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Activation of the Mouse IL-2 Gene by Okadaic Acid: Synergy with Interleukin-1

WERNER FALK,* KIRSTIN STRICKER,† GERALD PRAAST,† YASUMASA TSUKITANI,‡
PETER H. KRAMMER,† and MICHAEL STOECK†,§

ABSTRACT

Interleukin-1 (IL-1) has potent immunoregulatory and inflammatory functions. Its activity is mediated by an 80-kDa receptor on the cell surface and leads to activation of other genes. The underlying molecular events are largely unknown. We investigated the role of phosphatases in activation of the IL-2 gene in EL4 thymoma cells. We found that the protein phosphatase PP1 and PP2A inhibitor okadaic acid (OA) alone was able to significantly stimulate IL-2 production by the IL-1-responsive EL4 subline EL4 5D3 and also by the IL-1-nonresponsive EL4 subline EL4D6/76. In the IL-1-responsive cell line OA strongly synergized with phorbol myristate acetate (PMA) and IL-1. In the IL-1-nonresponsive cell line OA synergized with PMA but not with IL-1. Under suboptimal conditions of PMA/OA synergy an additional synergistic effect of IL-1 was shown. This was true for IL-2 and IL-6 production. Sphingomyelinase or sphingosine had no detectable effect. The kinetics of OA- and PMA-induced expression of IL-2 mRNA and IL-2 protein was different. PMA induced maximal expression between 6 and 12 h and was almost undetectable at 24 h. OA-induced expression was first obvious at 12 h and continued longer than 36 h. In both cases IL-1 caused no shift in kinetics, but potentiated the effects of the different tumor promoters. Utilizing IL-2 promoter-CAT constructs we showed in transfection experiments that the synergistic effect was also evident on the transcriptional level. We conclude from the data that phosphatases play an important role for IL-2 expression and that IL-1 can use additional pathways of activation that are different from events induced by PMA or OA.

INTRODUCTION

Interleukin-1 (IL-1) is a pleiotropic molecule that has immunoregulatory and inflammatory functions [for review see (1)]. IL-1 activity is mediated by the two distantly related agonists IL-1α and IL-1β and a receptor antagonist (IL-1Ra) with structural relation to the agonists. Agonists and antagonist bind an 80-kDa single-chain receptor (IL-1RI) primarily expressed on T cells and fibroblasts and a 67-kDa receptor (IL-1RII) mainly found on B cells and macrophages (1) with comparable affinity. IL-1RII is not involved in signal transduction (2,3). Despite many attempts over the last years the mechanism of signal transduction in cells after exposure to IL-1 remains poorly understood (4,5). After binding of IL-1 the complex is internalized and, depending on the cell type, transported to the nucleus from where it can be recovered in active form (6,7). There is some indication that this process is required for activity.

We have reported that an IL-1RI-positive EL4 T cell subline EL4D6/76 incapable of internalization does not transmit a signal to the IL-2 promoter (8). Recently, Grenfell *et al.* have shown that mutation of the putative nuclear targeting sequence in IL-1 decreased IL-1 activity (9). On the other hand, large parts of the cytoplasmic domain of the IL-1RI could be removed without abolishing internalization, although the IL-1 response was inhibited (10,11). For the IL-1RII on B cells no such information is available since nuclear translocation was not observed (12). The nature of second messenger events is

*Department of Internal Medicine I, University of Regensburg, 93042 Regensburg, Germany.
†Forschungsschwerpunkt Tumorimmunologie, Deutsches Krebsforschungszentrum, 69120 Heidelberg, Germany.
‡Fujisawa Pharmaceutical Co., Tokyo, Japan.
§Present address: Byk Gulden Pharmaceuticals, 78467 Konstanz, Germany.

controversial. Much of this controversy may be due to the finding that only the IL-1RI was shown to be capable of signaling, whereas the IL-1RII only binds IL-1 (2,3). It was shown in some systems that cAMP levels were elevated after exposure to IL-1 (13,14) and that proteinkinase A could be an important mediator (15), whereas in other systems no or insufficient changes of cAMP levels were reported (16–19). The same controversy holds true for the involvement of proteinkinase C (20,21). The simultaneous involvement of both pathways was also shown (22,23).

