MOLECULAR AND CELLULAR BIOLOGY, Apr. 1996, p. 1842–1850 Vol. 16, No. 4
0270-7306/96/$04.00+0

Protein Kinase C-θ Isoenzyme Selective Stimulation of the Transcription Factor Complex AP-1 in T Lymphocytes

GABRIELE BAIER-BITTERLICH,[1] FLORIAN ÜBERALL,[1] BIRGIT BAUER,[2] FRIEDRICH FRESSER,[2] HELMUT WACHTER,[1] HANS GRUNICKE,[1] GERD UTERMANN,[2] AMNON ALTMAN,[3] AND GOTTFRIED BAIER[2]*

Institute for Medical Chemistry and Biochemistry[1] and Institute for Medical Biology and Human Genetics,[2] University of Innsbruck, A-6020 Innsbruck, Austria, and La Jolla Institute for Allergy and Immunology, La Jolla, California 92037[3]

Received 10 July 1995/Returned for modification 18 September 1995/Accepted 4 January 1996

T-lymphocyte stimulation requires activation of several protein kinases, including the major phorbol ester receptor protein kinase C (PKC), ultimately leading to induction of lymphokines, such as interleukin-2 (IL-2). The relevant PKC isoforms which are involved in the activation cascades of nuclear transcription factors involved in IL-2 production have not yet been clearly defined. We have examined the potential role of two representative PKC isoforms in the induction of the IL-2 gene, i.e., PKC-α and PKC-θ, the latter being expressed predominantly in hematopoietic cell lines, particularly T cells. Similar to that of PKC-α, PKC-θ overexpression in murine EL4 thymoma cells caused a significant increase in phorbol 12-myristate 13-acetate (PMA)-induced transcriptional activation of full-length IL-2–chloramphenicol acetyltransferase (CAT) and NF-AT–CAT but not of NF-IL2A–CAT or NF-κB promoter–CAT reporter gene constructs. Importantly, the critical AP-1 enhancer element was differentially modulated by these two distinct PKC isoenzymes, since only PKC-θ but not PKC-α overexpression resulted in an ≈2.8-fold increase in AP-1–collagenase promoter CAT expression in comparison with the vector control. Deletion of the AP-1 enhancer site in the collagenase promoter rendered it unresponsive to PKC-θ. Expression of a constitutively active mutant PKC-θ A148E (but not PKC-α A25E) was sufficient to induce activation of AP-1 transcription factor complex in the absence of PMA stimulation. Conversely, a catalytically inactive PKC-θ K409R (but not PKC-α K368R) mutant abrogated endogenous PMA-mediated activation of AP-1 transcriptional complex. Dominant negative mutant Ha-RasS17N completely inhibited the PKC-θ A148E-induced signal, identifying PKC-θ as a specific constituent upstream of or parallel to Ras in the signaling cascade leading to AP-1 transcriptional activation.

Molecular cloning and biochemical studies identified protein kinase C (PKC) enzymes as members of a distinct family of serine/threonine protein kinases, playing critical roles in the regulation of cellular differentiation and proliferation of diverse cell types (reviewed in reference 36). In an attempt to find PKC isoforms that are involved in growth control and/or activation of T lymphocytes, we have identified PKC-θ (5), whose human gene locus was recently mapped to chromosome 10p15 (15). PKC-θ is characterized by a unique tissue distribution, i.e., in skeletal muscle, lymphoid organs, and hematopoietic cell lines, particularly T cells (4, 5, 10, 34, 39, 53), and by isoenzyme-specific activation requirements and substrate preferences in vitro (4). PKC-θ undergoes cytosol-to-membrane translocation in T cells stimulated with phorbol esters (4), implying that this isoform is likely to be involved in T-cell activation pathways. The unique expression and functional properties of PKC-θ suggest that it may play a specialized role in T-cell signaling pathways.

T-cell activation results in the expression of interleukin-2 (IL-2), an autocrine growth factor that is a critical stimulus for the growth and differentiation of B and T lymphocytes. Pharmacological and biochemical studies indicate that activation of two major signaling pathways, one of which can be triggered by phorbol esters (such as phorbol 12-myristate 13-acetate [PMA]) and the other of which can be triggered by Ca^{2+} ionophores, is required for induction of IL-2 (reviewed in reference 51). A substantial amount of work over the past several years has shown the requirement of cooperative interactions of several transcription factors, including AP-1, NF-κB, NF-AT, and NF-IL2A (Oct-1), with the minimal inducible promoter/enhancer region of the IL-2 gene (11).

Several lines of evidence point to AP-1 as a critical transcription factor for IL-2 regulation. AP-1 is a dimer of different members of the Fos (c-Fos, FosB, Fra-1, Fra-2, and FosB2) and Jun (c-Jun, JunB, and JunD) family of proteins (1). AP-1 thereby interacts with the IL-2 regulatory region directly (25, 26, 33, 47) and also indirectly as a component of NF-AT and NF-IL2 (37, 50). AP-1 activity is regulated by de novo synthesis of Jun and Fos proteins, as well as by posttranslational modifications such as phosphorylation and dephosphorylation (1, 8, 9, 30, 43, 48). Two potential AP-1-binding sites have been identified in the mouse and human IL-2 enhancer region at −150 bp (proximal AP-1) and −180 bp (distal AP-1). These elements show sequence similarity to the consensus AP-1 enhancer sequence and have been studied by deletional, mutational, and gel shift analyses (14, 18, 25, 40). Most of these data support an important role for AP-1 in IL-2 transcription, especially as a result of the interaction with the proximal enhancer site (25).

PKC has been implicated in the activation of AP-1 in T lymphocytes, as demonstrated by studies involving PKC-specific pharmacological inhibitors (24, 28) or PKC down-regulation by chronic phorbol ester treatment (26, 52). Nevertheless, specific PKC isoforms responsible for AP-1 transcriptional activation and mode of action remain to be identified. Studies

* Corresponding author. Mailing address: Institute for Medical Biology and Human Genetics, University of Innsbruck, Schoepfstr. 41, A-6020 Innsbruck, Austria. Phone: 43 (512)/507-3451. Fax: 43 (512)/507-2861.

presented here include overexpression of wild-type and mutant PKC isoforms, i.e., dominant negative "kinase-dead" mutants PKC-θ K409R and PKC-α K368R, as well as pseudosubstrate mutants PKC-θ A148E and PKC-α A25E, which are mutationally activated and therefore independent of mitogen or receptor stimulation for enzymatic function. We report that although both PKC isoenzymes are involved in the activation of the IL-2 promoter, overexpression of only PKC-θ (but not PKC-α) delivers a signal contributing to AP-1 enhancer activation in a Ras-dependent fashion. These studies implicate PKC-θ as a specific constituent of the signaling cascade that is involved in T-cell activation and functions with other signaling cascades to induce AP-1 gene transcription.

MATERIALS AND METHODS

Reagents and plasmids. PMA was purchased from Molecular Probes Inc., Eugene, Oreg. Phytohemagglutinin and phosphatidylserine (PtdSer) were purchased from Sigma Chemical Co., St. Louis, Mo. pIL-2/0–CAT, containing 2,000 bp of the human IL-2 promoter upstream of the chloramphenicol acetyltransferase (CAT) reporter gene (47); $^{-73/+60}$Col-CAT, consisting of sequences from −73 to +60 of the human fibroblast collagenase gene (8); and pZIPneo-RasL61 (8) were obtained from M. Karin. Synthetic multimers of the NF-IL2A and NF-AT elements of the IL-2 promoter upstream of the CAT gene (14, 45) were kind gifts of G. R. Crabtree. Additionally, an NF-κB–CAT construct (42) was obtained from N. D. Perkins. Plasmid pEF-RasN17 was constructed by shuffling the full-length coding regions of pEXV3RasN17 (23) into expression plasmid pEF-neo (a kind gift from Y. Liu), downstream and under the control of the EF-1α promoter (35). Plasmid pRc/CMV-Xenopus PKC-ζ (13) was a kind gift from J. Moscat. Human PKC-θ, rat PKC-ε, and bovine PKC-α wild-type cDNAs were subcloned into the cytomegalovirus (CMV) expression vector pRc/CMV (Invitrogen) essentially as described previously (4) or subcloned in pEF-neo, respectively. Site-directed mutagenesis of PKC cDNA was performed with the Transformer system (Clontech, Palo Alto, Calif.) as described by the manufacturer. The *Pvu*I selection primer (5′-CGGTCCTCCGTTCGTTGTCAG-3′) and the mutagenic primers PKC-θ K/R^{409} (5′-TTTTCGCAATAAGGGCCTTAA AG-3′), PKC-θ A/E^{148} (5′-GCCGGGGTGAAATCAAGCAGGC-3′), PKC-α K/R^{368} (5′-GTACGCCATCAGAATTCTGAAGAAGG-3′), PKC-α A/E^{25} (5′-CGCAAAGGGGAGCTCAGGCAGAAGAAC-3′), and PKC-ε A/E^{159} (5′-CGCCAAGGGGAGGTCAGGCGCA-3′) were used in this study.

