Mitogenic Signaling Pathways Regulating Expression of c-myc and Ornithine Decarboxylase Genes in Bovine T-Lymphocytes†

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ABSTRACT: Expression of the c-myc and ornithine decarboxylase (ODC) genes is elevated early after mitogenic activation of T-lymphocytes, and regulation of the two genes seems to be coupled to transmembrane signaling pathways that are in part different. The evidence is consistent with protein kinase C (PKC) being both necessary and sufficient to induce expression of the ODC gene in response to treatment of T-cells with either the mitogen concanavalin A (Con A) or biologically active phorbol esters. Furthermore, there seems to be no involvement of events dependent on calmodulin (CaM) in the regulation of ODC in these cells. The situation with c-myc is more complex. In contrast to ODC, transcription of this gene is not stimulated by treatment of resting T-cells with phorbol esters alone, but the cells respond to phorbol esters in combination with the calcium ionophore ionomycin. Induction of the c-myc gene by Con A is inhibited by CaM antagonists. These results are consistent with a model in which transcriptional activation of the c-myc gene in resting T-lymphocytes requires two signals, one from PKC and the other involving CaM.

The growth of resting animal cells is regulated by the interaction of polypeptide growth factors with specific receptors on their surface. The binding of these factors in turn initiates changes in ion fluxes and metabolic reactions that are thought to be part of the signaling pathways controlling cell proliferation (Rozengurt, 1986). Elevation of cytosolic free Ca^{2+} (Tsien et al., 1982; Moolenaar et al., 1984, 1986) and activation of PKC[1] (Rodriguez-Pena & Rozengurt, 1985; Blackshear et al., 1985) are two events that occur rapidly after growth factor binding. Depending on the cell type and perhaps the mitogen involved, elevation of cytosolic Ca^{2+} is due to either mobilization of endogenous stores (Moolenaar et al., 1984) or stimulation of uptake of exogenous Ca^{2+} (Tsien et al., 1982; Moolenaar et al., 1986). PKC is thought to be activated synergistically by Ca^{2+} and diacylglycerol (Wolf et al., 1985); the latter arises from the signal-induced hydrolysis of membrane inositol phospholipids (Berridge, 1987).

These early signaling events are thought to initiate regulatory cascades, which modulate the expression of genes whose products are necessary for progression through the G_1 phase of the cell cycle (Stiles, 1985; Denhardt et al., 1986). The relationships between these early signaling pathways and gene expression are of interest and are clearly fundamental to our understanding of the regulation of cell growth. Results from other laboratories have suggested that in some cell lines PKC has an important role in this process but that growth-associated genes may be regulated by other undefined signaling pathways as well (Coughlin et al., 1985; Kaibuchi et al., 1986; Hovis et al., 1986; Stumpo & Blackshear, 1986; Blackshear et al., 1987; McCaffrey et al., 1987). CaM has also been shown to be important in the progression of cells through the cell cycle (Rasmussen & Means, 1987). The present study investigates the interrelationships between PKC- and CaM-regulated events in controlling the expression of two important growth-regulated genes, the protooncogene c-myc and ODC, in primary cultures of normal bovine T-lymphocytes. The evidence is consistent with the regulation of ODC by mitogen in these cells being purely through a PKC-linked pathway and the control of c-myc being through a synergistic interaction between PKC and CaM.

Materials and Methods

Materials. Con A (grade IV), 5,6-dichlorobenzimidazole riboside (DRB), trifluoperazine dihydrochloride (TFP), and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W-7) were purchased from Sigma. Ionomycin and 12-*O*-tetradecanoylphorbol 13-acetate were from Calbiochem. Indo 1 acetoxymethyl ester was obtained from Molecular Probes (Junction City, OR), and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) was purchased from Seikagaku America (St. Petersburg, FL).

Cell Culture. Bovine T-lymphocytes were purified from suprapharyngeal lymph nodes and cultured as previously described (Seyfried & Morris, 1983). Cells were stimulated with 18 μg of Con A/mL of culture medium.