It is clear that phosphorylation events do occur in many cells after binding of IL-1. Whether this phosphorylation is essential for internalization or is one step in the signaling cascade cannot be concluded from the data available. Different substrates for IL-1-dependent phosphorylation have been identified. Phosphorylation of a 65-kDa protein was shown in peripheral blood mononuclear cells (19). The 27-kDa heat shock protein in fibroblasts and HepG2 cells (24) and the EGF receptor in fibroblasts and KB cells were found to be phosphorylated (25,26). In AtT-20 pituitary cells a group of 19- to 20-kDa cytosolic proteins was found to be phosphorylated (27), as well as talin in fibroblasts (28) and a 41-kDa cytosolic protein in K562 cells (29). In the IL-1-responsive D10.A Th2 helper cell line binding of the IL-1R activated tyrosine kinases, which resulted in the phosphorylation of a set of proteins of 38, 75, 97, and 115 kDa (30). In Chinese hamster ovary cells transfected with the IL-1RI the receptor itself was phosphorylated after IL-1 stimulation (31,32). IL-1-dependent alteration of the phosphorylation levels of microtubule-associated protein-2 kinases was also reported (33). Later steps in the signaling cascade seem to involve the activation of the nuclear binding proteins AP-1 (34,35) and NFκB (36). However, the role of AP-1 as mediator of IL-1 activity in T cells is controversial. In addition, we have shown that IL-1 specifically stimulated an NFκB-like nuclear factor IL-1 NF in EL4 cells (37). It was also shown recently that a new second messenger pathway might be involved in IL-1 signal transduction. Cleavage of sphingomyelin and increase in intracellular ceramide accompanied IL-1 activity in EL4 cells and sphingomyelinase synergized with PMA in these cells to induce IL-2 production (38).

To further investigate the role of phosphorylation events after binding of IL-1 that lead to IL-2 secretion we have employed the non-phorbol-type tumor promoter okadaic acid (OA). OA is a specific inhibitor of protein phosphatases PP1 and PP2A (39) and has pronounced effects in several aspects of T cell activation (40–43). AP-1 may be a possible mediator of IL-1 activity in T cells. It was shown that the binding activity and the expression of AP-1 were modulated by OA alone or in synergistic fashion with PMA (44,45). Furthermore, OA increased the stability of IL-2 mRNA in human T lymphocytes (46) and was able to mimic many effects of IL-1 in primary human fibroblasts (47). Recently, it was shown that OA-sensitive phosphatases play a role in signaling IL-2 synthesis. It was reported that IL-1 leads to increased phosphorylation of PKC-β whose action was potentiated by OA (48). We show in this report that the expression of interleukin-2 (IL-2) by the IL-1-responsive EL4 5D3 cells was stimulated by OA on the protein and mRNA level and that OA synergized with PMA and with IL-1. We show in addition that the IL-2 promoter in EL4 was synergistically stimulated by OA and IL-1. We therefore conclude that the level of protein phosphorylation is an important requisite for IL-1 activity, but that the events controlled by phosphatases and kinases are at least in part qualitatively different from the IL-1-mediated signal in EL4.

MATERIALS AND METHODS

Cells and cell culture

The selection of the EL4 subclones EL4 5D3 and EL4D6/76 was described by von Hoegen *et al.* (8). EL4 6.1 cells were obtained from Dr. R. McDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland. Cells were cultured in RPMI 1640 containing 10% FCS, 3×10^{-5} *M* 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin in humidified air at 37°C with 5% CO_2. If not indicated otherwise, washed cells were adjusted to a final density of 10^6 per ml and incubated for the indicated times with the indicated concentrations of reagents in culture medium at 37°C in humidified air at 37°C with 5% CO_2. At the end of the incubation period cell free supernatants were removed and tested for IL-2 or IL-6. Alternatively, cells were collected by centrifugation and total cytoplasmic RNA was prepared.