Transient transfection of COS-1 cells and partial purification of recombinant His_6-tagged PKC isoenzymes. COS-1 cells were seeded at a density of 10^6 cells per 10-cm-diameter dish containing Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C. One day later, the cells were transfected with 20 μg of circular plasmid DNA per dish by lipofectamine treatment (Gibco-BRL, Gaithersburg, Md.) as described by the manufacturer. At 48 h posttransfection, cells were lysed in 1 ml of buffer A (150 mM NaCl, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 1% Nonidet P-40, 50 μg each of aprotinin and leupeptin per ml, 1 mM phenylmethylsulfonyl fluoride). The six-histidine-tagged PKC proteins were purified, and the kinase activity of recombinant PKC or control preparations was determined by measuring ^{32}P transfer from [γ-^{32}P]ATP into the [A25S]PKC substrate, the synthetic peptide RFARKGSLRQKNVY (representing the pseudosubstrate sequence of PKC-α [22] with an alanine-to-serine substitution), by using the PKC assay system described recently (4).

Immunoblot analysis. Immunoblot analysis with the isoenzyme-specific antibodies anti-PKC-α and anti-PKC-θ was performed as described previously (4). Isoenzyme-specific antibodies anti-PKC-ε and anti-PKC-ζ were purchased from Gibco-BRL and used as recommended by the vendor.

Transient EL4 thymoma and COS-1 cell transfections. Transfection of EL4 cells by the DEAE-dextran method and PMA (5 ng/ml unless indicated otherwise) stimulation were performed as previously described (4). Briefly, 2×10^7 murine EL4 thymoma cells were cotransfected in duplicate with 5 μg of an enhancer/promoter-CAT reporter gene construct, 1 μg of pCMVβ, a β-galactosidase expression vector (Clontech), and 20 μg of distinct wild-type or mutant PKC expression plasmids or a vector control, respectively. The cells were resuspended in 10 ml of complete medium and 24 h later were stimulated in the absence or presence of PMA for an additional 24 h. The cells were harvested, protein concentrations were determined by the Bradford assay (Bio-Rad, Melville, N.Y.), and the enhancer/promoter activation was measured by determining the expression of the CAT reporter protein by a CAT enzyme-linked immunosorbent assay (ELISA; Boehringer Mannheim Biochemicals, Mannheim, Germany) as specified by the manufacturer. For normalization of transfection efficiencies, the level of β-galactosidase reporter protein was measured in the same cell extracts by using a β-galactosidase ELISA (5 Prime→ 3 Prime, Boulder, Colo.). Transient cotransfection of COS-1 cells with a promoter-reporter CAT plasmid, pCMVβ, and the indicated PKC expression vectors was done with Lipofectamine (Gibco-BRL). Subconfluent cultures of COS-1 cells were transfected and were starved in Dulbecco's modified Eagle's medium–0.5% fetal calf serum for 18 h prior to stimulation with 100 ng of PMA per ml for an additional 24 h. Cells were lysed and CAT expression was determined as described above. The results shown are obtained with different preparations of expression plasmids and represent the mean and standard error of the mean from three representative experiments done in duplicate.

TABLE 1. Kinase activities of recombinant PKC wild-type or mutant proteins in vitro[a]

Recombinant PKC enzyme	Cofactor[b]	Enzyme activity (%)[c]
None	EGTA	3 ± 2
None	PtdSer/PMA/Ca^{2+}	2 ± 3
PKC-θ wt	EGTA	30 ± 7
PKC-θ wt	PtdSer/PMA/Ca^{2+}	100 ± 5
PKC-θ K409R	EGTA	2 ± 2
PKC-θ K409R	PtdSer/PMA/Ca^{2+}	2 ± 2
PKC-θ A148E	EGTA	70 ± 9
PKC-θ A148E	PtdSer/PMA/Ca^{2+}	100 ± 19
PKC-ε wt	EGTA	25 ± 15
PKC-ε wt	PtdSer/PMA/Ca^{2+}	100 ± 20
PKC-ε A159E	EGTA	65 ± 14
PKC-ε A159E	PtdSer/PMA/Ca^{2+}	100 ± 25
PKC-α wt	EGTA	18 ± 4
PKC-α wt	PtdSer/PMA/Ca^{2+}	100 ± 10
PKC-α K368R	EGTA	3 ± 2
PKC-α K368R	PtdSer/PMA/Ca^{2+}	2 ± 2
PKC-α A25E	EGTA	80 ± 15
PKC-α A25E	PtdSer/PMA/Ca^{2+}	100 ± 14

[a] COS-1 cells were transfected with various PKC cDNA expression constructs, and His_6-tagged PKC was partially purified as described in Materials and Methods. Purified PKC or vector control preparations were assayed in an enzymatic assay in the absence or presence of the indicated cofactor combinations. pRc/PKC-ζ wt has been extensively characterized by Diaz-Meco et al. (13).

[b] The concentrations of cofactors used are 280 μg of PtdSer per ml, 10 μM PMA, and 1 mM $CaCl_2$. To measure PKC activity in the absence of Ca^{2+}, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (1 mM final concentration) was added instead of $CaCl_2$.

[c] To correct for differences in transfection efficiencies, enzyme activities are expressed as a percentage of cofactor-dependent phosphorylation of the [A25S]PKC peptide, which was determined separately for kinase-active PKC preparations in each experiment. Data are means ± standard deviations.

RESULTS

Functional characterization of recombinant mutant PKC isoenzymes. Mutant PKC isoenzyme cDNAs were generated by site-directed mutagenesis of the cDNA, and their biochemical characteristics (in comparison with those of the wild-type protein) were studied with recombinant PKC protein preparations in vitro. COS-1 cells were transfected with the appropriate PKC expression constructs or a vector control, and the recombinant PKC was purified by exploiting the COOH-terminal six-histidine (His_6) fusion tag and analyzed in standard PKC kinase assays against substrate [A25S]PKC peptide in the absence or presence of known PKC cofactors including PtdSer, PMA, and Ca^{2+} as described previously (4). The results are summarized in Table 1. Mutant isoforms in which Lys at the ATP-binding site is replaced with Arg (PKC-θ K409R and PKC-α K368R) are enzymatically inactive (and therefore

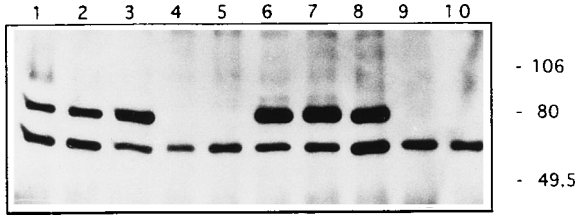
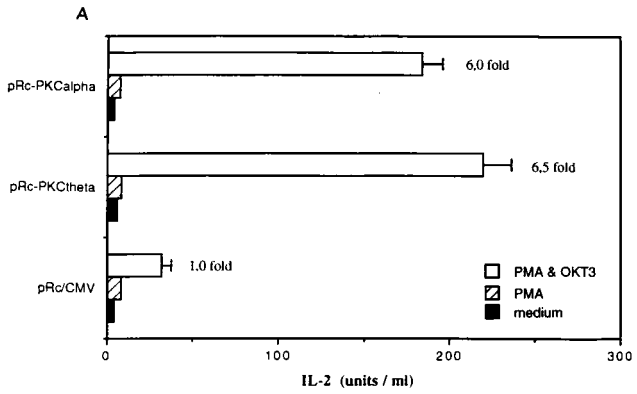


FIG. 1. Immunoblot analysis of recombinant PKC-α or PKC-θ preparations from transiently transfected COS-1 cells. Lysates from COS-1 cells transfected 48 h earlier with His_6-tagged pRc/PKC-θ K/R (lane 1), pRc/PKC-θ A/E (lane 2), pRc/PKC-θ wt (lane 3), pRc/PKC-α K/R (lane 6), pRc/PKC-α A/E (lane 7), pRc/PKC-α wt (lane 8), pTag/Lck (lane 4) (2), pTag/Fyn (lane 9) (2), or the pRc/CMV control vector (lanes 5 and 10) were purified with an Ni^{2+}-chelating resin and subjected to immunoblotting with a mixture of anti-PKC-α and anti-PKC-θ antibodies, followed by goat anti-rabbit immunoglobulin G-peroxidase conjugate and use of a chemiluminescence detection kit (ECL reagent). The sizes of the molecular mass standards are shown (in kilodaltons).



