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Regulation of IL-4 lymphokine gene expression and cellular proliferation in murine T helper type II cells

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Activation of T cells results in the production of lymphokines and cellular proliferation. Protein kinase C (PKC) plays a key role in this process. It has been shown that this enzyme is essential to elicit a response to Con A or specific antigen in CD4$^+$ T helper type 1 (Th 1) cells that secrete IL-2. We have now explored the signal transduction pathway that leads to transcription of the IL-4 gene and proliferation in murine CD4$^+$ T helper type 2 (Th 2) cells. Surprisingly, we have found in two independently derived Th 2 clones that neither cellular proliferation nor IL-4 lymphokine production is affected by blocking or depletion of PKC. This differential mechanism of signal transmission leading to cellular activation implies a new distinction between murine Th 1 and Th 2 cells.

Introduction

The activation of T cells by specific antigen, mitogen, or monoclonal antibodies directed against certain cell surface proteins induces the hydrolysis of phosphatidylinositol 4′5′, biphosphate (PIP_2), mediated by phospholipase C (Wilson *et al.*, 1984; Bennett and Crooke, 1987). This hydrolysis leads to the production of second messengers such as diacylglycerol (DAG) and inositol triphosphate ($InsP_3$) (Berridge, 1987). $InsP_3$ increases the concentration of intracellular calcium ($[Ca^{2+}]_i$) (Carafoli, 1987), whereas DAG is an essential cofactor for the activation of the enzyme protein kinase C (PKC) (Kikkawa and Nishizuka, 1986). It has been postulated that the combination of both effects leads to production of lymphokines, expression of lymphokine receptors, and cellular proliferation (Mills *et al.*, 1985; Isakov *et al.*, 1987).

In the murine system two types of CD4$^+$ T helper (Th) cells have been defined, namely Th 1 and Th 2 cells, that differ in their lymphokine production and immune function (reviewed by Mosmann and Coffman, 1987). Whereas Th 1 cells produce IL-2, γ-IFN, and lymphotoxin on activation, Th 2 cells secrete IL-4 and IL-5 (Cherwinsky *et al.*, 1987). Recently, it has been reported that in Th 1 cells PKC is essential for IL-2 production and proliferation in response to T cell receptor (Tcr)-mediated activation but not for proliferation in response to exogenous IL-2 (Mills *et al.*, 1988; Valge *et al.*, 1988).

The mechanism of signal transduction in Th 2 cells after Tcr-mediated stimulation has not been elucidated yet. Using two different systems to block the function of PKC, we have found that this enzyme plays a different regulatory role in Th 2 than in Th 1 cells; namely, neither IL-4 lymphokine production nor cellular proliferation is dependent on the presence of PKC in Th 2 cells. These results support the idea that the differentiation of CD4$^+$ T cells into Th 1 or Th 2 cells is an event that occurs at the level of second messengers generated by the activation of differential signaling pathways.

Results

Calcium ionophores induce IL-4 mRNA in D10 cells

We were interested in determining what signals lead to IL-4 mRNA expression in the prototype Th 2 cell, D10.G4.1 (D10). There is general agreement that activation of T cells through the Tcr leads to an increase in $[Ca^{2+}]_i$ as well as activation of PKC. This physiological activation signal can be mimicked by the use of ionophores in conjunction with phorbol esters (Truneh *et al.*, 1985). We cultured resting D10 cells for 6 h with these agents and measured IL-4 mRNA expression in a Northern blot. As shown in Figure 1, ionomycin alone (but not PMA alone) is able to induce IL-4 message. Synergy is seen with the combination of the two agents, resulting in IL-4 mRNA levels similar to those induced by Con A stimulation. Another Ca^{2+} ionophore, A23187, also induced IL-4 steady-state message in D10 cells in a dose-dependent manner, supporting the idea that the IL-4 gene is positively regulated by a Ca^{2+}-dependent pathway.

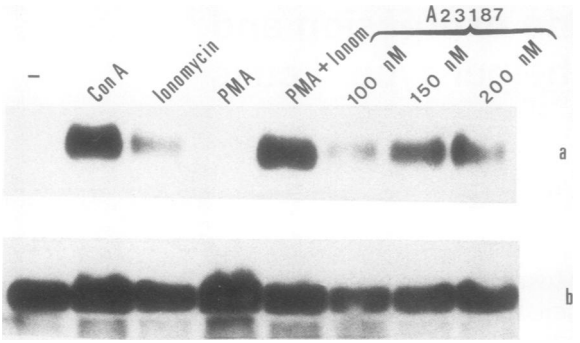

Figure 1. **IL-4 mRNA expression in D10 cells after stimulation with Con A (5 mg/ml), ionomycin (0.5 μg/ml), PMA (10 ng/ml), PMA plus ionomycin, or A23187.** Total RNA was extracted 6 h after activation, run on a Northern gel, and hybridized with (a) IL-4 cDNA and (b) β-Actin cDNA.

It is possible that a high level of $[Ca^{2+}]_i$ leads to activation of PKC (for review see Nishizuka, 1984). For this reason, we analyzed the effect of calcium ionophores in the presence of the agent H-7 (1-(5-isoquinolinyl-sulfonyl)-2-methylpiperazine dihydrochloride), which has been reported to be a potent inhibitor of PKC (Hidaka *et al.,* 1984). The chemical analogue HA1004 was used as control; it is an efficient inhibitor of cAMP- and cGMP-dependent kinases but a poor inhibitor of PKC. As can be seen in Figure 2, H-7 had no effect on the level of IL-4 mRNA induced by ionomycin. These results strongly suggest that IL-4 message can be induced by elevated $[Ca^{2+}]_i$ levels in the absence of PKC activation.