Reagents

Phorbol myristate acetate (PMA), MTT, sphingosine, and sphingomyelinase from *Bacillus cereus* or from *Staphylococcus aureus* were all purchased from Sigma, Deisenhofen. Okadaic acid was obtained from Fujisawa Pharmaceutical Co., Tokyo, Japan, or purchased from BIOTREND Chemikalien, Köln. Human recombinant IL-1α was kindly provided by Drs. A. Stern and P. Lomedico (Hoffmann-LaRoche, Nutley, NJ). The specific activity was 5×10^6 U/mg protein as determined by the lymphocyte activating factor assay. It was used at a concentration of 10 U/ml.

Cytokine assays

IL-2 activity in supernatants was quantitated by the ability to support growth of the IL-2-dependent cell line W2 as described (49). Units of IL-2 are based on a standard purchased from Biogen (Geneva, Switzerland; Batch No. RNB85738/09Y). IL-2 titers were measured at concentrations of OA that had no adverse effect on the assay procedure. IL-6 activity in supernatants was quantitated by the ability to support growth of the IL-6-dependent cell line B9. One U/ml was defined as the concentration of IL-6 yielding half-maximal proliferation of 3000–4000 cells per well after 72 h of culture. In both assays, either [^{3}H]thymidine incorporation or MTT conversion was used for measurement of proliferation. Both methods gave comparable results.

RNA extraction and hybridization procedures

Cytoplasmic RNA was prepared by the method of Gough (50). RNA was denatured with formaldehyde and separated in a 1% agarose gel and blotted on positively charged nylon membranes (BNBZF3R, Pall, Dreieich, FRG). UV-fixed blots were stained with methylene-blue to ensure efficient transfer and equal loading of lanes. Probe labeling and hybridization were

performed as described (49). The probe for mouse IL-2 was generously provided by A. Schimpl (Würzburg, FRG) and contains the complete exon 4 of the IL-2 gene. Hybridized blots were exposed to X-ray films for 1 to 3 days.

Transfections

EL4 5D3 cells were grown to a density of about 4×10^5 cells/ml. Cells (1.4×10^7) were transfected with 10 μg pIL 2-293 plasmid (37) DNA in a final volume of 1.2 ml using the DEAE Dextran procedure (51). Two days after transfection cells were divided into aliquots and stimulated as above. After 15 h of stimulation the cell lysates were assayed for chloramphenicol acetyltransferase (CAT) activity as described. The conversion of [^{14}C]chloramphenicol to its acetylated forms was quantified using an automatic thin-layer chromatography linear analyzer (Berthold, Wildbad, Germany). The protein concentration of cell extracts was measured according to Bradford and was used to normalize CAT activity. In addition, the plasmid pTKCAT21A was used as standard for transfection efficiency in each experiment. Plasmid pBLCAT2 was used as negative control and was uninducible after transfection but showed a very low basal transcriptional activity (37).

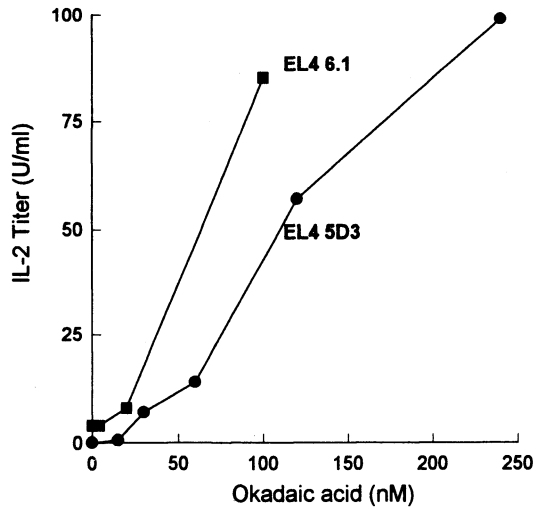

FIG. 1. Stimulation of EL4 5D3 and EL4 6.1 cells with OA. Cells were cultured at 10^6/ml in culture medium with the indicated concentrations of OA for 18 h. Cell-free supernatants were removed and the IL-2 concentration was measured with an IL-2-dependent cell line as described in Materials and Methods.