RNA Isolation and Analysis. Total cellular RNA was isolated (Chirgwin et al., 1979) from cultured cells. RNA was separated by electrophoresis in agarose gels in the presence of formaldehyde and blotted to nitrocellulose (Maniatis et al., 1982). Care was taken to load equal amounts of RNA per lane in each experiment (3–5 μg); in this way, the values of mRNA levels obtained were normalized to total cellular RNA, which did not change in bovine T-cells over the time period of interest (Fillingame & Morris, 1973). The blots were hybridized to nick-translated double-stranded plasmid inserts. The amount of hybridization was quantitated by densitometric scanning of autoradiographs that had been exposed within the linear range of the film. In some experiments mRNA was examined by dot–blot analysis (Thomas, 1983).

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[1] Abbreviations: CaM, calmodulin; Con A, concanavalin A; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; ODC, ornithine decarboxylase; PKC, protein kinase C; TFP, trifluoperazine; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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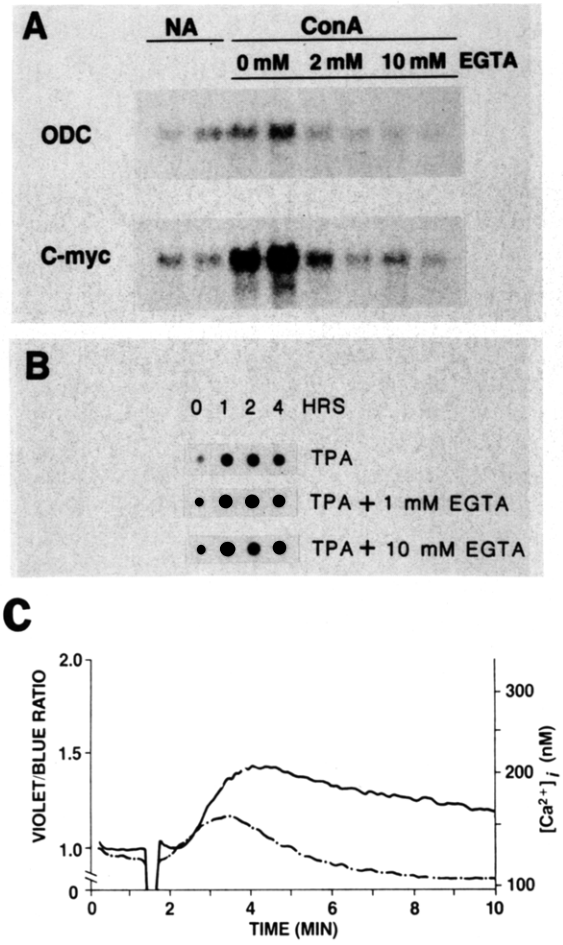

FIGURE 1: Effect of chelation of exogenous Ca^{2+} on Con A induced or TPA-induced mRNA induction and on intracellular free calcium. (A) EGTA was added at final concentrations of 0, 2, and 10 mM, as indicated, at the time of addition of Con A to the cultures. RNA was then isolated at 1 and 2 h after activation (the first and second lanes of each pair, respectively) and Northern blot analysis performed. The first two lanes, marked "NA", are RNA from duplicate resting cultures. (B) Resting lymphocyte cultures were treated with 30 nM TPA and 0, 1, or 10 mM EGTA, as indicated. RNA was prepared at the indicated times, and dot-blots were analyzed for ODC mRNA. (C) Intracellular free calcium determined by flow cytometry from the violet/blue Indo 1 fluorescence ratio (ordinate) as a function of time (abscissa). The break in the tracing at 1.5 min indicates the point of addition of Con A. In one experiment (—) only Con A was added, and in the other (-·-) 5 mM EGTA was added at zero time.