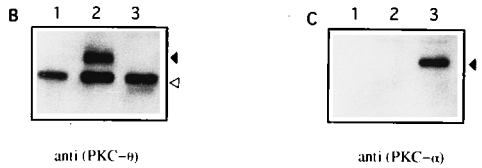


FIG. 2. Enhanced IL-2 production in PKC-θ- and PKC-α-overexpressing Jurkat E6-1 cells. Jurkat cells were stably transfected with the PKC-θ and PKC-α linearized DNA plasmid constructs by electroporation in a BTX T820 ElectroSquarePorator (BTX Biotech Inc., San Diego, Calif.), at 320 V and with five repetitive pulses each for 99 μs, and G418-resistant cell masses (1 mg of free acid G418 per ml of medium) were expanded and analyzed for activation-induced IL-2 secretion. Cells transfected with nonrecombinant pRc/CMV vector plasmid served as controls to exclude potential vector-dependent effects. (A) IL-2 titers in test culture supernatants were determined in a conventional 24-h bioassay by measuring the proliferation (i.e., [^{3}H]thymidine uptake) of an IL-2-dependent indicator T-cell line (CTLL-2), and secreted IL-2 was quantitated by comparison with a recombinant IL-2 standard as described previously (3). (B and C) Western blot analysis of Ni^{2+}-chelating resin-precipitated protein fractions derived from pRc/CMV- (lanes 1), pRc/PKC-θ (lanes 2), or pRc/PKC-α (lanes 3)-transfected and PMA/OKT3-stimulated Jurkat cells (2.5×10^6 cell equivalents per lane), followed by immunodetection with the corresponding antiserum as indicated. The arrowhead indicates the position of PKC proteins. The open arrowhead in panel B indicates an unidentified protein recognized by anti-PKC-θ serum.

termed kinase dead), and mutant isoforms in which Ala in the pseudosubstrate sequence is replaced with Glu (PKC-ε A159E, PKC-θ A148E, and PKC-α A25E) to introduce a negative charge that mimics the presence of a phosphate at this location are constitutively activated, since these mutants show elevated basal kinase activity (65, 70, and 80%, respectively) in the absence of any cofactors (Table 1).

Immunoblot analysis with PKC isoform-specific antisera detected ~80-kDa (PKC-θ) or ~82-kDa (PKC-α) proteins, respectively, in COS-1 cells transfected with the corresponding PKC expression plasmids but not in cells transfected with pTag-p56^{lck}, pTag-p59^{fyn} (2), or a vector control (Fig. 1). A reduced level of mutant PKC-θ A148E and PKC-α A25E per cell equivalent (ca. 25 to 30% of wild-type levels) was consistently obtained after cell lysis and Ni^{2+}-chelating resin purification, presumably because of enhanced sensitivity to proteolysis or translocation to 1% Nonidet P-40 lysis buffer-nonextractable cellular compartments (data not shown). To compensate for this reduced yield, three times more COS-1 cell equivalents (in comparison with PKC wild-type preparations) of the A/E mutants were used in the Western blot (immunoblot) analysis in Fig. 1. Transient COS-1 cell transfection with pRc/PKC-ε wild-type (wt) and pRc/PKC-εA159E mutant expression plasmids did demonstrate high PKC-ε protein expression, as shown by immunoblot analysis with PKC-ε-specific antibodies (data not shown). pRc/PKC-ζ wt plasmid demonstrated high expression in COS-1 cell transfection assays, as previously described by Diaz-Meco et al. (13). Furthermore, COS-1 cell-derived recombinant PKC-ζ wt protein demonstrated high enzymatic activity in vitro in a standard immunocomplex kinase assay (data not shown).

IL-2 production in stably transfected human leukemic T-cell lines. IL-2 promoter activation requires antigen receptor engagement plus an accessory signal usually supplied by the antigen-presenting cell. T-cell activation in a human leukemic T-cell line, Jurkat E6-1, can be mimicked by agents that bypass the receptors, such as PMA and soluble anti-human CD3 monoclonal antibody OKT3. Jurkat cells were stably transfected with the pRc/CMV vector control, or His_6-tagged PKC-θ and PKC-α pRc/CMV cDNA plasmid constructs and G418 drug-resistant cell mass populations were expanded and analyzed for activation-induced IL-2 cytokine secretion. The expression of transfected PKC-θ and PKC-α was assessed by immunoblotting of Ni^{2+}-chelating resin precipitates, employing PKC isoform-specific antibodies (Fig. 2B and C). Comparable levels of recombinant PKC were detected in PMA/OKT3-stimulated Jurkat cells, representing approximately fourfold overexpression relative to the levels of endogenous PKC-θ or PKC-α isoforms, respectively. Recombinant expression from the CMV promoter, however, was significantly lower in nonactivated Jurkat E6-1 cells, suggesting that the CMV immediate-early promoter, driving the expression of PKC transgenes in pRc/CMV, requires activation signals for efficient transcription (1a).

IL-2 titers in test culture supernatants were determined as described previously (3). Figure 2A demonstrates that overexpression of either PKC-θ or PKC-α in Jurkat cells promotes a marked increase in PMA/OKT3-induced IL-2 production (6.5- or 6.0-fold over the vector control, respectively; PMA and soluble monoclonal antibody OKT3 at final concentrations of 10 ng/ml and 2 μg/ml, respectively). PMA alone did not stimulate IL-2 production in any of the transfected Jurkat cell populations. These results indicate that both PKC-θ and PKC-α provide signals in the pathway leading to IL-2 production.

Transient overexpression of distinct PKC isoforms in murine EL4 thymoma cells. Next, we decided to use transient-transfection assays to further investigate the cellular signaling cascade(s) involving these two representative PKC family members. Requirements for activation of an IL-2 promoter/CAT reporter construct transfected into T-cell lines were