IL-4 mRNA is superinduced in Con A–activated D10 cells in the absence of functional PKC

From the previous results we conclude that activation of PKC alone is insufficient to induce detectable levels of IL-4 mRNA, but activation results in synergy when used in conjunction with ionophores. We therefore tested the role of PKC in the expression of the IL-4 gene after Tcr-mediated activation of Th 2 cells; namely, we compared IL-4 steady-state message in Con A–stimulated D10 cells in the presence of the PKC inhibitor H-7 or the analogue HA1004. The mitogen Con A has been shown by immunoprecipitation to bind to the Tcr proteins (Chilson and Kelly-Chilson, 1989) and to stimulate murine T cells at least in part through the Tcr (Valge *et al.,* 1988). Total RNA was extracted 3 and 24 h after addition of Con A. Figure 3 shows a Northern blot of the resulting mRNAs hybridized with an IL-4 probe. Three hours after activation, the IL-4 transcript level is clearly lower in cells cultured in the presence of H-7 compared with HA1004 (Figure 3A). However, after 24 h, D10 cells have significantly more IL-4 mRNA in the presence than in the absence of H-7 (Figure 3B). These findings suggest that PKC plays a dual role in IL-4 mRNA accumulation. Initially it has a positive effect, whereas later on it negatively influences IL-4 message levels.

IL-4 mRNA is induced in PKC-depleted, Con A–activated D10 cells

To confirm the results obtained with the agent H-7, we attempted to chronically deplete D10 cells of functional PKC. This can be achieved in most cell types by prolonged exposure to phorbol esters such as PMA (Mizuguchi *et al.,* 1987; Nishimura *et al.,* 1987). For this purpose we incubated D10 cells with 200 nM PMA for 48 h and then measured the total activity of PKC in a cell-free system in the presence of phosphatidyl-serine, Ca^{2+}, and PMA. The results are shown in Figure 4A. PKC phospho-transferase activity of $[\gamma^{32}P]$-ATP into histone was measured in cell extracts of control and PMA-pretreated D10 cells. In three separate experiments the control PKC activity ranged between 34.7 and 38.8 pmol/min/mg of protein, whereas the activity in PMA-pretreated cells was between 1.0 and 2.01 pmol/min/mg of protein. The cellular viability after PMA pretreatment was 100% (data not shown).

The enzyme PKC seems to be the substrate for phorbol esters (Bell, 1986). The binding sites

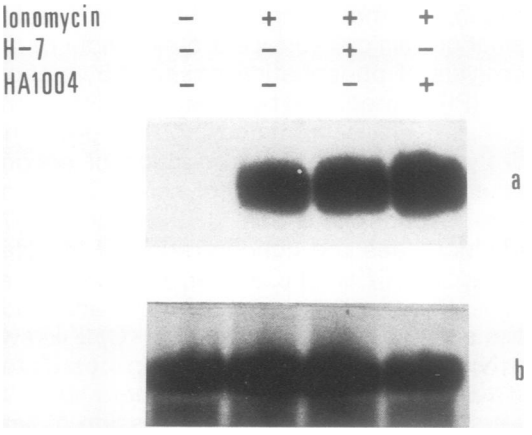

Figure 2. **Effect of H-7 and its analogue HA1004 on ionomycin-induced IL-4 mRNA expression.** D10 cells were stimulated for 6 h with ionomycin (0.5 μg/ml) in the presence or the absence of either H-7 (25 μM) or HA1004 (25 μM). Total RNA was extracted and 20 μg per lane were run on a Northern gel. The blots were hybridized with (a) IL-4 cDNA and (b) β-Actin cDNA.