RESULTS

Okadaic acid stimulates IL-2 production in EL4 cells

It has been shown by many investigators that for activation of the IL-2 gene in cells of the T cell lineage activation of the PKC pathway is required. We therefore investigated whether inhibition of dephosphorylation would lead to IL-2 gene transcription. The non-phorbol tumor promoter OA has specificity for the protein phosphatases pp1 and pp2a and was shown to be active in T cells in inducing AP-1 activity and expression (44,45). The IL-1 responsive EL4 5D3 and EL4 6.1 cells (8) were stimulated with increasing concentrations of OA for 24 h. Supernatants were then removed and tested for IL-2 concentration in a bioassay. Figure 1 shows that the response of both cell lines was dose dependent. Concentrations higher than 250 n*M* were toxic to the cells and morphological signs of toxicity were already evident at 100 n*M* concentrations (data not shown).

Synergy of OA with PMA and IL-1

Since PMA as activator of PKC induces IL-2 production in EL4 cells it was tested whether OA and PMA synergized as costimulators. EL4 5D3 cells were stimulated for 24 h in varying concentrations of PMA and OA and mixtures thereof. As shown in Fig. 2A strong synergy was observed. Optimal stimulation for IL-2 production was achieved at 60 n*M* OA plus 16 ng/ml of PMA with a stimulation index [(PMA + OA)/PMA + OA] of 69. All other combinations were less effective. The reason for the decline of the dose–response curves at higher concentrations of OA at a given concentration of PMA was not investigated further. On visual inspection of the culture wells it correlated with the number of dead cells.

It was shown by us and others that in certain EL4 cell lines PMA and IL-1 synergize for IL-2 production. We therefore tested whether this was also true for the combination of OA and IL-1. EL4 5D3 cells were stimulated for 24 h with graded concentrations of OA with or without 10 U/ml of rhIL-1α. As can be seen in Fig. 2B there was a strong synergistic effect at an optimal concentration of OA of 60 n*M*. Also, as in Fig. 2A, further increase of OA concentration led to decreasing production of IL-2.

Expression of IL-6 protein was also synergistically stimulated by IL-1 in the presence of OA or PMA. Cells were stimulated for 24 h with OA, PMA, or IL-1 and mixtures thereof. IL-2 and IL-6 secreted into the supernatants were measured in bioassays. As shown in Table 1, IL-1 alone did not stimulate secretion. PMA and OA alone were effective. Their combination with IL-1 had the expected synergistic effect for both cytokines. The combination of OA and PMA also acted synergistically. Interestingly, under the conditions of the assay, the combination of all three stimuli showed a very strong synergy for both cytokines, pointing to the interpretation that IL-1, OA, and PMA, respectively, were each using a different signaling pathway.

Time course of activation by okadaic acid

To investigate the mode of action of okadaic acid more precisely, time course experiments were performed. At the initiation all cultures were stimulated with the indicated agents and the respective culture supernatants were harvested at the indicated times (3, 6, 12, 24, and 48 h) and their IL-2 concentration was measured (Fig. 3, right panel). A big difference in the kinetics of the response becomes evident from the Fig. 3. Whereas the response to PMA reached almost maximal levels at 12 h, the response to OA developed much slower. Only low titers were detected at 12 h, but steadily increasing titers were monitored at 24 and 48 h. The addition of IL-1 did not change the kinetics of the response. Also notable was the fact that the synergistic effect of IL-1 with PMA was already obvious at 6 h

