibits ( $\sim$ 50% inhibition) the nuclear transcription of the human IL-2 gene as determined by nuclear run-off assays (8). To determine whether this inhibition is mediated through interference with the transcriptional activity of the IL-2 promoter, Jurkat cells were transiently transfected with plasmid IL-2 CAT which contains the IL-2 promoter region driving the expression of CAT gene (see Materials and Methods). Cells were stimulated with ionomycin and PMA in the presence or absence of PGE $_2$ , 8 Br-cAMP, forskolin, or CT. All four cAMP elevating agents reduced the CAT activity of the IL-2 promoter plasmid. Fig. 1 A shows the results using PGE $_2$  as the cAMP elevating agent. In four different experiments PGE $_2$  inhibited the activity of the IL-2 promoter in a dose-dependent fashion. Inhibition of IL-2 promoter activity by PGE $_2$  was partial even at suprapharmacologic doses (60% inhibition with  $10^{-4}$  M PGE $_2$ ). Partial inhibition of IL-2 promoter activity was not due to degradation of its inhibitor isobutylmethylxanthine (0.5–5 mM) did not affect the level of inhibition (not shown). Similar results were ob-



**Figure 1.** PGE $_2$  specifically inhibits the Ca $^{2+}$ - and PKC-mediated trans-activation of the IL-2 promoter. Jurkat cells were transiently transfected with the plasmid IL-2 CAT which contains the IL-2 promoter driving the expression of CAT gene (A), or the control plasmid SV40 CAT which contains the SV40 enhancer and promoter regions linked to the CAT gene (B). 24 h after transfection, cells were incubated for  $\sim$ 30 min with PGE $_2$  ( $10^{-6}$ – $10^{-9}$  M) and then stimulated with ionomycin and PMA. Cell extracts were prepared 12 h later and assayed for CAT activity. Acetylated and unacetylated chloramphenicol were separated by thin layer chromatography. Conversion of [ $^{14}$ C]chloramphenicol to its acetylated forms was quantified using a Betagen Betascope. Results (expressed as percentage of maximum activity) represent the mean ( $\pm$  2 SD) of four independent experiments.

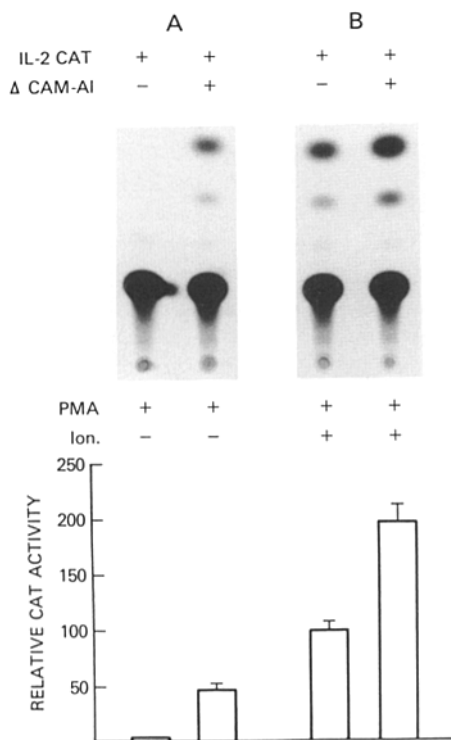
tained with the other cAMP elevating agents used. Specifically, the doses needed to obtain 50% inhibition were as follows: PGE<sub>2</sub>, 10<sup>-6</sup> M; 8BrcAMP, 10<sup>-3</sup> M; forskolin, 10<sup>-4</sup> M; and CT, 1 ng/ml. The inhibitory effect of cAMP on IL-2 promoter activity was specific since the activity of an unrelated promoter (SV40 CAR, control vector) was not affected (Fig. 1 B).

***ΔCaM-AI trans-Activates the IL-2 Promoter in Synergy with PMA.*** To investigate the role of calcineurin in the activation of the IL-2 promoter, we cotransfected a plasmid containing the IL-2 promoter linked to a CAT gene along with plasmid ΔCaM-AI into Jurkat cells. Cotransfection of ΔCaM-AI, in the presence or absence of 0.7 μg/ml ionomycin, did not affect the IL-2 promoter activity (not shown). Stimulation with PMA alone did not increase IL-2 promoter activity in vector-treated cells, but did so only in the presence of ΔCaM-AI (Fig. 2 A).