Recombinant DNA Hybridization Probes. The c-myc probe was generated from a mouse genomic DNA clone (Bernard et al., 1983) by subcloning the *Pst*I fragment containing most of the second exon. A mouse ODC cDNA clone was obtained by subcloning the *Sal*I/*Hin*dIII fragment from plasmid pOD48 (McConologue et al., 1984).

Analysis of Intracellular Free Calcium. Cells were loaded with the acetoxymethyl ester of Indo 1 by incubation in a 3 μM solution for 40 min at 37 °C. Analysis and calibration of Indo 1 fluorescence were performed by flow cytometry as previously described (Rabinowitch et al., 1986).

RESULTS

Influence of Ca^{2+} and CaM Antagonists on Expression of c-myc and ODC mRNAs. The elevation of cytosolic Ca^{2+} is one of the earliest events observed after mitogenic activation of T-lymphocytes (Tsien et al., 1982). This is illustrated in the case of bovine T-cells in Figure 1C, using the fluorescent calcium indicator, Indo 1, for the determination. The first significant increase from the resting intracellular level of 130 nM was observed at about 1 min after addition of mitogen; this value reached a maximum of 210 nM by about 3 min and remained significantly elevated during the 10-min course of the experiment. When exogenous Ca^{2+} was chelated with excess EGTA, only a transient and diminished elevation of intracellular Ca^{2+} occurred (Figure 1C). This small increase in the absence of free exogenous calcium has been seen by others in a variety of cell types and has been attributed to mobilization of intracellular calcium stores (Rasmussen et al., 1984).

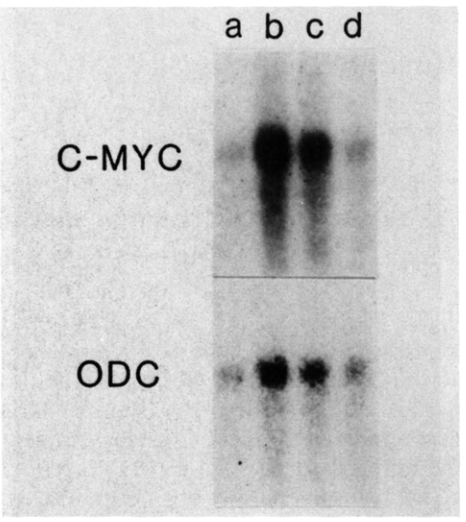

FIGURE 2: Influence of TFP on the induction of c-myc and ODC mRNAs by Con A. Cells were treated as indicated below for 2 h, and the RNA was analyzed by Northern blots. Lane a, no treatment; lane b, Con A alone; lanes c and d, Con A and 10 or 20 μM TFP, respectively.

The influence of exogenous Ca^{2+} on the mitogenic regulation of the c-myc and ODC genes was examined. Addition of EGTA to the culture medium blocked the elevation of c-myc and ODC mRNAs normally seen at 1 and 2 h after treatment of the cells with Con A (Figure 1A). This effect was not due to generalized toxicity caused by the chelating agent, since induction of ODC mRNA by phorbol esters in these cells was unaffected by the presence of EGTA (Figure 1B). The more rapid and intense induction of ODC by TPA (Figure 1B), as compared to Con A (Figure 1A), was seen before (White et al., 1987). It therefore appears that sustained elevation of cytosolic Ca^{2+} is required for the increases of both mRNAs in response to mitogenic activation.

The phenothiazine derivative TFP inhibited the elevation of both c-myc and ODC mRNAs (Figure 2) when added to the cells at concentrations previously reported to be pharmacologically active (Schatzman et al., 1981). As well as being a potent antagonist of CaM-regulated reactions, TFP also has been reported to inhibit phosphorylation reactions catalyzed by PKC (Schatzman et al., 1981). The naphthalene sulfonamide W-7 is a more selective inhibitor, showing K_i values of 12 μM and 110 μM for inhibition of CaM-dependent and PKC-catalyzed phosphorylations, respectively (Kobayashi et al., 1979; Tanaka et al., 1982). A clear differential effect of W-7 on the expression of c-myc and ODC was observed (Figure 3). At concentrations of the drug below 45 μM, there was strong inhibition of induction of c-myc with no effect on the level of ODC mRNA. This result suggests an involvement of CaM in the growth regulation of c-myc in these cells. At 60 μM W-7, ODC expression was also significantly inhibited (Figure 3), consistent with a role for PKC in the regulation of this gene. We were unable to detect inhibition of total RNA synthesis by TFP or W-7, on the basis of [^{3}H]uridine incor-