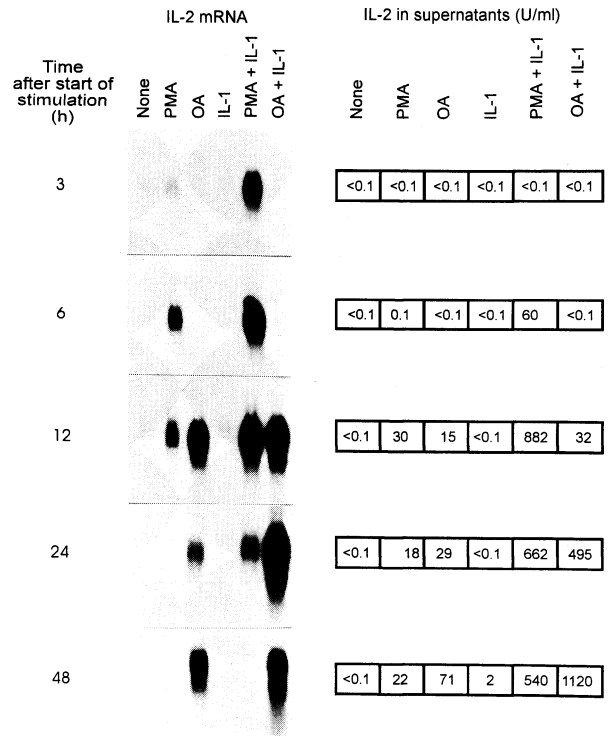

FIG. 3. Kinetics of synergistic activation of EL4 5D3 cells for IL-2 mRNA and IL-2 protein expression. Cells were incubated in separate cultures for 3, 6, 12, 24, and 48 h with PMA, OA, or IL-1 alone or with the combinations of PMA + IL-1 or OA + IL-1. The cell density was 10^6 per ml except at 48 h where cell density was reduced to 5×10^5 per ml. At the indicated times cells were collected by centrifugation. From the cell pellet RNA was prepared and subjected to Northern analysis. The left half of the figure shows the autoradiographs of the blots after probing with a mouse IL-2 probe. Equal loading of the blots was ascertained by methylene-blue staining. All signals had approximately equal intensity except the OA stimulation signal at 24 h, which had about 0.5× the intensity of the other signals. The right half of the figure shows the IL-2 titers in the supernatants of the same cells measured as described in Materials and Methods. The concentrations of the reagents were PMA 10 ng/ml; OA 120 n*M*; IL-1 10 U/ml.

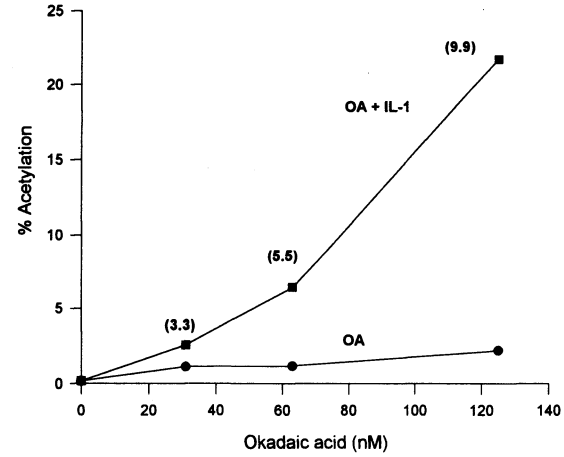

FIG. 4. OA and IL-1 synergistically activate the mouse IL-2 promoter. EL4 5D3 cells were transfected with pIL2-293 that contains the minimal promoter sequence from the mouse IL-2 gene in front of the CAT gene as reporter gene. Two days later cells were divided in aliquots and stimulated for 18 h with the indicated concentrations of OA with or without IL-1 (10 U/ml). CAT activity in the lysates of the cells was quantitated as described in Materials and Methods. The numbers in brackets represent the stimulation index SI = (OA + IL-1)/OA. IL-1 alone had no effect above background.

cells were stimulated with optimal doses of PMA + OA with or without IL-1 and Northern blots were hybridized with an IL-2 probe. At 6 h clearly more IL-2 mRNA was detected in the presence of IL-1 than in its absence. There was no detectable difference at later times. When the hybridization signals were evaluated by densitometry the stimulation index at 6 h was 3.1 and 1.0 at 18 h and 24 h, respectively. This again suggested a qualitative difference in the signals delivered by OA and IL-1.

Since sphingomyelin was shown to be involved in IL-1 signaling in EL4 cells, we were inclined to postulate that IL-1 and sphingomyelinase should act either additively or synergistically with PMA or OA. Using sphingomyelinases from 2 different sources and sphingosine in combination with PMA, OA and/or IL-1 EL4 5D3 cells were stimulated at densities ranging between 0.8×10^6/ml and 1.5×10^6/ml for 18 to 24 h and the IL-2 produced was measured in a bioassay. Sphingomyelinase was used at concentrations from 1 U/ml to 10^{-5} U/ml and sphingosine at 50 and 10 μ*M*. In all experiments PMA or OA showed strong synergy with IL-1. However, neither at optimal nor at suboptimal concentrations of these reagents did we detect any activity of sphingomyelinase or sphingosine.