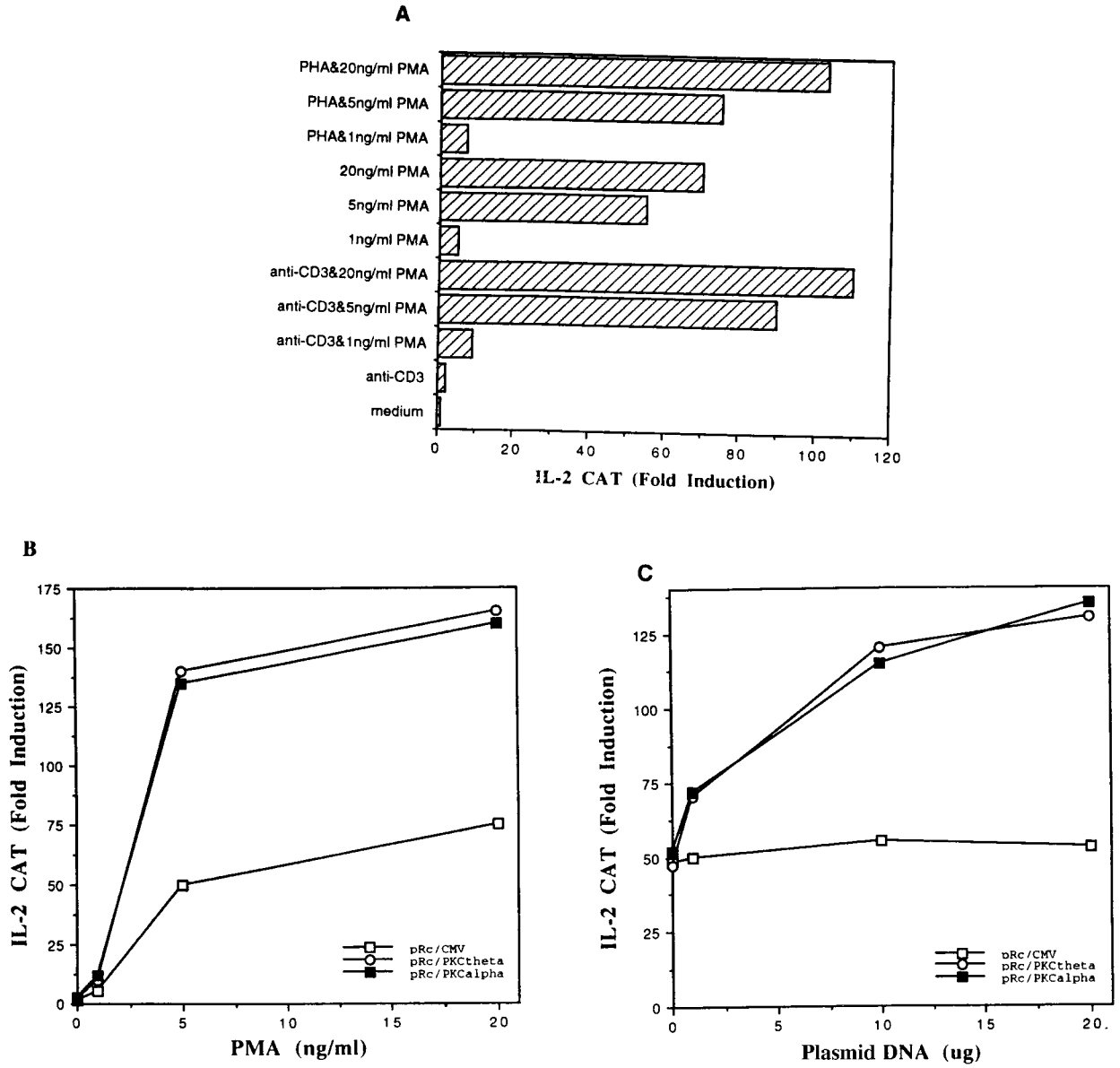


FIG. 3. Enhanced PMA-induced IL-2–CAT gene expression in EL4 thymoma cells by transient overexpression of PKC-θ or PKC-α. (A) EL4 cells were transfected with 5 μg of pIL-2/0–CAT. At 24 h posttransfection, the cells were incubated for another 24 h with the various amounts of PMA, 100 μg of anti-(mouse CD3) 2C11 monoclonal antibody per ml used to coat plates, 5 μg of phytohemagglutinin (PHA) per ml, or medium (as a control), as indicated. (B) EL4 cells were transfected with 5 μg of pIL-2/0–CAT plus 20 μg of pRc/PKC-θ, 20 μg of pRc/PKC-α, or 20 μg of the pRc/CMV vector control. At 24 h posttransfection, the cells were stimulated for 24 h with different amounts of PMA, as indicated. (C) EL4 cells were transfected with 5 μg of pIL-2/0–CAT plus different amounts of pRc/PKC-θ, pRc/PKC-α, and pRc/CMV vector control plasmid DNAs as indicated; however, the total amount of plasmid DNA used for each transfection was previously adjusted to 20 μg with empty vector pRc/CMV. At 24 h posttransfection, cells were stimulated for 24 h with 5 ng of PMA per ml. In all panels, at 48 h posttransfection, cells were harvested and CAT expression was determined as described in Materials and Methods.

shown to faithfully reflect those of the endogenous IL-2 gene (31, 44). We used the well-studied murine EL4 thymoma cell line, since in contrast to Jurkat E6-1 cells (Fig. 2A), phorbol ester alone, a well-characterized PKC activator, is capable of inducing high-level IL-2 gene expression in this cell line (6, 7, 31, 44) in the absence of a second signal such as phytohemagglutinin or anti-CD3 antibody. To test activation of the IL-2-promoter, murine EL4 thymoma cells were transfected with 5 μg of pIL-2/0-CAT, stimulated for 24 h with the indicated stimuli, harvested, and analyzed for CAT expression. The PMA-induced transcriptional activity of pIL-2/0-CAT was significant and ≥60% of that induced by stimulation with immobilized antibodies to CD3 plus PMA or phytohemagglutinin plus PMA (Fig. 3A).

To investigate the potential role of PKC-θ and PKC-α in induction of the IL-2 gene, EL4 cells were transiently cotransfected with cDNA constructs of PKC-θ or PKC-α and the pIL-2/0-CAT reporter (PKC expression plasmid was used in fourfold molar excess to statistically ensure cotransfection in each cell, which harbors the promoter/CAT reporter gene construct) and cells were subsequently stimulated for 24 h with PMA or left unstimulated, lysed, and assayed for CAT expression. To allow a comparison between different experiments, which vary with regard to transfection efficiencies, the different

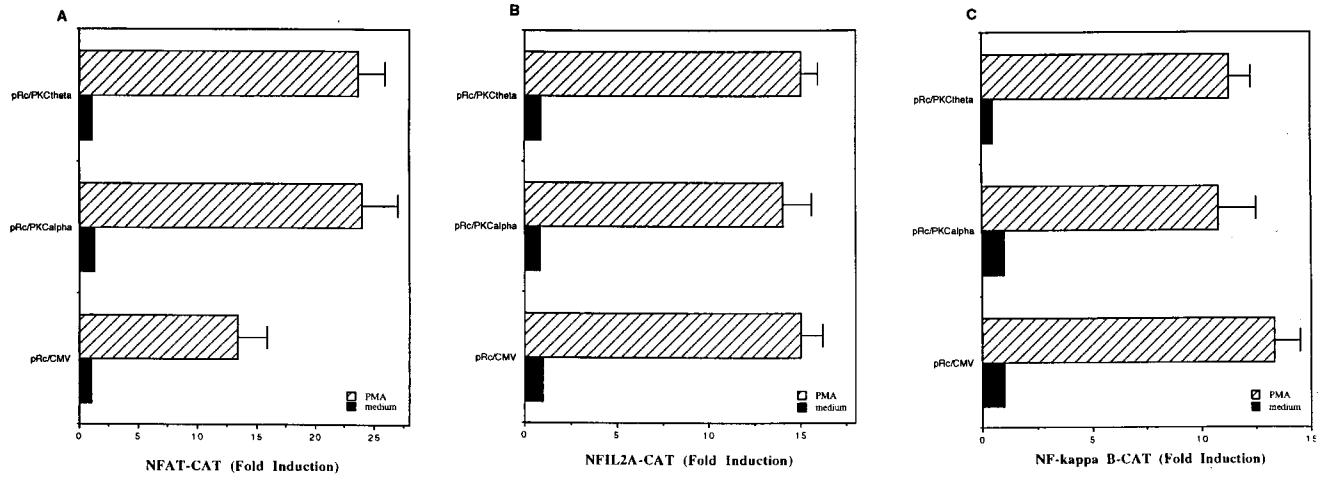


FIG. 4. Effect of transient overexpression of PKC-θ or PKC-α on NF-AT, NF-IL2A and NF-κB–CAT enhancer/promoter CAT reporter elements in EL4 thymoma cells. EL4 cells (2×10^7 per assay point) were transfected with 5 μg of NF-AT–CAT (A), 5 μg of NF-IL2A–CAT (B), or 5 μg of NF-κB-CAT (C) plus 20 μg of either pRc/PKC-θ, pRc/PKC-α, or pRc/CMV vector control plasmid DNA as indicated. At 24 h posttransfection, the cells were stimulated in the absence (medium control) or presence of 5 ng of PMA per ml for an additional 24 h. At 48 h posttransfection, EL4 cells were harvested, and CAT expression was determined as described in Materials and Methods.

cell populations to be tested were cotransfected with 1 μg of a β-galactosidase expression plasmid, pCMVβgal, and results of enhancer/promoter activation (i.e., CAT reporter protein expression) were normalized to the corresponding β-galactosidase reporter. PMA titration indicated that concentrations as low as 1 ng of PMA per ml caused a detectable increase in transcriptional activity. Cotransfection with either PKC-θ or PKC-α augmented the PMA-induced IL-2 CAT transcriptional activity (Fig. 3B), and this effect increased as a function of the amount of PKC expression plasmid (Fig. 3C). Maximal PKC-induced effects were obtained with 20 μg of PKC expression plasmid and 5 ng of PMA per ml (e.g., 2.7- or 2.8-fold for PKC-θ and PKC-α, respectively), and these conditions were subsequently used in the following experiments. This enhanced transcriptional activation of IL-2 CAT was completely dependent on the kinase activity of PKC, since inactive kinase-dead PKC-θ K409R and PKC-α K368R did not demonstrate any stimulatory effect on PMA-induced IL-2 CAT expression (data not shown). These results are consistent with the effects of overexpressed PKC-α or PKC-θ on IL-2 production in stably transfected Jurkat cells (Fig. 2A) and demonstrate that PKC-α and PKC-θ enhance IL-2 production through a direct effect on IL-2 promoter transcription, suggesting that their enzymatic activity is likely to influence the transcriptional activity of one or more of the nuclear binding factors regulating IL-2 gene expression.

To examine the response of defined enhancer elements within the IL-2 promoter to PKC-α or PKC-θ overexpression, EL4 cells were transiently transfected with IL-2 promoter-derived enhancer/promoter reporter gene constructs. Synthetic multimers of the NF-IL2A and NF-AT elements (14, 45), an NF-κB–CAT construct (29, 42), and $^{-73/+60}$Col-CAT, a construct composed of a CAT reporter gene under the control of a collagenase (Col) promoter that contains a single AP-1 phorbol ester response element (8, 48) were used for this purpose.

As shown in Fig. 4, transient overexpression of both PKC-θ and PKC-α in EL4 thymoma cells caused an ≈2.0-fold increase in the PMA-induced transcriptional activation of the NF-AT promoter-CAT (Fig. 4A) reporter gene but did not significantly affect PMA-induced transcriptional activation of NF-IL2A (Fig. 4B) or NF-κB promoter-CAT reporter genes (Fig. 4C). Similarly, a control Rous sarcoma virus-CAT promoter-reporter element was not significantly modulated in its PMA-dependent transcriptional activity by PKC-θ and PKC-α overexpression compared with the vector control (data not shown). Thus, PKC-θ and PKC-α overexpression was not a general stimulator of transcription.