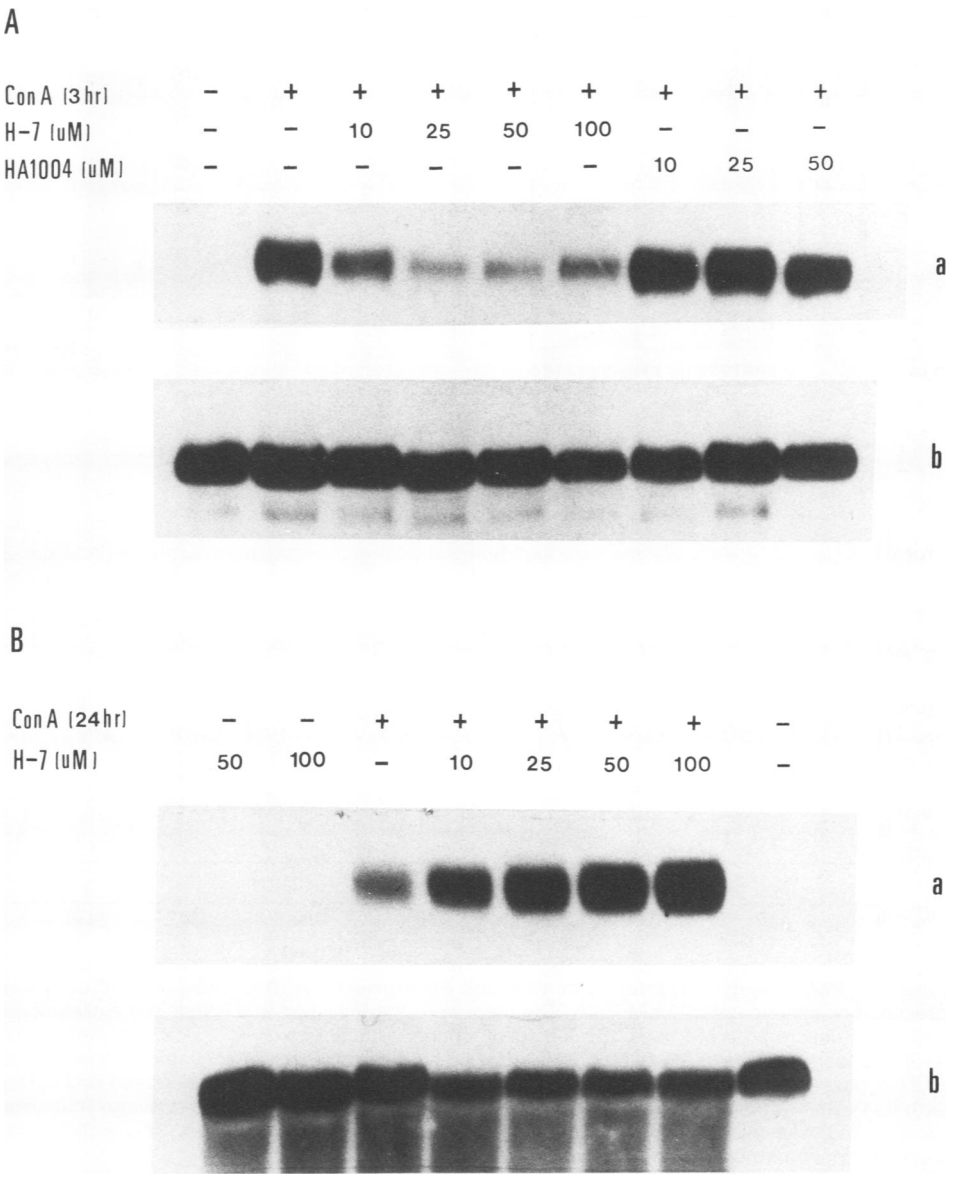

Figure 3. **Effect of H-7 and HA1004 on the IL-4 mRNA expression in Con A–stimulated D10 cells.** D10 cells were stimulated with Con A in the presence or absence of increasing doses of either H-7 or HA1004. Total RNA was extracted 3 h (A) and 24 h (B) after activation and run on a Northern gel. The blots were hybridized with (Aa and Ba) IL-4 cDNA and (Ab and Bb) β-Actin cDNA.

for phorbol esters can be estimated by the use of [^{3}H] phorbol dibutyrate (PDB) (Sando *et al.*, 1981). We therefore measured the level of [^{3}H] PDB binding in PMA-pretreated and control D10 cells in the presence or absence of increasing amount of the cold competitor PMA (Figure 5). The results show that PMA-pretreated cells express no significant binding sites for PDB. Although in control cells a dose-dependent inhibition of binding of radioactive PDB is seen in the presence of cold PMA, no such inhibition is observed in PMA-pretreated cells. These data clearly indicate that pretreatment of D10 cells with a high dose of PMA leads to depletion of PKC protein.

Functional depletion of PKC was demonstrated by the finding that, in PMA-pretreated D10 cells, the synergistic effect of PMA plus ionomycin on IL-4 message is lost (Figure 4B). We used a second approach to test for the functional depletion of PKC in PMA-pretreated D10 cells; namely, we showed that these cells are unable to phosphorylate an 80-kDa protein, a process that depends on activation of PKC (Rozengurt *et al.*, 1983; Friedrich *et al.*, 1989). As can be seen in Figure 6, PMA-pretreated D10 cells fail to phosphorylate this 80-kDa protein when activated with PMA (lane 3) compared with control cells (lane 2). Similarly, the phosphorylation of this 80-kDa protein is dramatically