DISCUSSION

The initiation of T cell activities in immune responses requires the activity of IL-1 as a costimulator. We have previously shown that CD4$^-$CD8$^-$ thymocytes were costimulated by

TABLE 2. LACK OF SYNERGY BETWEEN OA AND IL-1 IN EL4D6/76 CELLS

Stimulus[a]		*IL-2 titer*[b]	
PMA (ng/ml)	*OA (nM)*	*No IL-1*	*+ IL-1*[c]
—	30	<0.1	<0.1
—	60	1	1
16	—	2	3
16	30	61	43
16	60	520	565

[a]IL-1-nonresponsive EL4D6/76 cells were stimulated at a concentration of 10^6/ml for 20 h with the stimuli at the indicated concentrations.

[b]IL-2 titers in the supernatants were estimated using an IL-2-dependent cell line as described in Materials and Methods.

[c]IL-1 was used at a concentration of 10 U/ml.

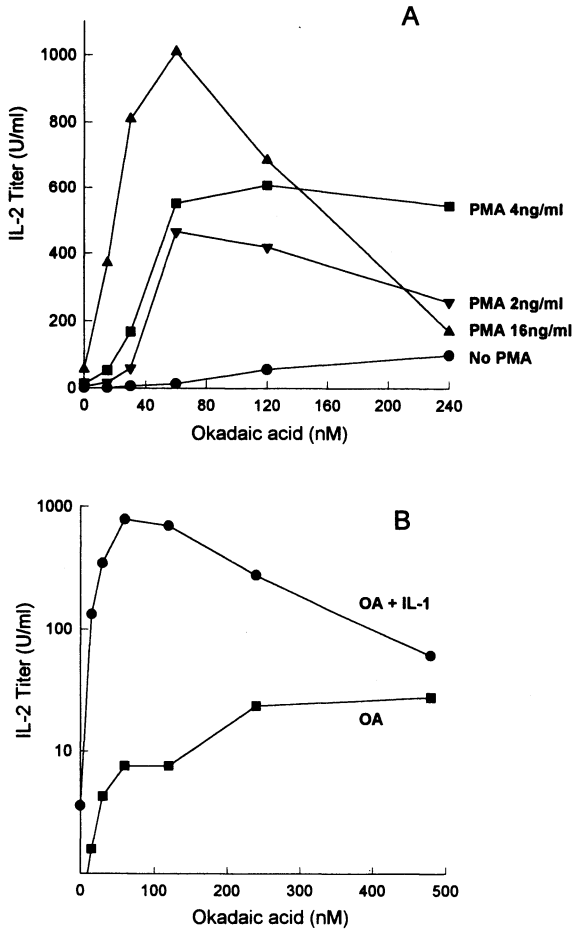

FIG. 2. Synergistic stimulation of EL4 5D3 cells by OA and PMA or IL-1 for IL-2 production. EL4 5D3 cells were incubated at a density of 10^6/ml for 24 h in 1 ml cultures. Cell-free supernatants were removed and the IL-2 concentration was measured with an IL-2-dependent cell line as described in Materials and Methods. (A) For stimulation OA and PMA were used alone at the indicated concentrations. For test of synergy OA and PMA were mixed so that the indicated final concentrations were achieved. (B) The indicated concentrations of OA with or without 10 U/ml of IL-1 were used for stimulation. Controls: no stimulation <0.1 U/ml; PMA (10 ng/ml) 19 U/ml; PMA + IL-1 (10 U/ml) 1126 U/ml.

and fully developed at 12 h. This again suggested that OA and IL-1 can use independent signal transduction pathways.

To determine whether the activities of OA were reflected at the transcriptional level Northern blots were prepared using total cytoplasmic RNA from cells that were stimulated as shown in Fig. 3. The blots were hybridized to an IL-2 probe and autoradiographed. As shown in Fig. 3 (left panel), the differences between the different modes of stimulation were even more striking. Stimulation with PMA + IL-1 gave rise to the appearance of significant amounts of IL-2 mRNA already at 3 h, demonstrating the very quick synergistic effect of IL-1. No IL-2 mRNA was seen in cells stimulated with OA or OA + IL-1 until after 12 h stimulation. Whereas the effect of PMA stimulation had mostly disappeared at 24 h, OA-induced production of IL-2 mRNA expression was still detected at 48 h.