Overexpression of PKC-θ but not PKC-α leads to an enhanced PMA-dependent stimulation of $^{-73/+60}$Col-CAT in EL4 cells. Since the activation of AP-1 CAT expression in response to PMA depends largely on the copy number of the AP-1 enhancer (20, 49), we decided to use $^{-73/+60}$Col-CAT (8), which contains only a single copy of an AP-1-binding site, to study AP-1 transcriptional activation under more physiological conditions. It was previously shown that the collagenase promoter $^{-73/+60}$Col-CAT, but not synthetic promoters containing multiple AP-1 sites, accurately reflects AP-1 transcriptional activity in T lymphocytes (49).

Interestingly, and in contrast to the previous promoter elements used, PKC-θ but not PKC-α cotransfection induced a significant (2.85-fold) increase in the PMA-dependent transcriptional activity of $^{-73/+60}$Col-CAT over that of the PMA-stimulated pRc/CMV vector control (Fig. 5A). To account for the dependence of the PKC-α isoform on Ca^{2+} as cofactor, the Ca^{2+} ionophore A23187 was used for stimulation; however, PKC-α overexpression did not synergize with A23187 (1 μg/ml) alone or A23187 (1 μg/ml) in combination with PMA (5 ng/ml) to induce $^{-73/+60}$Col-CAT promoter/reporter gene expression in comparison with a vector control (data not shown). Deletion of the AP-1 enhancer sequence rendered $^{-73/+60}$Col-CAT unresponsive to PKC-θ-generated signals, since the $^{-60/+60}$Col-CAT reporter (lacking an AP-1-binding site) was induced by PMA to a lesser degree and the PKC-θ-specific effect was abolished (Fig. 5B).

Neither overexpression of PKC-ε nor overexpression of PKC-ζ (a phorbol ester-nonresponsive isoenzyme) had a significant effect on the PMA-induced transcriptional activation of $^{-73/+60}$Col-CAT in EL4 cells (data not shown). Therefore, of these four representative PKC isoforms, PKC-θ is the only PKC isoenzyme capable of significantly enhancing PMA-dependent AP-1 transcriptional activation in EL4 cells. Ectopic expression of PKC-θ in COS-1 cells was used to confirm the

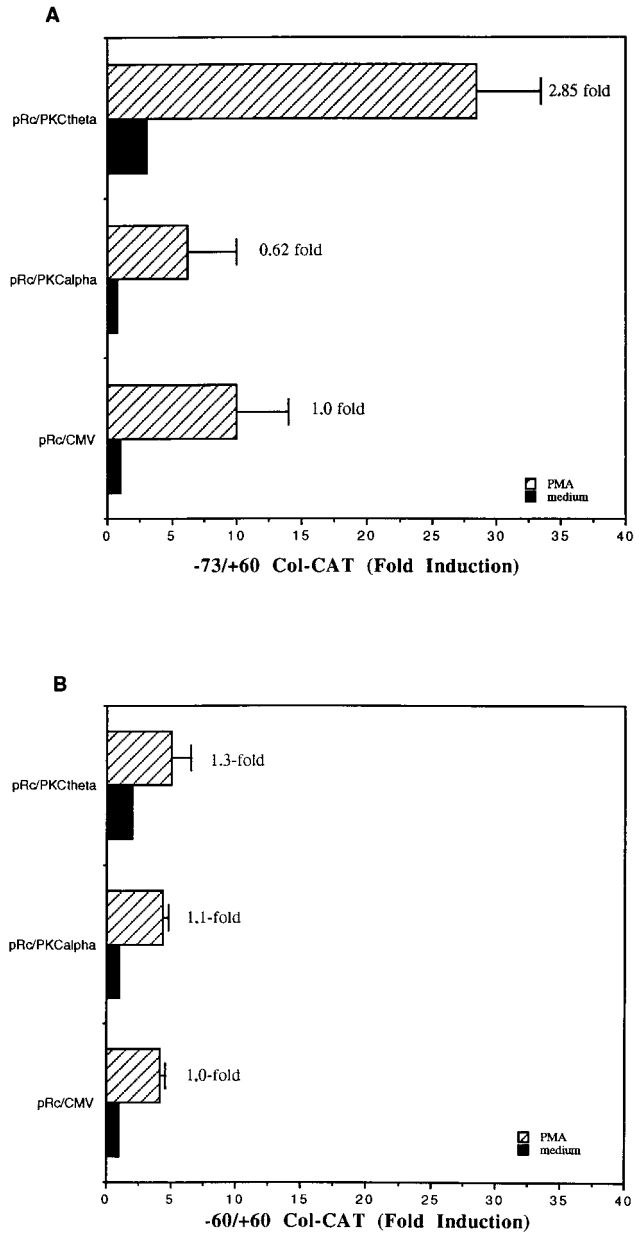


FIG. 5. Overexpression of PKC-θ enhances PMA-dependent stimulation of AP-1 CAT in EL4 thymoma cells. EL 4 cells (2×10^7 per assay point) were cotransfected with 5 μg of the $^{-73/+60}$Col-CAT (A) or $^{-60/+60}$Col-CAT (B) reporter plasmid plus 20 μg of the indicated pRc/PKC-θ or pRc/PKC-α wild-type expression or pRc/CMV vector control plasmid. At 24 h after transfection, the cells were left unstimulated (medium control) or were stimulated with 5 ng of PMA per ml for an additional 24 h, and CAT expression was determined as described in Materials and Methods.

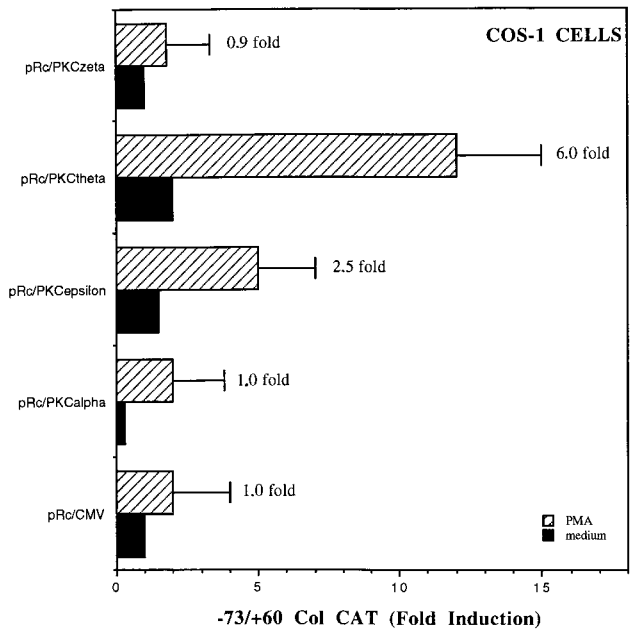


FIG. 6. Ectopic expression of PKC-θ wild type in COS-1 cells enhances PMA-dependent stimulation of AP-1 CAT. COS-1 cells were cotransfected with 5 μg of the $^{-73/+60}$Col-CAT reporter plasmid plus 20 μg of the indicated pRc/PKC-ζ, pRc/PKC-θ, pRc/PKC-ε, or pRc/PKC-α wild-type expression plasmid or the pRc/CMV vector control. At 18 h after serum starvation, the cells were left unstimulated (medium control) or were stimulated with 100 ng of PMA per ml for an additional 24 h, and CAT expression was determined as described in Materials and Methods.

PKC-θ isoenzyme-specific stimulation of AP-1 CAT in a nonhematopoietic cell line. By comparison with PKC-α, PKC-ε, and PKC-ζ, PKC-θ induced a markedly higher increase in PMA-responsive AP-1 CAT expression (6.0-fold) relative to that of the vector control (Fig. 6).

Expression of a constitutively activated PKC-θ A148E mutant induces PMA-independent transcriptional AP-1 activation. To demonstrate that PKC-θ by itself is sufficient to induce AP-1 CAT expression, we expressed the activated form PKC-θ A148E in resting EL4 cells. Expression of PKC-θ A148E resulted in a significant increase in AP-1 CAT expression in the absence of PMA stimulation when compared with that in PKC-ε A159E, PKC-α A25E, or vector control transfections (Fig. 7). This activation level, however, was suboptimal, since it could be further increased by PMA stimulation (Fig. 7), indicating either a submaximal activation status of PKC-θ A148E (as suggested by the a basal activity of only ≈70% of this mutant enzyme in vitro [Table 1]) or the requirement of additional, PKC-θ-independent PMA-responsive signaling events for full activation of AP-1 CAT transcription in these cells. Transient expression of oncogenic RasL61, a constitutively active Ras mutant, induced basal AP-1 CAT expression ~5-fold (compared with the ~14-fold induction over basal activity by PKC-θ A148E), and no significant synergistic collaboration of PKC-θ A148E and RasL61 on AP-1 CAT expression in transient-coexpression experiments in resting EL4 cells could be observed (data not shown).