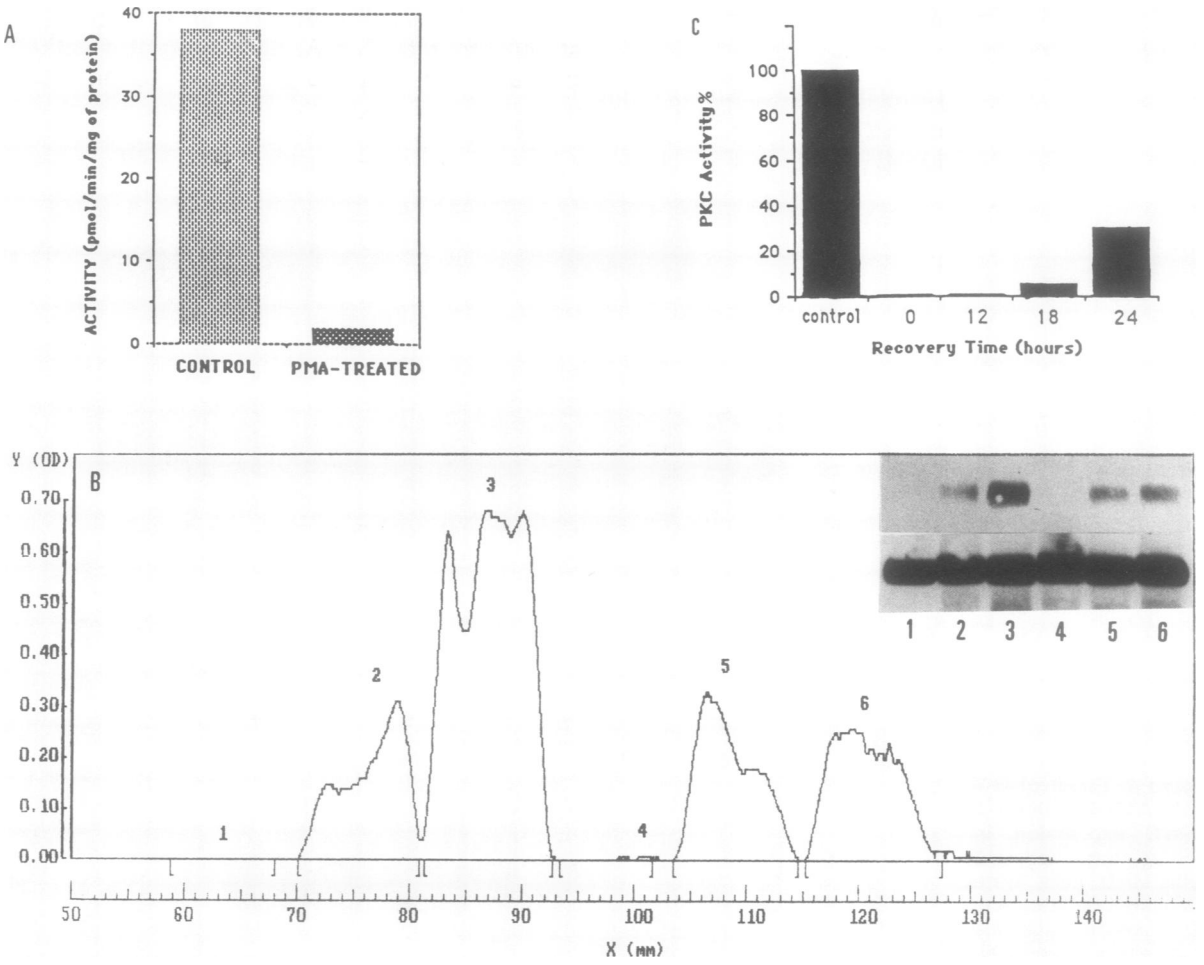

Figure 4. **PKC activity in lysates of control and PMA-pretreated D10 cells.** (A) D10 cells were incubated for 48 h in the presence or absence of 200 nM PMA. The cells were then washed and lysed. The extracted protein was assayed for PKC activity as described in Methods. (B) Control of functional depletion of PKC. D10 cells were incubated in the presence (lanes 4–6) or absence (lanes 1–3) of 200 nM PMA for 48 h, and subsequently activated with ionomycin (0.5 μg/ml) (lanes 2 and 5) or PMA plus ionomycin (lanes 3 and 6) for 6 h. Unstimulated cells are shown in lanes 1 and 4. Northern blots were hybridized with IL-4 cDNA (above) and with β-Actin cDNA (below). (C) Kinetics of PKC recovery. D10 cells were pretreated with PMA for 48 h and then washed and recultured in fresh medium containing no PMA. PKC activity was measured at 0, 12, 18, and 24 h after pretreatment.

decreased when control D10 cells are activated with PMA in the presence of H-7, compared with the analogue HA1004 (lanes 4 and 5). The dose of H-7 used was able to inhibit the proliferation of D10 cells in response to PMA (E. Muñoz, A.M. Zubiaga, and B.T. Huber, manuscript submitted). In addition to the 80-kDa protein, other proteins are phosphorylated in response to PMA. This pattern of phosphorylation has been seen repeatedly in several experiments (Muñoz *et al.*, 1990).

The time course of the PKC free state was established in PMA-pretreated D10 cells. As can be seen in Figure 4C, PKC activity starts to recover by 24 h after removal of PMA (~30% compared with control cells).

The levels of IL-4 mRNA were therefore compared in Con A–stimulated control and PKC-depleted D10 cells. Total RNA was isolated 3, 24, and 48 h after activation. As can be seen in Figure 7, results very similar to those for the drug H-7 were obtained. After 3 h, the IL-4 mRNA level is clearly lower in PMA-pretreated compared with control D10 cells. However, after 24 h, PMA-pretreated D10 cells have significantly more IL-4 mRNA than D10 control cells. Forty-eight hours after Con A stimulation, very low levels of IL-4 mRNA are seen in control D10 cells, whereas PMA-pretreated D10 cells still show a high level of IL-4 message. Quantitation of the IL-4 mRNA was carried out by densitometry read-

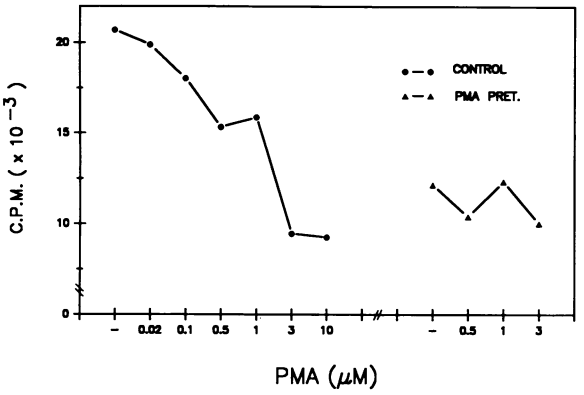

Figure 5. **Phorbol ester binding sites in D10 cells.** Cells (10^6/ml) were pretreated with 200 nM PMA for 48 h. The cells were then used to determine their phorbol ester binding sites in the presence of 20 nM of [^{3}H] PDB and increasing doses of cold PMA as described in Methods. The results are the mean of three independent experiments. Standard deviations were <15% in all points.

ings in relation to the level of the β-actin message.

Overexpression of IL-4 message in PKC-depleted D10 cells is not due to stabilization of mRNA

To analyze the mechanism leading to increased IL-4 mRNA expression in PKC-depleted D10 cells, we compared the IL-4 message in PMA-pretreated and control D10 cells that were stimulated with Con A. Six hours after activation, actinomycin D (10 μg/ml) was added to the cultures, and total RNA was extracted at the indicated timepoints thereafter. As can be seen in Figure 8, the half-life of the Con A–induced IL-4 mRNA in PKC-depleted D10 cells is shorter than in control D10 cells. In a separate experiment we have determined that the half-life of this lymphokine is 12–14 h in normal D10 cells (data not shown). These results clearly indicate that the IL-4 lymphokine superinduction observed in Con A–activated, PKC-depleted D10 cells is not due to stabilization of the message. It is likely, therefore, that prolonged IL-4 transcription occurs in the absence of PKC.