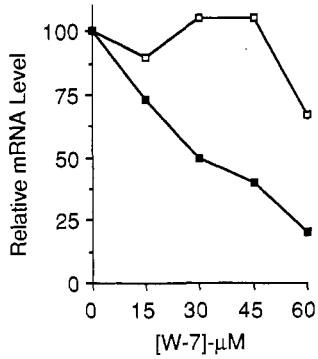

FIGURE 3: Influence of W-7 on the levels of ODC and c-myc mRNAs after treatment with Con A. Cells were stimulated with Con A for 2 h in the presence of the indicated concentrations of W-7. RNA was extracted and analyzed for ODC (□) and c-myc (■) by Northern blots.

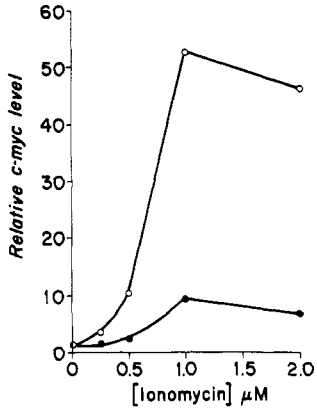

FIGURE 4: Induction of c-myc mRNA by ionomycin and TPA. Either ionomycin alone (●) or ionomycin together with 30 nM TPA (○) was added to resting lymphocyte cultures, and at 2 h RNA was extracted and analyzed.

poration in bovine lymphoblasts; similar experiments showed H-7, a selective inhibitor of PKC (Hidaka et al., 1984), to be a general inhibitor of RNA synthesis and therefore not appropriate for studies of gene expression (M. L. Allen and D. R. Morris, unpublished results).

Further evidence that Ca^{2+} has a role in regulating the activity of the c-myc gene in resting T-cells comes from studies with the Ca^{2+} ionophore ionomycin. Ionomycin by itself caused an elevation of c-myc mRNA of ca. 10-fold (Figure 4). The influence of the ionophore was maximal at about 1.0 μM, a concentration that produced an average increase in intracellular Ca^{2+} similar to that promoted by Con A (data not shown). Ionomycin, when added in combination with TPA, elicited an even stronger induction of c-myc, reaching an mRNA elevation of over 50-fold at optimal ionomycin concentration (Figure 4). This influence of TPA was strongly synergistic with ionomycin, since the phorbol ester by itself had little or no effect on the level of c-myc mRNA [see Table I and also White et al. (1987)]. Ionomycin, added together with TPA, exerted no influence on ODC mRNA level beyond that seen with TPA alone (data not shown).

The stability of the Ca^{2+}-mediated event, once executed, was investigated in the experiment shown in Figure 5. Cells were stimulated with Con A for 3 h to maximally induce c-myc mRNA. Either W-7 or an RNA polymerase II inhibitor, DRB, was added to the cells, and the level of c-myc mRNA was followed as a function of time. In the presence of DRB, after a short lag, the decay of the mRNA began with a half-life of about 10 min. Similar results were obtained with actinomycin D (not shown). In contrast, in cells treated with 50 μM

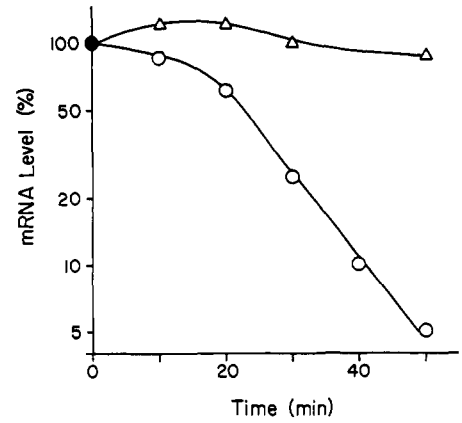

FIGURE 5: Influence of W-7 and DRB on the induced level of c-myc mRNA. Cells were cultured in the presence of Con A and, at 3 h, either W-7 (△, 50 μM) or DRB (○, 60 μg/mL) was added. RNA was extracted and analyzed at zero time (●) and at the times indicated after drug addition.