Expression of kinase-dead PKC-θ K409R mutant abrogates PMA-mediated AP-1 transcriptional activation in EL4 cells. Consistent with the above finding, a kinase-dead PKC-θ K409R mutant (whose expression was driven by the CMV immediate-early promoter in the pRc/CMV plasmid) significantly inhibited endogenous PMA-mediated activation of AP-1 CAT expression in EL4 cells (Fig. 8A). Almost complete abrogation of the PMA signal could be obtained by the inhibitory PKC-θ K409R mutant once PKC-θ K409R expression was driven by the stronger EF-1α promoter (Fig. 8B), which further increased the recombinant expression level of PKC-θ K409R in EL4 cells ≈fivefold (1a). Again, this effect was isoenzyme specific, since similar PKC-α K368R mutant expression plasmids did not demonstrate a significant effect in this particular signaling pathway (Fig. 8). Transcriptional activity of NF-IL2A–CAT was unaffected by EF-1α promoter-driven expres-

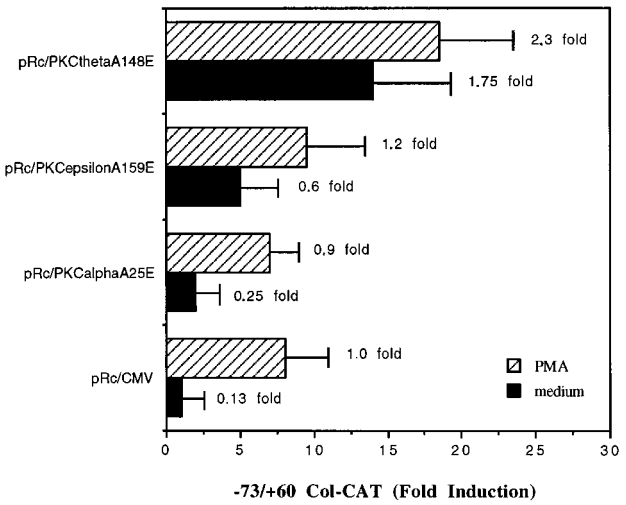
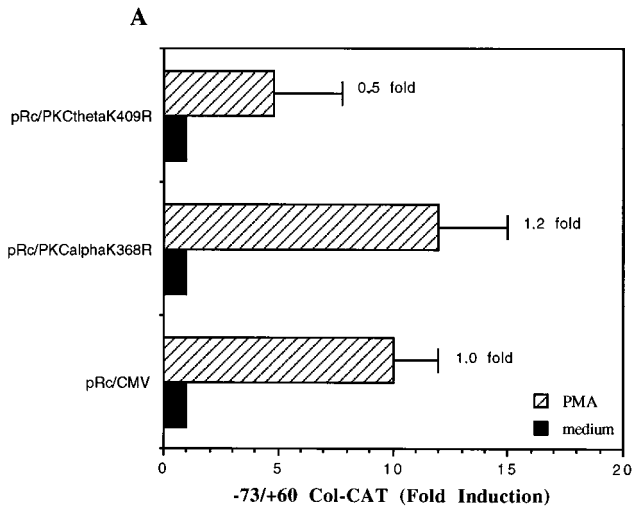


FIG. 7. Constitutively activated PKC-θ A148E mutant can replace PMA in stimulation of AP-1 CAT in resting EL4 cells. EL4 cells (2×10^7 per assay point) were cotransfected with 5 μg of the $^{-73/+60}$Col-CAT reporter plasmid plus 20 μg of the indicated pRc/PKC-θ A148E, pRc/PKC-ε A159E, pRc/PKC-α A25E, or pRc/CMV vector control plasmid. At 24 h after transfection, the cells were left unstimulated or were stimulated with 5 ng of PMA per ml for an additional 24 h, and CAT expression was determined as described in Materials and Methods. The relative fold induction values over that for the PMA-stimulated pRc/CMV vector control are given.



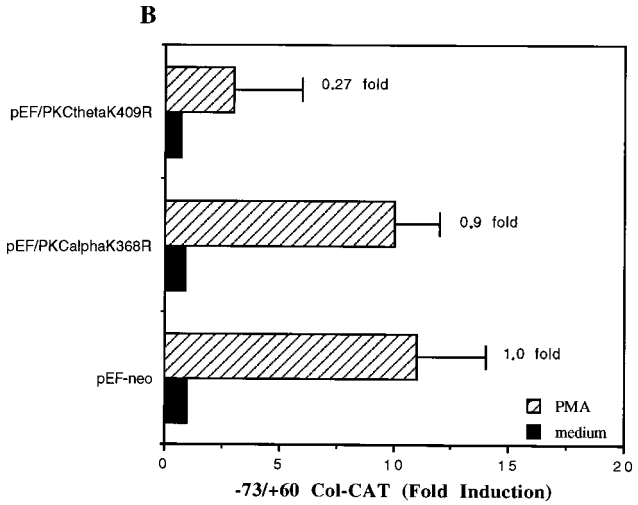


FIG. 8. Dominant negative PKC-θ K409R mutant blocks PMA in stimulation of AP-1 CAT in EL4 cells. EL 4 cells (2×10^7 per assay point) were cotransfected with 5 μg of the $^{-73/+60}$Col-CAT reporter plasmid plus 20 μg of the indicated pRc/PKC-θ K409R, pRc/PKC-α K368R, and pRc/CMV (A) or pEF/PKC-θ K409R, pEF/PKC-α K368R, and pEF-neo vector control plasmids (B). At 24 h after transfection, the cells were left unstimulated or were stimulated with 5 ng of PMA per ml for an additional 24 h, and CAT expression was determined as described in Materials and Methods.

sion of the inhibitory PKC-θ K409R mutant (data not shown), demonstrating that PKC-θ K409R is not a general inhibitor of transcription.

Dominant negative Ha-RasN17 blocks the PKC-θ A148E-mediated AP-1 CAT signal in EL4 cells. To determine the role of Ras in the PKC-θ-mediated signal leading to AP-1 CAT transcription, cotransfection of dominant-negative mutant Ha-RasN17, which blocks the activation of normal Ras protein, with PKC-θ at a ratio of 1:1 was performed. The plasmid pEF/RasN17 used in these experiments has been previously characterized (19). As shown in Fig. 9, coexpression of dominant-negative mutant Ha-RasN17 completely inhibited PMA-dependent PKC-θ wild-type and PKC-θ A148E-induced, PMA-independent AP-1 CAT signals, placing PKC-θ either upstream of or parallel to Ras in the signaling cascade.

DISCUSSION

The fact that T cells express several different species of PKC isoenzymes makes it difficult to determine the specific cellular functions of individual isoenzymes. Therefore, the involvement of PKC, a major phorbol ester receptor, in the transcriptional regulation of phorbol ester-inducible genes was determined via overexpression of distinct and representative PKC isoenzymes. In the present studies, three distinct forms of PKC-α or PKC-θ constructs, encoding (i) wild-type PKC, (ii) inhibitory kinase-dead PKC, and (iii) pseudosubstrate mutant PKC protein, were used; the latter produce cofactor-independent and therefore constitutively activated kinases. These mutant PKC isoenzymes with different biological and biochemical properties provide useful tools for investigation of the signal transduction events in the cell involving the given PKC isoform. Similar mutations in the kinase domain for PKC-α, PKC-ε, and PKC-ζ isoenzymes or in the regulatory pseudosubstrate domain for PKC-α, PKC-δ, PKC-ε, PKC-η, and PKC-ζ isoenzymes have been successfully created (12, 13, 16, 17, 21, 32, 38, 41).

The IL-2 gene promoter, most of the transcription factors that activate it, and the signals that regulate its activity are well characterized. Therefore, measurements of IL-2 gene activation represent a physiologically relevant end point assay to investigate the effect(s) of overexpressed PKC isoenzyme-mediated perturbations in the T-cell-signaling pathway. Potential effects of representative PKC isoforms on gene regulation have been analyzed in transient-cotransfection studies in which distinct PKC wild-type or mutant cDNA expression constructs described above and promoter/CAT reporter genes were cotransfected into the same cell. Using this experimental assay system, we were able to examine the activation of each PKC isoenzyme individually in intact cells, in direct comparison with the vector control. The observed ≈2.5- to 3-fold increase in, e.g., IL-2/CAT expression in PKC-overexpressing EL4 cells suggests that the PMA-induced transcriptional activity is ex-