Blocking of PKC does not affect IL-4 lymphokine secretion or proliferation of Th 2 cells in response to specific antigen

To analyze the role of PKC in IL-4 release after antigen recognition, we stimulated D10 cells with conalbumin in the context of syngeneic spleen cells as antigen-presenting cells, in the presence of either the PKC inhibitor H-7 or the analogue HA1004. Culture supernatants were collected 24 h later and tested in a bioassay on IL-4–responsive HT-2 indicator cells. As can be seen in Figure 9A, significant amounts of IL-4 protein can be released in the presence of H-7. Similar results were obtained with D10 cells ac-

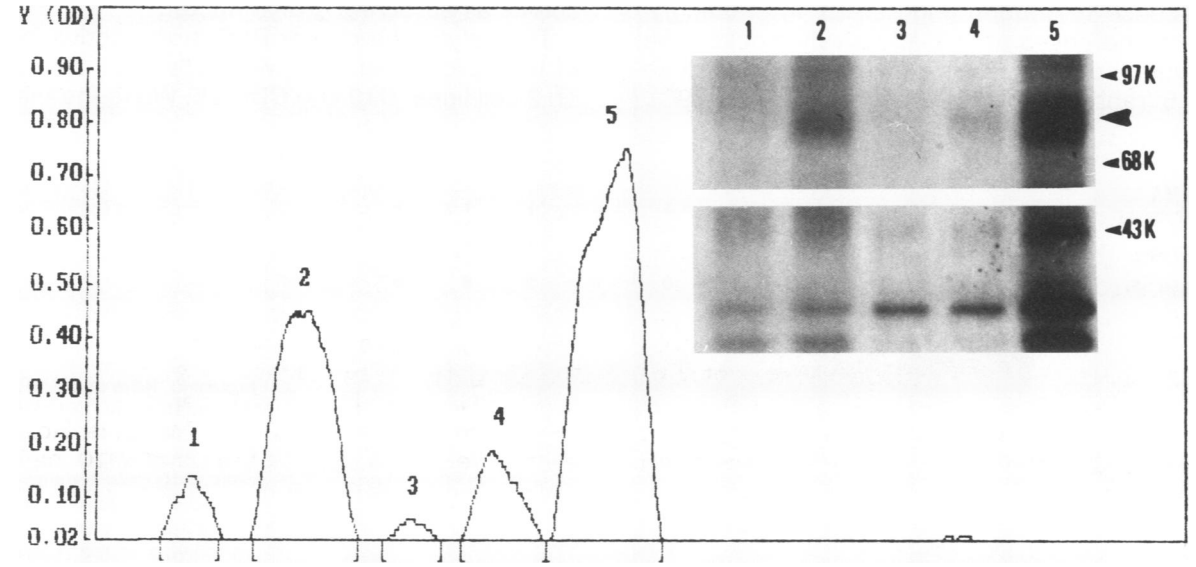

Figure 6. **Protein (80-kDa) phosphorylation in D10 cells.** ^{32}P-labeled PMA-pretreated (lane 3) and control (lanes 1, 2, 4, and 5) cells were cultured for 30 min in the presence of media (lane 1), 50 ng/ml PMA (lanes 2 and 3), 50 ng/ml PMA plus 50 μM H-7 (lane 4), or 50 ng/ml PMA plus 50 μM HA1004 (lane 5). Proteins were extracted and electrophoresed in a 10% SDS-polyacrylamide gel.

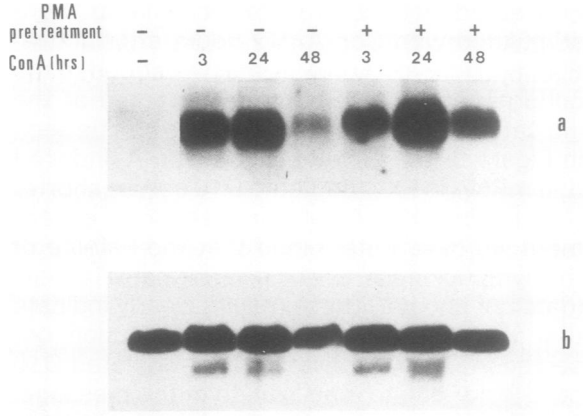

Figure 7. **IL-4 mRNA expression in control or PMA-pretreated D10 cells activated with Con A.** Total RNA was extracted at different timepoints after activation and run on a Northern gel: (a) IL-4; (b) β-Actin. Relative ratios between IL-4 and β-Actin signals were calculated by densitometry scanning. Values are (lane 1) 0.0; (lane 2) 5.7; (lane 3) 7.7; (lane 4) 0.2; (lane 5) 2.8; (lane 6) 8.7; and (lane 7) 2.8. (Min. ratio, 0.0; Max. ratio, 10.0.)

tivated by Con A. These findings were confirmed in another Th 2 clone, CDC.25; namely, the following values were obtained in an HT-2 bioassay: 1) CDC25 alone, 4995 ± 513; 2) CDC25 + APC + Ag, 55 093 ± 5922; 3) as 2 plus H-7 (10 μM), 71 721 ± 3459; 4) as 2 plus H-7 (25 μM), 70 211 ± 9113; and 5) as 2 plus H-7 (50 μM), 46 528 ± 914.

Furthermore, H-7 at doses from 10 to 25 μM did not block the proliferation of D10 cells in response to antigen (Figure 9B). Interestingly, the same doses of H-7 effectively blocked the antigen-specific proliferation of the Th 1 clone D1.5 (rabbit γ globulin/IA^d specific) (Figure 9C), as well as the Con A–induced proliferative response of splenic T cells (data not shown). The H-7 analogue HA1004 did not affect antigen-induced proliferation of either cell type. In addition, antigen-mediated proliferation was not inhibited in D10 cells depleted of PKC by high doses of PMA (data not shown).