W-7, a concentration of the drug sufficient to inhibit c-myc induction by ca. 75% when added at zero time (Figure 3), the induced level of c-myc message was maintained in this experiment for 50 min and in other experiments for as long as 4 h. To test for the possible stabilization of c-myc mRNA by W-7, DRB was added to 3-h stimulated cells, as in Figure 5, in the presence and absence of 50 μM W-7, and the cultures were harvested 40 min later. In both cultures, the level of c-myc had declined to less than 10% that at zero time, providing no evidence for an influence of W-7 on mRNA decay (data not shown). Thus, we conclude that elevated transcriptional activity of the c-myc gene, once established in these cells, is relatively independent of continued execution of the CaM signal.

Influence of Down-Regulation of PKC on Expression of c-myc and ODC mRNAs. Although phorbol esters are well-known activators of PKC (Nishizuka, 1984), prolonged treatment of cells with high concentrations of TPA leads to dramatic down-regulation of intracellular levels of the enzyme (Rodriguez-Pena & Rozengurt, 1985; Blackshear et al., 1985). This approach has recently been shown to deplete PKC in bovine lymphocytes (Grove & Mastro, 1988). The physiological effects of PKC down-regulation are demonstrated in Figure 6A; overnight incubation of the cells with 300 nM TPA abolishes the induction of ODC mRNA by additional exposure of the cells to the phorbol ester, thus confirming the efficacy of the treatment. Furthermore, PKC down-regulation prevents the elevation of ODC mRNA induced by the mitogen Con A (Figure 6B). This result clearly implicates PKC as an important component of the mitogenic signaling pathway that regulates ODC mRNA level under these conditions.

The influence of PKC depletion on the regulation of c-myc mRNA is more complex. The Northern blot presented in Figure 6C suggests only a partial inhibition of mitogen-regulated c-myc induction at 2 h after stimulation. An identical pattern was found with cells harvested at 1 and 3 h after mitogen addition (data not shown). This result is expanded in quantitative terms in Table I. The induction of c-myc mRNA is inhibited by only about 40%, while that of ODC mRNA was completely abolished in the same experiment (Figure 6B). Additionally, it can be seen that the induction of c-myc by ionomycin alone is unaffected by PKC down-regulation, while the synergistic effect of TPA in combination with the ionophore is completely lost (Table I). In conclusion, it appears that PKC plays an important role in mitogenic regulation of c-myc expression, but in contrast to ODC, a