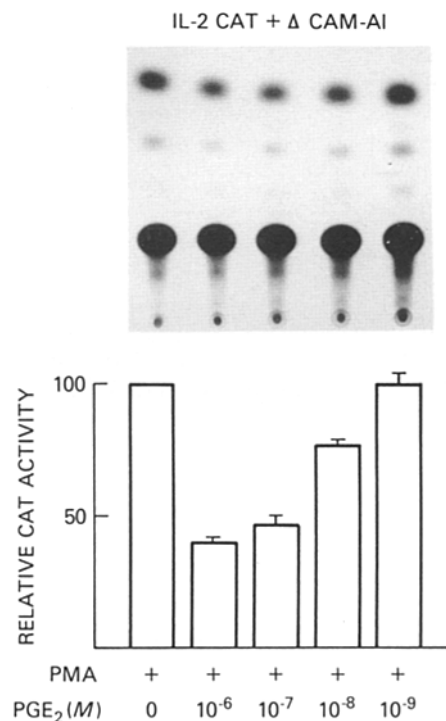
The combination of ionomycin and PMA was tested next. In the absence of ΔCaM-AI, IL-2 promoter activity was twice that of cells stimulated with PMA alone in the presence of ΔCaM-AI (Fig. 2 B). Overexpression of calcineurin by means of ΔCaM-AI further induced IL-2 promoter activity (by 1.7-

to 2.3-fold) suggesting that in addition to calcineurin, other Ca<sup>2+</sup>-dependent pathways are also involved in IL-2 promoter activity (Fig. 2 B). These results confirm that ΔCaM-AI can partially substitute for the Ca<sup>2+</sup>-requirements of IL-2 promoter activation as reported previously (11, 12), and that its overexpression further induces IL-2 promoter activity.

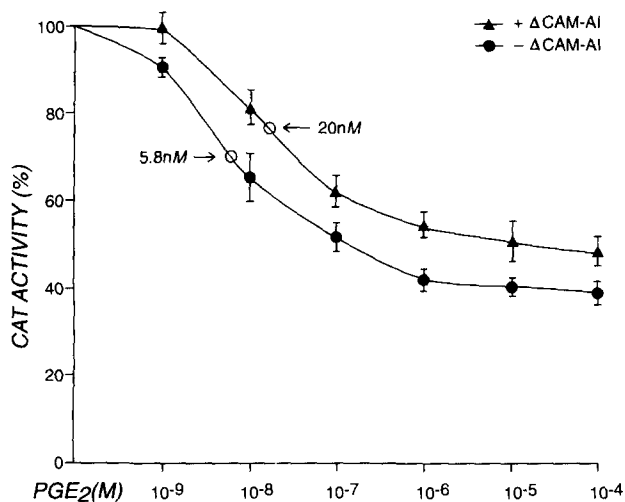
***cAMP Inhibits the Calcineurin-dependent trans-Activation of the IL-2 Promoter.*** Having demonstrated that calcineurin can partially substitute for the Ca<sup>2+</sup>-dependent pathways of T cell activation, we next examined whether cAMP antagonizes its action on IL-2 promoter activity. As shown in Fig. 3, PGE<sub>2</sub> inhibited the IL-2 promoter activity induced by the synergistic actions of ΔCaM-AI and phorbol ester. Similar dose-dependent inhibition was also observed when other cAMP elevating agents were used (not shown). We have previously shown that PKC-dependent pathways of T cell activation are not inhibited by cAMP (8). Thus, inhibition of IL-2 promoter activity by cAMP in this experiment, can be attributed to cAMP-mediated events inhibiting the activity of either the calcineurin per se, or more distant sites of the calcineurin-dependent pathway of T cell activation. It is of interest that inhibition of IL-2 promoter activity in this experiment (where ΔCaM-AI substitutes for the Ca<sup>2+</sup>-dependent pathway for its activation) is partial and comparable



**Figure 2.** The mutant calcineurin plasmid ΔCaM-AI acts in synergy with phorbol esters to mediate the *trans*-activation of the IL-2 promoter. Jurkat cells were transiently transfected with 5 μg of plasmid IL-2 CAT alone or with the plasmid ΔCaM-AI (15 μg). Cells were stimulated with PMA alone (A), or in combination with ionomycin (B). Cell lysates were examined for CAT activity and analyzed as described in Fig. 1. Results (expressed as percentage of activity observed when cells were stimulated with ionomycin and PMA in the absence of ΔCaM-AI) are representative of three independent experiments (mean ± 2 SD).



**Figure 3.** PGE<sub>2</sub> inhibits the ΔCaM-AI and PMA induced *trans*-activation of IL-2 promoter. Jurkat cells were cotransfected with plasmids IL-2 CAT (5 μg) and ΔCaM-AI (15 μg) and stimulated with PMA alone in the presence or absence of PGE<sub>2</sub> (10<sup>-6</sup>-10<sup>-9</sup> M). Cell lysates were examined for CAT activity as described in Fig. 1. Results (expressed as percentage of maximum activity) are representative of three independent experiments (mean ± 2 SD).



**Figure 4.**  $\Delta$ CaM-AI decreases the sensitivity of IL-2 promoter to PGE<sub>2</sub>. Jurkat cells were cotransfected with plasmid IL-2 CAT (5  $\mu$ g) in combination with  $\Delta$ CaM-AI (15  $\mu$ g) (+  $\Delta$ CaM-AI or its vector -  $\Delta$ CaM-AI) and stimulated with ionomycin and PMA in the presence of PGE<sub>2</sub> (10<sup>-4</sup>–10<sup>-9</sup> M). Lysates were analyzed for CAT activity as described in Fig. 1. CAT activity is plotted as percent activity in lysates of transfected cells that were not treated with PGE<sub>2</sub>. Results represent five independent experiments (mean  $\pm$  SD). Arrows indicate the doses of PGE<sub>2</sub> required to obtain 50% of the maximum inhibitory effect.

to the inhibition observed in Fig. 1, whereas IL-2 promoter activity was under the influence of all Ca<sup>2+</sup>-dependent pathways (see Discussion).