Discussion

The proliferation of T cells is dependent on the action of T-cell growth factors, which are secreted by activated T cells and can act in a paracrine or autocrine manner (Cantrell and Smith, 1984; Kupper *et al.,* 1987). IL-2 and IL-4 are the main autocrine growth factors produced by the CD4$^+$ Th 1 and Th 2 subsets, respectively (Lichtman *et al.,* 1987). Stimulation of the Tcr by monoclonal antibodies, lectins, or specific antigen activates a signaling pathway that leads to secretion of these lymphokines. There is strong experimental evidence that activation of PKC correlates closely with IL-2 production (Weiss *et al.,* 1984; Imboden *et al.,* 1985). Furthermore, it has been shown in normal peripheral T cells as well as one Th 1 cell line that the absence of PKC activation prevents IL-2 transcription after Tcr-mediated stimulation (Yamamoto *et al.,* 1986). Under these conditions Th 1 cells do not proliferate except when exogenous IL-2 is supplied (Valge *et al.,* 1988). It is likely, therefore, that the lack of proliferation is due to the inability to produce IL-2, which serves as autocrine growth factor.

To analyze the role of PKC in the activation of IL-4–producing cells, we tested two cloned Th 2 cell lines, D10 and CDC.25. We found that

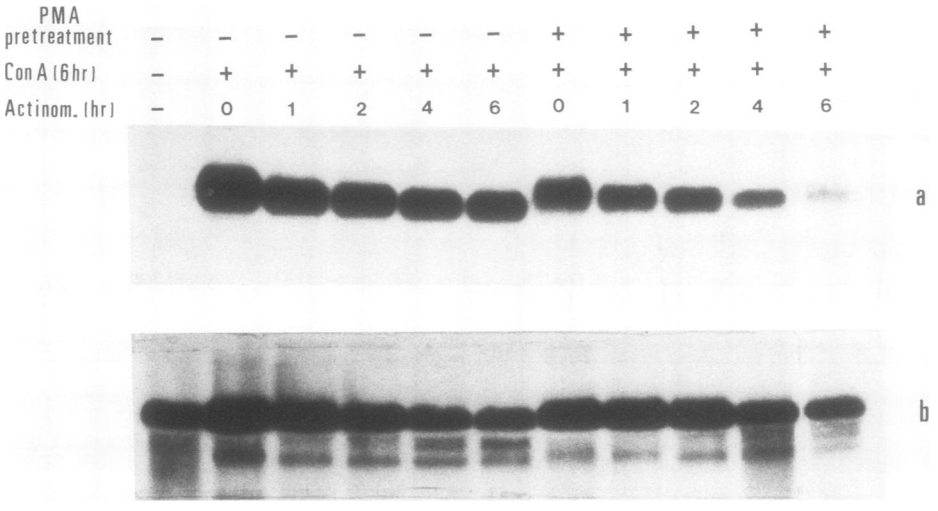

Figure 8. **Rate of mRNA degradation in control and PMA-pretreated Con A–activated D10 cells.** D10 cells were activated with Con A for 6 h, and actinomycin D (10 μg/ml) was added. Total RNA was extracted at various timepoints thereafter and run on a Northern gel: (a) IL-4; (b) β-Actin.

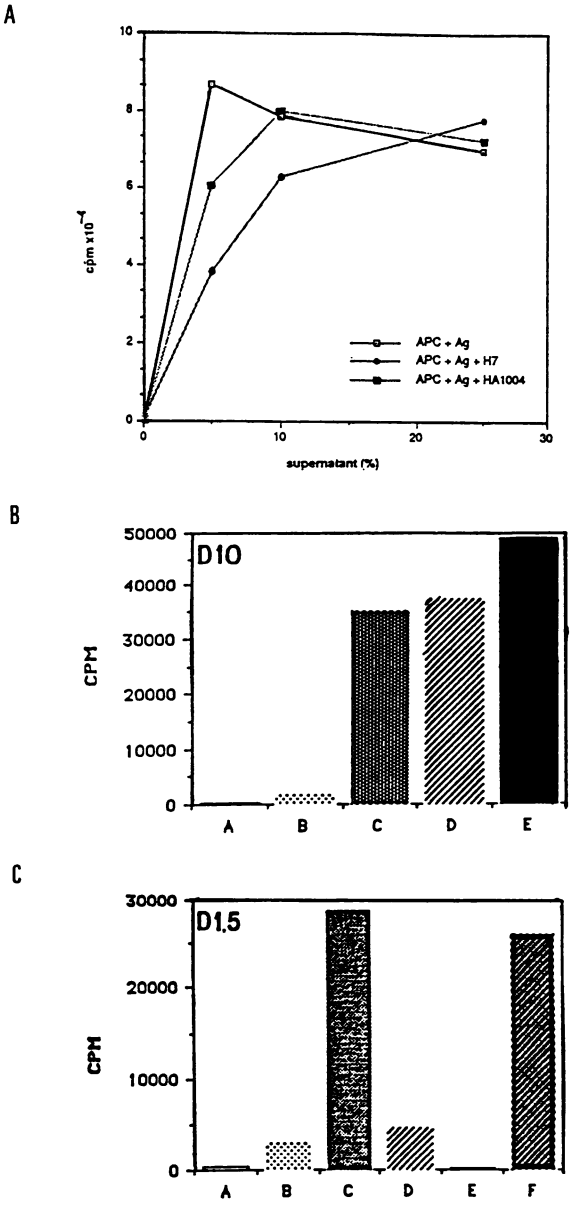

Figure 9. (A) IL-4 production of antigen-activated D10 cells in the presence or absence of either H-7 (25 μM) or HA1004 (25 μM). Supernatants collected after 24 h were assayed on HT-2 indicator cells. (B) Proliferation of conalbumin-activated D10 cells in the presence or absence of either H-7 or HA1004. (a) Unstimulated T cells; (b) +APC; (c) +APC + Antigen (200 μg/ml); (d) APC + Antigen + H-7 (10 μM); (e) +APC + Ag + H-7 (25 μM). (C) Proliferation of rabbit IgG activated D1.5 Th 1 cells. (Lanes a–e) same as in Figure 7B; (f) +APC + Antigen + HA1004 (25 μM).

activation of PKC is not required for these cells to secrete IL-4 and proliferate in response to antigen.