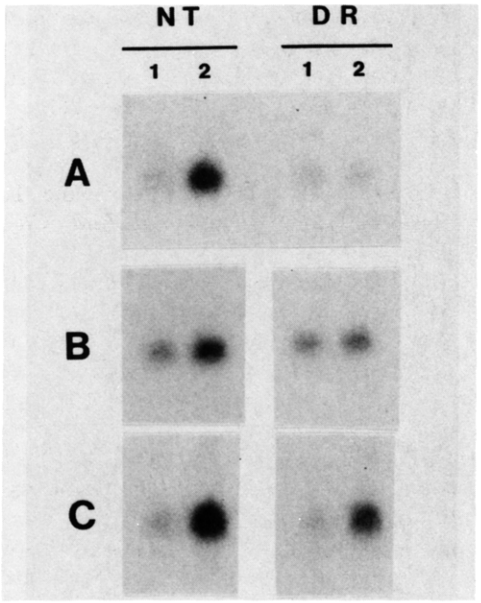

FIGURE 6: Influence of PKC down-regulation on expression of ODC and c-myc mRNAs. Resting T-cells were cultured for 18 h in the presence ("DR") or absence ("NT") of 300 nM TPA, washed, and resuspended in fresh medium. Cultures were then stimulated for 2 h with either 30 nM TPA (panel A) or Con A at 18 μg/mL (panels B and C). RNA was extracted, and Northern blots were prepared. Lanes 1 contain RNA from unstimulated cultures and lanes 2 from stimulated cells. Blots were probed for ODC (panels A and B) and c-myc (panel C).

Table I: Influence on c-myc Expression of PKC Down-Regulation by Preincubation with TPA[a]

	rel c-myc mRNA level[b]	
treatment	no preincubn[c]	preincubd[d]
no addition	1.0	1.0
Con A	4.9	2.9
ionomycin	9.8	11.6
ionomycin + TPA	37	9.3
TPA	1.8	1.3

[a] Cells were treated as indicated for 2 h, and RNA was extracted and analyzed by electrophoresis and Northern blotting. Concentrations: Con A, 18 μg/mL; ionomycin, 1.0 μM; TPA, 30 nM. [b] c-myc mRNA level was determined by densitometric scanning of autoradiograms as described under Materials and Methods. Values are normalized to those in the resting cultures ("no addition"). [c] Cells were treated as indicated with no preincubation with TPA. [d] Cells were cultured for 18 h in the presence of 300 nM TPA, washed, and transferred to fresh medium, and then treated as indicated.

second signal linked to Ca^{2+} also seems to be required.

Discussion

Our previous results (White et al., 1987) strongly suggested that activation of expression of the c-myc and ODC genes in T-lymphocytes involved, at least in part, different pathways of mitogenic signal transduction. Specifically, ODC mRNA was strongly induced by treatment of resting cells with TPA, while phorbol esters alone had little or no effect on expression of c-myc under these conditions. Additionally, the intracellular Ca^{2+} antagonist, Cd^{2+}, was a potent inhibitor of c-myc induction by mitogen but had no effect on ODC expression over the same concentration range. These results suggested, but did not prove, a model where ODC was controlled only by a PKC-linked signaling pathway with no involvement of CaM, while c-myc expression was regulated, at least in part, by a pathway involving CaM. The results presented in this paper substantiate and extend this hypothesis.

The interpretation of the present results concerning expression of the ODC gene seems straightforward. The fact that the gene is induced by treatment of the cells with TPA alone (White et al., 1987) suggests that activation of PKC is sufficient by itself for elevation of ODC mRNA levels. Additionally, PKC is strongly implicated in the regulation of ODC by mitogenic lectin, on the basis of inhibition by both TFP and down-regulation of the kinase. Since induction of ODC by Con A is not influenced by either Cd^{2+} (White et al., 1987) or W-7, one is led also to conclude that the mitogen-induced elevation of intracellular Ca^{2+}, although required (Figure 1), does not mediate ODC induction through CaM. Hovis et al. (1986) obtained a somewhat different result with mitogen-activated NIH 3T3 cells, in which down-regulation of PKC revealed only a partial requirement for PKC in ODC expression, implying the possible existence of another parallel signaling pathway in this cell type. We have no evidence for this putative second signal transduction pathway linked to ODC expression in T-lymphocytes.