**Overexpression of  $\Delta$ CaM-AI Renders Cells More Resistant to the Inhibitory Effects of PGE<sub>2</sub>.** Transfection of Jurkat cells with calcineurin expression plasmids increases the calcineurin concentrations and superinduces the IL-2 promoter activity (Fig. 2). Thus, in the presence of excess calcineurin, one might expect we would predict that the IC<sub>50</sub> for cAMP-dependent inhibition would increase. Cells were cotransfected with the IL-2 promoter and either plasmid  $\Delta$ CaM-AI, or its expression vector alone, and stimulated with ionomycin and PMA. However, in the presence of  $\Delta$ CaM-AI the IC<sub>50</sub> (defined here as the concentration required to obtain 50% of the maximum inhibitory effect) for PGE<sub>2</sub> increased by  $\sim$ 3.4-fold (5.8 nM vs. 20 nM) (Fig. 4). In this experiment, the dose of PGE<sub>2</sub> required to obtain a substantial inhibitory effect on IL-2 promoter activity ( $\sim$ 10<sup>-8</sup> M), is comparable to the dose of PGE<sub>2</sub> that is necessary to elevate cAMP in Jurkat cells (22) suggesting that its effect is physiologically relevant and mediated through cAMP. These results provided further evidence to support the notion that cAMP inhibits calcineurin-dependent pathways of T cell activation.

## Discussion

In this study we have demonstrated that agents that increase intracellular cAMP in Jurkat T cells (22) also inhibit

IL-2 promoter activity by counteracting calcineurin-dependent pathways of T cell activation. In fact, one of these agents (PGE<sub>2</sub>) did so at effector concentrations that are likely to occur physiologically, suggesting a central role for cAMP in mediating these effects.

The partial inhibition of the Ca<sup>2+</sup> and/or calcineurin-dependent activation of IL-2 promoter activity is intriguing. Data presented here and elsewhere (11) have suggested that in addition to calcineurin, other Ca<sup>2+</sup>-dependent pathways that do not utilize calcineurin participate in IL-2 gene transcription. However, partial inhibition of IL-2 promoter activity was also observed in experiments where calcineurin alone substituted for the Ca<sup>2+</sup> signal. Calcineurin is essential for IL-2 promoter activity and its inhibition by cyclosporin A effectively blocks IL-2 gene transcription (11, 12, 21). The partial inhibition of IL-2 promoter activity by cAMP elevating agents observed in these experiments suggests that their effect may be exerted at more distal sites in the calcineurin-regulated pathway. Alternatively, calcineurin may activate several discrete *trans*-activating pathways (23) and the partial inhibition by PGE<sub>2</sub> may reflect selective blockades of some, but not all, of these signaling events.

The small, but reproducible, shift in PGE<sub>2</sub> sensitivity caused by overexpression of calcineurin may indicate a role of cAMP-dependent kinase substrates in modulating gene transcription. Calcineurin has been shown to dephosphorylate inhibitor-1 (I-1) and a related gene product, the dopamine and cAMP regulated phosphoprotein (DARPP) (15, 16). Both of these proteins, once phosphorylated by PKA, inhibit protein phosphatase-1 (PP-1), a relatively broad-specificity enzyme (18); the dephosphorylation of I-1 and DARPP-32 by calcineurin activates PP-1, thus promoting the dephosphorylation of substrates for PKA. Similarly, dephosphorylation of the R<sub>II</sub> subunit of PKA by calcineurin facilitates its reassociation with the catalytic subunit to reform the inactive holoenzyme, further antagonizing the action of cAMP. These mechanisms operating either alone or in combination may allow signals that act via Ca<sup>2+</sup> to attenuate the action of cAMP (18, 24). It is conceivable that analogous mechanisms may be involved in downregulating IL-2 promoter activity.

In addition to PGE<sub>2</sub> released by activated macrophages at sites of inflammation, stimulation of T cells through the CD2 (25) or CD3 (26) cell surface molecules also increases cAMP level. cAMP may represent an important mechanism to rapidly attenuate the transcription of IL-2 once the inducing stimulus is removed (25). Consistent with these immunosuppressive effects of PGE<sub>2</sub> in vitro are the findings that misoprostol, a PGE<sub>1</sub> analogue, reduces the incidence of acute rejection in renal transplant recipients (27). These studies provide evidence for crosstalk between the Ca<sup>2+</sup>- and the cAMP-pathways in human T cells and identify the calcineurin pathway as an essential component of this interaction. cAMP elevating agents may provide a useful tool to further dissect the molecular events associated with T cell activation.

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