Activation of PKC by PMA is insufficient to induce steady-state IL-4 mRNA in D10 cells. In contrast, ionophores are able to induce IL-4 mRNA accumulation, suggesting that a Ca^{2+} calmodulin–dependent kinase is involved in IL-4 gene activation. In fact, we have found that the expression of the IL-4 gene in Con A–activated D10 cells is blocked by the calmodulin antagonist W-7 (Muñoz, Zubiaga, and Huber, unpublished observation). Furthermore, the PKC inhibitor H-7 has no effect on the steady-state IL-4 message that is induced by Ca^{2+} ionophores. Interestingly, however, D10 cells lack a measurable Ca^{2+} influx in response to Con A (data not shown), suggesting that they do not have a PIP_2 turnover (Kuno and Gardner, 1987). On the other hand, it has been reported that these cells can hydrolyze phosphatidylcholine (PC) (Dinarello *et al.*, 1989). The phosphatidic acid generated from the PC hydrolysis (Bocckino *et al.*, 1987) has been shown to mobilize intracellular calcium (Murayama and Ui, 1987).

PMA and ionophores act as cofactors, leading to similar levels of IL-4 message as in Con A–activated cells. These results suggest that activation of PKC by DAG as well as elevated levels of Ca^{2+} are necessary to obtain an optimal response. DAG could be generated in these cells through the hydrolysis of PC by phospholipase C (Besterman *et al.*, 1986; Daniel *et al.*, 1986).

The following kinetics of IL-4 mRNA after Con A activation are observed in D10 cells: it is detectable at 3 h, peaks at 6 h, and declines thereafter (Muñoz *et al.*, 1989). In the presence of the PKC inhibitor H-7 or in PMA-pretreated, PKC-depleted cells, however, the kinetics of IL-4 mRNA expression are different; namely, whereas lower levels of message are seen initially at 3 h, maximal levels are detected at 24 h and are still maintained at 48 h. Our results suggest, therefore, that initially the IL-4 gene is regulated positively by a pathway involving a Ca^{2+} calmodulin–dependent kinase in conjunction with PKC but that in a later phase PKC induces a negative feedback on IL-4 transcription. This implies a dual action of PKC in Th 2 cells, as has been proposed recently in other cellular systems (Nishizuka, 1988).

It has been suggested that the activation of PKC can negatively regulate the pathway of mRNA degradation and thereby stabilize mRNA (Shaw and Kamen, 1986). However, we have determined that the accumulation of IL-4 mRNA in Con A–activated, PKC-depleted D10 cells is not due to a stabilization of the IL-4 message. It is therefore likely that an increase in the rate of IL-4 gene transcription or prolonged transcription accounts for the observed effect. Thus,

negative regulation by PKC may occur at the transcriptional level.

An alternative explanation for the observed differences in steady-state IL-4 message in Con A–activated normal and PKC-depleted cells could be that in the latter case there is a delay in the initiation of IL-4 transcription, resulting in a shift in the kinetics of the detected response. This seems unlikely, because the same shift in the kinetics of IL-4 mRNA expression is seen in the presence of the PKC inhibitor H-7, which is present continuously during the activation period.

The IL-4 protein that is released by D10 cells in the absence of functional PKC can act as an autocrine growth factor, allowing cellular proliferation to proceed in response to antigen. This implies that activation induced by IL-4 acts over its specific receptor in a PKC-independent manner, as has been reported for IL-2 (Mills *et al.,* 1988). This possibility is supported by the experimental finding that the proliferation of HT-2 cells in response to rIL-4 cannot be inhibited by H-7 (data not shown). The effect of T cell growth factors could be mediated through phosphorylation of the 40S ribosomal S6 protein by a phospholipid-independent S6 kinase that probably is independent of PKC (Evans and Farrar, 1987).

Finally, it has been described that Th 2 cells are more dependent on IL-1 for proliferation than are Th 1 cells (Greenbaum *et al.,* 1988; Lichtman *et al.,* 1988). We have shown recently that IL-1 induces proliferation in a subline of D10 cells in a PKC independent manner (Muñoz, Zubiaga, and Huber, submitted for publication). This could imply that proliferation of Th 2 cells is regulated mainly by a PKC-independent pathway.

Overall these findings suggest that IL-2– and IL-4–producing cells use different signaling transduction pathways after Tcr-mediated activation.

Methods

Mice

Balb/c (H-2^d) and AKR/J (H-2^k) mice were purchased from the Jackson Laboratories (Bar Harbor, ME).

Media and reagents

Complete culture medium was RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 2 mM L-glutamine (GIBCO), 1 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 50 M 2-mercaptoethanol, antibiotics (GIBCO), and 10% fetal calf serum (Hyclone, Logan, UT). Ionomycin and A23187 were purchased from Calbiochem (La Jolla, CA). The protein kinase inhibitors H-7 and HA1004 were obtained from Seikagaku America (St. Petersburg, FL). (γ-^{32}P)ATP (6000 Ci/mmol), (α-^{32}P)dCTP (800 Ci/mmol), [^{3}H] Phorbol 12-13 dibutyrate (19.1 Ci/mmol) and carrier-free ^{32}P (8500 Ci/mmol) were purchased from New England Nuclear (Boston, MA). All other reagents were from Sigma Chemical (St. Louis, MO).

Cell lines

The generation and maintenance of the cloned Th 2 cell lines D10 (ATCC) (specific for conalbumin in the context of I-A^k) and CDC.25 (specific for rabbit IgG in the context of I-A^d), and the Th 1 cell line D1.5 (identical specificity as CDC.25), have been described (Kaye *et al.,* 1983; Tony *et al.,* 1985; Kurt-Jones *et al.,* 1987). Briefly, Th cell lines were stimulated every 2 wk with antigen and irradiated syngeneic splenocytes as APC. α-Methyl mannoside–treated supernatant from rat spleen cells that had been stimulated with Con A for 48 h was added to the culture medium as a source of lymphokines. For the IL-4 bioassays, the HT-2 indicator cell line, which proliferates in response to IL-2/IL-4, was used.