TABLE 1. SYNERGISTIC INDUCTION OF IL-2 AND IL-6 SECRETION BY OA, PMA, AND IL-1

Stimuli[a]	*IL-2 titers (U/ml)*[b]	*IL-6 titers (U/ml)*[c]
None	<0.1	<0.1
PMA (5 ng/ml)	3	1
OA (125 n*M*)	12	90
IL-1 (10 U/ml)	<0.1	<0.1
PMA + IL-1	26	40
OA + IL-1	66	3500
PMA + OA	494	2400
PMA + OA + IL-1	>2000	>22000

[a]EL4 5D3 cells were stimulated for 24 h at a concentration of 10^6 per ml with the indicated agents. When combined stimuli had the same concentrations as when used alone.

[b]IL-2 titers represent international standard.

[c]IL-6 titers were estimated as described in Materials and Methods. One unit per ml causes 50% maximal MTT conversion.

The late onset of IL-2 mRNA appearance and the persistence of IL-2 mRNA could be explained by posttranslational activity of OA leading to increased stability of IL-2 mRNA as reported for T cells (46). Alternatively, the transcriptional activity could be affected. To discriminate between the two possibilities and to investigate whether OA might be active on the transcriptional level, EL4 5D3 cells were transiently transfected with the plasmid pIL2-293 that contained the first 293 bp of the mouse IL-2 promoter in front of the CAT gene as reporter gene. Transfected cells were stimulated with graded doses of OA with or without IL-1. Eighteen hours later CAT activity was determined in the cell lysates. Figure 4 shows that OA alone only weakly stimulated CAT expression. Strong synergistic stimulation was observed in the presence of IL-1 leading to stimulation indices (SI) as high as 9.9, demonstrating strong activity of OA on the transcriptional level.

To further dissect the roles of IL-1 and OA IL-2 production of the IL-1-nonresponsive cell line, EL4D6/76 was tested after stimulation for 20 h with PMA, OA, and several combinations of these agents. As seen from Table 2, OA was able to weakly induce EL4D6/76 by itself but strongly synergized with PMA. There was no synergy of OA or PMA with IL-1 detectable. The IL-2 levels reached with PMA and OA together were also not further enhanced by the addition of IL-1. This lack of additive or synergistic effects suggests that OA may have some activities in common with IL-1 as reported, but does not substitute for the defect of EL4D6/76. This again indicates that IL-1 activates additional pathways.

In experiments evaluating the synergy between OA and PMA we had observed that at concentrations of PMA and OA yielding maximal production of IL-2 there was no additional effect of IL-1. To determine whether under such conditions both agents together completely replace IL-1 the time course of IL-1 activity under these conditions was investigated. Since OA by itself acted rather late but the synergy with IL-1 was observed very early after activation early time points were investigated. The data in Fig. 5 show that this indeed was the case. EL4 5D3

FIG. 5. Kinetic of IL-2 mRNA expression and IL-1-dependent synergy with PMA and OA at optimal stimulation conditions. EL4 5D3 cells at a density of 10^6/ml were stimulated for 6, 12, and 24 h with optimal concentrations of PMA (32 ng/ml) + OA (60 n*M*) with or without IL-1 (10 U/ml). Cells were collected by centrifugation and RNA was prepared. Northern blots were prepared and probed with an IL-2 probe as described in Fig. 3.