The situation with regard to c-myc expression in T-cells is more complex. In contrast to other cell types, including 3T3 fibroblasts and B-lymphocytes (Kelly et al., 1983; Smeland et al., 1985), phorbol esters by themselves show little or no influence on the level of c-myc in resting T-lymphocytes. And yet, the down-regulation experiments reported here argue that PKC is required for at least part of the response to mitogenic lectin. It seems, therefore, that an additional signal is needed either prior to or simultaneously with activation of PKC. The synergistic action of ionomycin and TPA suggests that signals from Ca^{2+} and PKC are sufficient for activation of c-myc expression. Inhibition by W-7 of the Ca^{2+} signal required for c-myc expression, together with our previous results showing inhibition by Cd^{2+} (White et al., 1987), implicates a calcium-sensitive protein, such as CaM, in the Ca^{2+} signal linked to c-myc expression in cells stimulated by lectin. The situation with regard to Ca^{2+}-binding proteins in cells is complex, and we cannot identify with certainty the protein involved in this signaling pathway. For example, other calcium-sensitive, phenothiazine-binding proteins are found in cells besides CaM (Carraway et al., 1987). A lymphocyte-specific Ca^{2+}-binding protein of M_r 68 000 has been described that is distinct from two better characterized proteins of similar size, calcimedin and calelectrin (Morse & Moore, 1988). It has not been reported whether this protein interacts with phenothiazines, such as TFP, or with naphthalenesulfonamides, such as W-7.

In addition to the synergistic interaction between Ca^{2+} and PKC, it is important to point out that ionomycin alone appreciably elevates c-myc mRNA level, in a fashion that is not sensitive to down-regulation of PKC. This implies that pharmacological activation of a Ca^{2+}-linked signal transduction pathway by itself is sufficient to induce expression of c-myc, albeit at a suboptimal level. At this point, it is not clear whether this PKC-independent, Ca^{2+}-linked pathway is the same as the one that participates in the ionomycin–TPA synergy.

In considering mechanisms for the two steps regulating c-myc expression, it is important to discuss the experiments involving the influence of W-7 on cells after activation with Con A (Figure 5). The apparent stability of the event mediated by the Ca^{2+} signal could arise from a modification of a transcription factor that is not readily reversible or from a stable change in the structure of the chromatin associated with the c-myc gene. We suggested earlier (White et al., 1987) that an alteration of chromatin structure could result from the known modulation of DNA topoisomerase II activity through the action of CaM-dependent protein kinase II (Sahyoun et al., 1986). Another candidate for signaling through CaM is

the activation of CaM-dependent protein kinase III by elevated cytosolic Ca^{2+} (Palfrey et al., 1987; Connelly et al., 1987). It is interesting to note that CaM and CaM-binding proteins have been identified in nuclei (Bachs & Carafoli, 1987). The stability of the CaM-mediated event provides one explanation for the fact that TPA by itself stimulates expression of the gene in some cell types, e.g., 3T3 cells, but has no effect in T-lymphocytes. The stable, CaM-dependent event could remain executed when 3T3 cells enter the resting condition, thus maintaining a state that is responsive to TPA. Further insight into this important difference between these cell types awaits additional knowledge concerning the molecular mechanisms of the CaM- and PKC-dependent regulatory steps.

We concluded previously that not only were the two types of genes represented by c-myc and ODC controlled by different mechanisms in T-cells, i.e., transcriptionally and posttranscriptionally, respectively, but that the two genes were also linked to different mitogenic signaling pathways (White et al., 1987). The present experimental approach has provided additional insight into the nature of these pathways. Con A is thought to bind to the T-cell antigen–receptor complex (Chilson et al., 1984), thereby activating the breakdown of phosphatidylinositol bis(phosphate) to diacylglycerol and inositol tris(phosphate) (Imboden & Stobo, 1985) and triggering the uptake of Ca^{2+} (Tsien et al., 1982), possibly through the action of inositol tris(phosphate) (Kuno & Gardner, 1987). We propose that simultaneous elevation of cytosolic Ca^{2+} and diacylglycerol synergistically activates PKC, as shown in a reconstituted system by Wolf et al. (1985). Activation of PKC in turn would lead to expression of the posttranscriptionally controlled type 2 genes, as represented in this study by ODC. Elevation of cytosolic Ca^{2+}, acting through a W-7-sensitive binding protein, would synergize with activated PKC in the two-step transcriptional activation of the type 1 genes, as proposed for c-myc.

Registry No. PKC, 9026-43-1; ODC, 9024-60-6; Ca, 7440-70-2.

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