Proliferation and IL-4 assays

Proliferative responses of the T cell clones were assessed by purifying live cells on a Ficoll-Paque gradient (Pharmacia, Piscataway, NJ) at least 2 wk after restimulation with antigen and irradiated spleen cells. Usually, 2.5×10^6/ml spleen cells, 200 μg/ml of antigen, and 2.5×10^5/ml T cells were cocultured in 200-μl aliquots in 96-well plates. The cultures were incubated for 72 h at 37°C, and [^{3}H]-TdR (0.5 μCi/well) was added for the final 12 h of culture. Radioactivity incorporated into DNA was measured by liquid scintillation counting.

To determine the IL-4 activity in supernatants of Th 2 cells that had been stimulated under different conditions, we collected supernatants after 24 h of culture and assessed in a bioassay on the HT-2 indicator cell line. Briefly, 2×10^4 HT-2 cells/well were cultured with increasing dilutions of supernatant for 24 h. During the last 6 h, they were pulsed with [^{3}H]-TdR, as described above.

RNA analysis

Resting D10 cells were cultured under different conditions as described. Total RNA was then extracted according to Auffray and Rougeon (1980), with some modifications. Briefly, 10^7 D10 cells were washed three times with cold phosphate-buffered saline (PBS), pelleted, and resuspended in 3 ml of 3M LiCl/6M urea. The suspension was homogenized with a 60-s pulse by the use of a polytron. The RNA was then precipitated at 4°C for 15 h and extracted twice with phenol/chloroform/isoamylalcohol. Twenty micrograms of RNA was electrophoresed on a Northern gel (1% agarose/2% formaldehyde/1× 3-(*N*-morpholino)propanesulfonic acid [MOPS]) and the gel blotted onto a nylon membrane (Biotrans Nylon membrane, ICN Biomedicals, Irvine, CA). The RNA was crosslinked according to the protocol developed by Church and Gilbert (1984). The blot was prehybridized in 5% sodium dodecyl sulfate (SDS), 100 mM NaCl, 50 mM sodium phosphate, and 1 mM EDTA for 2 h at 65°C and then hybridized for 12 h in a new aliquot of the same solution containing an α^{32}P random-primed probe (Boehringer Mannheim Biochemicals, Indianapolis, IN). The hybridized membrane was washed three times for 5 min each at 65°C in 5% SDS, 1× 150 mM sodium chloride/15 mM sodium citrate, pH 7.0 (SSC), and three times for 30 min each at 65°C in 0.1% SDS, 0.5× SSC. Autoradiographs were exposed at −70°C with enhancer screens.

Protein kinase C assay

Total cellular PKC activity was measured according to a method described by Farrar and Anderson (1985), with some modifications. Briefly, 10^7 D10 cells were incubated in complete medium for 48 h in the presence or absence of 200 nM PMA. The cells were then washed three times with Ca^{2+}- and Mg^{2+}-free PBS and the pellet resuspended in 1 ml of sonication buffer (20 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.5, 5 mM EDTA, 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 5 mM β-mercaptoethanol, 1% NP-40, 10 μg/ml pepstatin, 25 μg/ml apronitin, 10 μg/ml leupeptin, and 10 μg/ml soybean trypsin inhibitor) and sonicated with a Brandson sonicator. After 30 min of centrifugation at 13 000 × *g* at 4°C, the supernatant was collected and immediately used to determine the PKC activity. Aliquots of 50 μl were assayed for PKC activity in a total volume of 250 μl containing 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 100 μg histone (Sigma Type IIIS), 100 μM ATP, 1 μCi [γ^{32}P]ATP in the presence or absence of 100 μg phosphatidylserine and 10 ng PMA. Samples were incubated at 30°C for 20–30 min. The reaction was then stopped with 1 ml 20% ice-cold trichloroacetic acid (TCA) and filtered through nitrocellulose papers. After several washes with 20% TCA, radioactivity was measured in a Beckman counter. PKC activity was calculated by the use of the following formula: CPM (Ca^{2+} + PMA + phosphatidylserine) − CPM (Ca^{2+} only), and was expressed as pmol/min/mg protein. Protein determinations were carried out according to the Bradford method (1976).

Phorbol ester binding

D10 cells were cultured for 48 h in the presence or absence of 200 nM PMA. The cells were then washed twice with cold PBS and incubated (2 × 10^6/ml) for 60 min at 4°C in complete culture medium containing 20 nM [^{3}H] PDB, in the presence or absence of increasing doses of PMA. Cells were washed three times with cold PBS and the pellet incubated overnight in 2% SDS 37°C and counted in a beta counter by liquid scintillation.

Analysis of phosphoproteins

Analysis of protein phosphorylation was performed as previously described (Friedrich *et al.*, 1989), with minor modifications. Cells (5 × 10^6/ml) were incubated for 6 h at 37°C in phosphate-free RPMI containing carrier-free ^{32}P (50 μCi/ml). PMA and protein kinase inhibitors were added during the last 30 min. The cells were then spun at 1000 rpm for 5 min. The pellet was resuspended in 0.5 ml lysis buffer (1% Triton X-100, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EGTA, 2 mM PMSF and 5 μg/ml leupeptin) and incubated on ice for 20 min. The lysate was boiled for 10 min and centrifuged at 100 000 × *g* for 15 min. Proteins present in the supernatant were precipitated overnight with 2 vol of acetone and analyzed on a 10% SDS-polyacrylamide gel under reducing conditions. After electrophoresis the gel was soaked in 1 M KOH for 90 min at 56°C, fixed, dried, and exposed to a Kodak X-R film.

Densitometry

The densitometry analyses were performed with a laser densitometer from Bio-Rad (model 620).

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