IL-1 to express *de novo* functional IL-1 receptors (49). Others have shown that IL-1 is needed for the production of IL-2. Utilizing IL-1-responsive and IL-1-nonresponsive subclones of the murine thymoma EL4 we have shown that IL-1 enhances the activity of the IL-2 promoter and that this enhancement was due to the specific activation by IL-1 of the NFκB-like factor IL-1 NF that binds to the TCEd element of the promoter (37). Yet, relatively little is known about the messengers involved in this activation cascade. In this report we investigated whether serine/threonine phosphatases would be involved in IL-1 signaling. We found that in contrast to IL-1 alone the pp1 and pp2a serine/threonine phosphatase inhibitor OA alone was capable of inducing IL-2 production. Since there was indication in the literature showing qualitatively comparable effects of IL-1 and okadaic acid (44,45,47,52), we looked at the effect of combining the two reagents expecting synergistic or additive activities. We found strong synergy with respect to IL-2 or IL-6 production over a wide range of OA concentration. This led us to conclude that even though OA shares many effects with IL-1, some pathways induced by IL-1 may be different from the ones induced by OA. Several other lines of evidence also point to this interpretation. In contrast to OA, which activated EL4 cells very slowly compared to PMA IL-1 activity was rapidly demonstrable. In synergy with PMA or OA or both the costimulatory effect was evident at the earliest time points. OA was also active in the IL-1-nonresponsive cell line EL4 D6/76. Although OA mimics early effects of IL-1, these effects do not comprise all of the activities of IL-1 since one should have expected restoration of the defective IL-1 responsiveness of this cell line. However, it is possible that OA and IL-1 share some pathways later in the signaling cascade. It could be speculated that the presence of internalized IL-1 is required for activation of the IL-2 promoter. Since internalization of the IL-1/IL-1R complex alone was shown not to be sufficient for IL-2 promoter activation (11), it would be interesting to test whether intracellularly expressed IL-1 synergizes with OA or whether complementation and synergy between the truncated, internalized, but inactive IL-1R and OA exists. Furthermore, we tested whether the additional pathway of IL-1 activity could be the recently described sphingomyelin pathway, which would explain the synergistic effects shown (38). However, we found no activity of sphingomyelinase or sphingosine in our system. The discrepancies between our results and the published findings may be due to the EL4 cells used, which were not further characterized. Another possibility could be the difference in sensitivity of detection of produced IL-2. We used an IL-2-specific bioassay with a detection limit of 0.1 U/ml, whereas Mathias *et al.* tested IL-2 by ELISA (38).

It was shown by others that OA increased the stability of IL-2 mRNA in human T cells (46). The slow accumulation and the long duration of IL-2 mRNA expression after OA stimulation shown in this paper fits this finding. Nevertheless, it is unlikely that this is the mechanism of OA action. We found that the transcription of the transfected IL-2 promoter was enhanced by OA and that IL-1 synergistically increased this transcription. In addition, in experiments not shown in this paper, we found that in EL4 cells the IL-2 mRNA showed no detectable decay within 8 h. Therefore, it can be concluded that the activity of OA leads to increased transcription of the IL-2 gene.

The target sequences on the IL-2 promoter were not investigated. It was shown that OA stimulated expression and binding activity of the transcription factor AP-1 in EL4 (45). However, there was almost no synergy between OA and IL-1 in this respect, which is in contrast to the effect found on the promoter in our experiments shown in this report. There is also evidence by us and others that AP-1 is not a major contributor to the effective stimulation of the IL-2 promoter by IL-1 costimulation (37,53–55). It can be expected, therefore, that the increased transcription was due to activation of sites different from the AP-1 binding site.

It is also very unlikely that modulation of IL-1R expression plays a role in OA stimulation in contrast to the TNFR, where down-modulation and shedding was observed (56). Since less than 20 IL-1R per cell are sufficient for activation (3) up- or down-regulation of the approximately 2500 receptors may not effectively influence activation.

Taken together, we believe that a high level of cellular protein phosphorylation, which is brought about by the synergistic action of PMA and OA, is a prerequisite for high level IL-2 expression. We have also preliminary evidence that any change in steady-state phosphorylation can prime the cell for a second stimulus. We found synergy between staurosporine, an inhibitor of PKC action, and IL-1 in stimulation of IL-2 synthesis, although the amount of protein produced was low (1–3 U/ml). Undoubtedly, IL-1 itself induces phosphorylation processes that use the same pathways as the substrates of OA, but IL-1 also induces additional events. The observed nuclear translocation of the ligand–receptor complex might be of importance in this respect. It is conceivable that IL-1 itself and structures of the cytoplasmic part of the IL-1RI have to interact with the nuclear membrane to properly activate or induce the IL-1-specific

nuclear factor IL-1 NF. Experiments to tackle this question are underway.

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Address reprint requests to:
PD Dr. W. Falk
Department of Internal Medicine I
University of Regensburg
93042 Regensburg, Germany

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