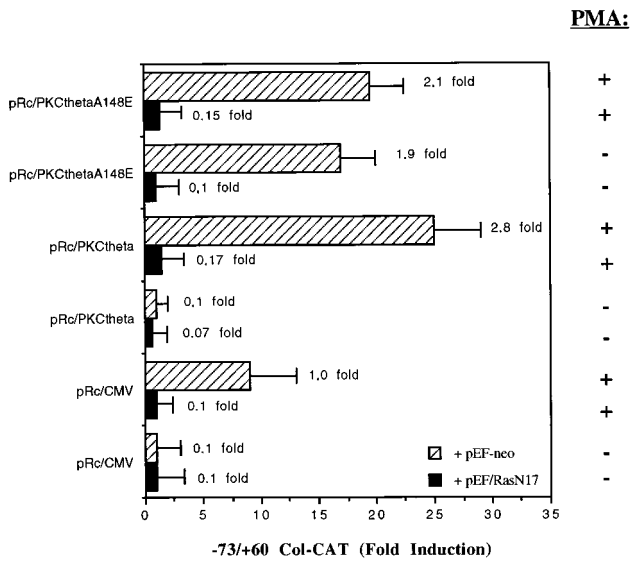


FIG. 9. Dominant negative mutant Ha-RasN17 blocks PMA-dependent PKC-θ wild-type and PMA-independent, PKC-θ A148E-mediated stimulation of AP-1 CAT in EL4 cells. EL 4 cells (2×10^7 per assay point) were cotransfected with 5 μg of the $^{-73/+60}$Col-CAT reporter plasmid, 10 μg of pRc/PKC-θ wild type, pRc/PKC-θ A148E, and pRc/CMV plus either 10 μg of pEF-neo or 10 μg of pEF/RasN17. As indicated on the right, at 24 h after transfection, the cells were stimulated with 5 ng of PMA per ml (+) or left unstimulated (−) for an additional 24 h, and CAT expression was determined as described in Materials and Methods. Relative fold induction values over that for the PMA-stimulated pRc/CMV vector control are given.

tremely sensitive to the given amount of PKC isoenzymes in the cell, suggesting that the level of distinct PKC isoenzymes may be a limiting factor in cellular activation events.

We conclude from these studies that PKC-θ is competent to mediate AP-1 CAT transcriptional activation and differs from PKC-α in its competence to participate in the AP-1 signaling cascade. The fact that transient transfection of PKC-α induces the activation of IL-2 or NF-AT CAT in a fashion similar to that of PKC-θ excludes the possibility that the level of PKC-α expression or its protein stability in intact cells is significantly lower than that of PKC-θ. It appears to be of physiological importance that the functions of these two representative PKC family members are on the one hand partially distinct (with regard to AP-1) but on the other hand partially overlapping (with regard to NF-AT, NF-κB, and NF-IL2A). Recent work indicates that PKC-δ, the closest relative of PKC-θ (the isoforms demonstrate a striking sequence similarity in the V1 and pseudosubstrate regions [for PKC amino acid alignments, see reference 5]), causes specific activation of AP-1 CAT in a Ras-dependent manner in NIH 3T3 fibroblasts (21). Consistent with our findings in the EL4 cell model, PKC-α and PKC-ε did not mediate significant AP-1 activation in NIH 3T3 cells (21). Recent data obtained by Genot et al. (16) demonstrate that of PKC-α, PKC-ζ, and PKC-ε, PKC-ε is the major PKC isoform responsible for AP-1 transcriptional activation in Jurkat cells when a synthetic AP-1 CAT, containing multiple copies of AP-1 sites, is used. However, a direct comparison of PKC-ε with either PKC-δ or PKC-θ is missing in this study. Considering this limitation, we did reproduce their findings. Apart from PKC-θ, overexpression of only PKC-ε (not PKC-α or PKC-ζ) caused a 2.5-fold increase in the expression of $^{-73/+60}$Col-CAT (which contains only a single copy of an AP-1-binding site) over a vector control in COS-1 cells (Fig. 6) and the mutationally activated PKC-ε A159E was sufficient to induce $^{-73/+60}$Col-CAT expression in nonstimulated EL4 cells, albeit at a rate significantly lower than that produced by PKC-θ A148E (Fig. 7).

The functional divergence of these representative PKC isoforms provides a rationale to further explain the presence of multiple PKC family members in a given cell. Furthermore, it will permit detailed functional dissection of the complex signaling cascades involving distinct PKC isoenzymes. Undoubtedly, more work is necessary to determine the precise mechanism utilized by PKC-θ to activate AP-1, but our data represent an important step toward the identification and characterization of molecules that operate downstream of PKC-θ and are involved in growth control and/or activation of T cells. The ability of PKC-θ to activate AP-1 transcriptional activity suggests that it may also be sensitive to negative signals that are involved in the induction of anergy (46), since the AP-1 complex may be a unique target in anergic T cells (27). Transgenic mouse models are currently in preparation in our laboratory for use in a study of PKC-θ-mediated AP-1 regulation and to further illuminate the physiological context of this process.

ACKNOWLEDGMENTS

We are grateful to G. R. Crabtree, M. Karin, N. D. Perkins, and J. Moscat for providing pIL-2/0-, NFIL-2A-, NF-AT-, $^{-73/+60}$Col-, $^{-60/+60}$Col-, and NFκB-CAT constructs and pRc/CMV-PKC-ζ.

This work was supported in part by grants from NIH (CA35299), the Austrian Dr. Legerlotz-Stiftung 1994, the Tyrolean D. Swarowski Förderung 1994 and 1995, and the Austrian Fond zur Förderung der wissenschaftlichen Forschung (projects P10530-MED, P10776-MED, and SFB00201, respectively).

REFERENCES

1. **Angel, P., and M. Karin.** 1991. The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation. Biochim. Biophys. Acta **1072:**129–157.
1a. **Baier, G.** Unpublished observations.
2. **Baier, G., G. Baier-Bitterlich, C. Couture, D. Telford, L. Giampa, and A. Altman.** 1994. An efficient expression, purification and immunodetection system for recombinant gene products. BioTechniques **17:**94–99.
3. **Baier, G., G. Baier-Bitterlich, D. Looney, and A. Altman.** 1995. Immunogenic targeting of synthetic human immunodeficiency virus type 1 peptide vaccines to antigen-presenting cells by chimeric anti-major histocompatibility complex class II and anti-secretory immunoglobulin D antibodies in vitro. J. Virol. **69:**2357–2365.
4. **Baier, G., G. Baier-Bitterlich, N. Meller, K. M. Coggeshall, D. Telford, L. Giampa, N. Isakov, and A. Altman.** 1994. Expression and biochemical characterization of PKC-θ. Eur. J. Biochem. **225:**195–203.
5. **Baier, G., D. Telford, L. Giampa, K. M. Coggeshall, G. Baier-Bitterlich, N. Isakov, and A. Altman.** 1993. Molecular cloning and characterization of PKC-θ, a novel member of the PKC gene family expressed predominantly in hematopoietic cells. J. Biol. Chem. **268:**4997–5004.
6. **Baldari, C. T., A. Heguy, and J. L. Telford.** 1993. Ras protein activity is essential for T-cell antigen receptor signal transduction. J. Biol. Chem. **268:**2693–2698.
7. **Baldari, C. T., A. Heguy, and J. L. Telford.** 1993. Calcium-dependent cyclosporin A-sensitive activation by the IL-2 promoter by $p56^{lck}$. J. Biol. Chem. **268:**8406–8409.
8. **Binetruy, B., T. Smeal, and M. Karin.** 1991. Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. Nature (London) **351:**122–127.
9. **Boyle, W. J., T. Smeal, L. H. K. Defize, P. Angel, J. R. Woodgett, M. Karin, and T. Hunter.** 1991. Activation of PKC decreases phosphorylation of c-Jun at sites that negatively regulate its DNA-binding activity. Cell **64:**573–584.
10. **Chang, J. D., Y. Xu, M. K. Raychowdhury, and A. Ware.** 1993. Molecular cloning and expression of a cDNA encoding a novel isoenzyme of PKC (nPKC). J. Biol. Chem. **268:**14208–14214.
11. **Crabtree, G. R.** 1989. Contingent genetic regulatory events in T lymphocyte activation. Science **243:**355–361.
12. **Dekker, L. V., P. McIntyre, and P. J. Parker.** 1993. Mutagenesis of the regulatory domain of rat PKC-η. J. Biol. Chem. **268:**19498–19504.
13. **Diaz-Meco, M. T., E. Berra, M. M. Municio, L. Sanz, J. Lozano, I. Dominguez, V. Diaz-Golpe, M. T. Lain de Lera, J. Alcami, C. V. Paya, F. Arenzana-Seisdedos, J. L. Virelizier, and J. Moscat.** 1993. A dominat negative protein kinase C ζ subspecies blocks NF-κB activation. Mol. Cell. Biol. **13:**4770–1775.

14. **Durand, D. B., J. P. Shaw, M. R. Bush, R. E. Replogle, R. Belagaje, and G. R. Crabtree.** 1988. Characterization of antigen receptor response elements within the interleukin-2 enhancer. Mol. Cell. Biol. **8:**1715–1724.
15. **Erdel, M., G. Baier-Bitterlich, C. Duba, N. Isakov, A. Altman, G. Utermann, and G. Baier.** 1995. Mapping of human PKC-θ gene locus to the short arm of chromosome 10 (10p15) by FISH. Genomics **25:**595–597.
16. **Genot, E. M., P. J. Parker, and D. A. Cantrell.** 1995. Analysis of the role of PKC-α, -ε and -ζ in T cell activation. J. Biol. Chem. **270:**9833–9839.
17. **Goode, N. T., and P. J. Parker.** 1994. A PMA-responsive PKC-ζ generated by fusion with the regulatory domain of PKC-δ. FEBS Lett. **340:**145–150.
18. **Granelli-Piperno, A., and P. Nolan.** 1991. Nuclear transcription factor that binds to elements of the IL-2 promoter: induction requirements in primary human T cells. J. Immunol. **147:**2734–2740.
19. **Gulbins, E., R. Bissonnette, A. Mahboubi, G. Baier, G. Baier-Bitterlich, C. Byrd, R. Kolesnick, D. Green, and A. Altman.** 1995. The Ras signaling pathway mediates Fas-induced apoptosis in T cells. Immunity **2:**341–351.
20. **Hata, A., Y. Akita, K. Suzuki, and S. Ohno.** 1993. Functional divergence of PKC family members. J. Biol. Chem. **268:**9122–9129.
21. **Hirai, S., Y. Izumi, K. Higa, K. Kaibuchi, K. Mizuno, S. Osada, K. Suzuki, and S. Ohno.** 1994. Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP-1/Jun by PKC-δ. EMBO J. **13:**2331–2340.
22. **House, C., and B. E. Kemp.** 1987. PKC contains a pseudosubstrate prototype in its regulatory domain. Science **238:**1726–1728.
23. **Howe, L. R., S. J. Leevers, N. Gomez, S. Nakielny, P. Cohen, and C. J. Marshall.** 1992. Activation of the MAP kinase pathway by the protein kinase raf. Cell **71:**335–342.
24. **Ioannides, C. G., R. S. Freedman, R. M. Liskamp, N. E. Ward, and C. A. O'Brian.** 1990. Inhibition of IL-2 receptor induction and IL-2 production in Jurkat cells by a novel peptide inhibitor of PKC. Cell. Immunol. **131:**242–248.
25. **Jain, J., V. E. Valge-Archer, and A. Rao.** 1992. Analysis of the AP-1 sites in the IL-2 promoter. J. Immunol. **148:**1240–1250.
26. **Jain, J., V. E. Valge-Archer, A. J. Sinskey, and A. Rao.** 1992. The AP-1 site at −150 bp, but not the NFκB site, is likely to represent the major target of PKC in the IL-2 promoter. J. Exp. Med. **175:**853–862.
27. **Kang, S. M., B. Bart, A. C. Tran, K. Brorson, R. H. Schwartz, and M. J. Lenardo.** 1992. Transactivation by AP-1 is a molecular target of T cell clonal anergy. Science **257:**1134–1138.
28. **Kvanta, A., E. Kontny, M. Jondal, S. Okret, and B. B. Fredholm.** 1992. Mitogen stimulation of T-cells increases c-Fos and c-Jun protein levels, AP-1 binding and AP-1 transcriptional activity. Cell. Signalling **4:**275–282.
29. **Leung, K., and G. J. Nabel.** 1988. HTLV-1 transactivator induces IL-2 receptor expression through a NFκB-like factor. Nature (London) **333:**776–778.
30. **Lin, A., J. Frost, T. Dend, N. Al-Alawi, T. Smeal, U. Kikkawa, T. Hunter, D. Brenner, and M. Karin.** 1992. Casein kinase II is a negative regulator of cJun DNA binding and AP-1 activity. Cell **70:**777–789.
31. **Macchia, G., C. T. Baldari, A. Massone, and J. L. Telford.** 1990. A role for PKC activity in interleukin-1 (IL-1) induction of IL-2 gene expression but not in IL-1 signal transduction. Mol. Cell. Biol. **10:**2731–2737.
32. **Maramutsu, M., K. Kaibuchi, and K. Arai.** 1992. PKC mutants in the autoinhibitory region exhibit two distinct properties. FEBS Lett. **311:**75–79.
33. **Meugge, K., T. M. Williams, J. Kant, M. Karin, R. Chiu, A. Schmidt, U. Siebenlist, H. A. Young, and S. K. Durum.** 1989. IL-1 costimulatory activity on the IL-2 promoter via AP-1. Science **246:**249–251.
34. **Mischak, H., J. Goodnight, D. W. Henderson, S. Osada, S. Ohno, and J. F. Mushinski.** 1993. Unique expression pattern of protein kinase C-θ: high mRNA levels in normal mouse testes and in T-lymphocytic cells and neoplasms. FEBS Lett. **326:**51–55.
35. **Mizushima, S., and S. Nagata.** 1990. pEF-BOS, a powerful mammalian expression vector. Nucleic Acids Res. **18:**5322.
36. **Nishizuka, Y.** 1992. Intracellular signaling by hydrolysis of phospholipids and activation of PKC. Science **258:**607–614.
37. **Northrop, J. P., K. S. Ullman, and G. R. Crabtree.** 1993. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific NF-AT complex. J. Biol. Chem. **268:**2917–2923.
38. **Ohno, S., Y. Konno, Y. Akita, A. Yano, and K. Suzuki.** 1990. A point mutation at the putative ATP-binding site of PKC-α abolishes the kinase activity. J. Biol. Chem. **265:**6296–6300.
39. **Osada, S., K. Mizuno, T. C. Saido, K. Suzuki, T. Kuroki, and S. Ohno.** 1992. A new member of the PKC family, nPKC-θ, predominantly expressed in skeletal muscle. Mol. Cell. Biol. **12:**3930–3938.
40. **Paliogianni, F., A. Raptis, S. S. Ahuja, S. M. Najjar, and D. T. Boumpas.** 1993. Negative transcriptional regulation of human IL-2 gene by glucocorticoids through intereference with nuclear transcription factors AP-1 and NF-AT. J. Clin. Invest. **91:**1481–1490.
41. **Pears, C. J., G. Kour, C. House, B. E. Kemp, and P. J. Parker.** 1990. Mutagenesis of the pseudosubstrate site of PKC leads to activation. Eur. J. Biochem. **194:**89–94.
42. **Perkins, N. D., R. M. Schmid, C. Duckett, K. Leung, N. R. Rice, and G. J. Nabel.** 1992. Distinct combinations of NFκB subunits determine the specificity of transcriptional activation. Proc. Natl. Acad. Sci. USA **89:**1529–1533.
43. **Pulverer, B. J., J. M. Kyriakis, J. Avruch, E. Nikolakaki, and J. R. Woodgett.** 1991. Phosphorylation of c-Jun mediated by MAP-kinases. Nature (London) **353:**670–674.
44. **Rayter, S. I., M. Woodrow, S. C. Lucas, D. A. Cantrell, and J. Downward.** 1992. p21^{ras} mediates control of IL-2 gene promoter function in T cell activation. EMBO J. **11:**4549–4556.
45. **Schreiber, S. L., and G. R. Crabtree.** 1992. The mechanism of action of cyclosporin A and FK506. Immunol. Today **13:**136–142.
46. **Schwartz, R. H.** 1990. A cell culture model for T lymphocyte clonal anergy. Science **248:**1349–1356.
47. **Serfling, E., R. Barthelmäs, I. Pfeuffer, B. Schenk, S. Zarius, R. Swoboda, F. Mercurio, and M. Karin.** 1989. Ubiquitous and lymphocyte-specific factors are involved in the induction of the moue IL-2 gene in T-lymphocytes. EMBO J. **8:**465–473.
48. **Smeal, T., B. Binétruy, D. A. Mercola, M. Birrer, and M. Karin.** 1991. Phosphorylation of c-Jun on serines 63 and 73 is required for oncogenic and transcriptional cooperation with Ha-Ras. Nature (London) **354:**494–496.
49. **Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah.** 1994. JNK is involved in signal integration during costimulation of T lymphocytes. Cell **77:**727–736.
50. **Ullman, K., J. Northrop, A. Admon, and G. Crabtree.** 1993. Jun family members are controlled by a calcium-regulated, cyclosporin A-sensitive signalling pathway in activated T lymphocytes. Genes Dev. **7:**188–196.
51. **Ullman, K., J. Northrop, C. L. Verweij, and G. Crabtree.** 1990. Transmission of signals from the TCR to the genes responsible for cell proliferation and immune function: the missing link. Annu. Rev. Immunol. **8:**421–435.
52. **Valge, V. E., J. G. P. Wong, B. M. Datlof, A. J. Sinskey, and A. Rao.** 1988. PKC is required for responses to TCR ligands but not to IL-2 in T cells. Cell **55:**101–109.
53. **Wang, F., U. P. Naik, Y. H. Ehrlich, Z. Freyberg, S. Osada, S. Ohno, T. Kuroki, K. Suzuki, and E. Kornecki.** 1993. A new protein kinase C, nPKCη, and nPKCθ are expressed in human platelets: involvement of nPKCη and nPKCθ in signal transduction by PAF. Biochem. Biophys. Res. Commun. **191:**240–246.