The widely used phosphatase 1 and 2A inhibitor okadaic acid is one of the many stimuli activating transcription factor NF-κB in cultured cells. Phosphorylation of IκB-α, one of NF-κB’s inhibitory subunits, is a prerequisite for IκB degradation and the subsequent liberation of transcriptionally active NF-κB. This observation suggested that the phosphorylation status of IκB is influenced by an okadaic acid-sensitive phosphatase. In this study, we provide evidence that the effect of okadaic acid on NF-κB activation is indirect and dependent on the production of reactive oxygen intermediates rather than the inhibition of an IκB-α phosphatase. Okadaic acid was found to be a strong inducer of cellular H$_2$O$_2$ and superoxide production in two distinct cell lines. The structurally unrelated phosphatase inhibitor calyculin A also induced oxidative stress. The delayed onset of reactive oxygen production in response to okadaic acid correlated with the delayed activation of NF-κB. Moreover, NF-κB induction was optimal at the same okadaic acid concentration that caused optimal H$_2$O$_2$ production. Both reactive oxygen intermediates production and NF-κB activation were inhibited by the antioxidant pyrrolidine dithiocarbamate and 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate, a Ca$^{2+}$ chelator. Future experiments using phosphatase inhibitors in intact cells must consider that the compounds can act as strong inducers of oxidative stress, which provides one explanation for their tumor-promoting activity.

**A hallmark of the higher eukaryotic transcription factor NF-κB is its rapid post-translational activation by a plethora of distinct, mostly pathogenic stimuli, including viral and bacterial infections, inflammatory cytokines, UV and γ radiation, and oxidants (reviewed in Liou and Baltimore (1993), Baeuerle and Henkel (1994), and Siebenlist et al. (1994)). Many of these stimuli cause oxidative stress in cells, i.e. there is an increased production of ROIs, such as superoxide, hydrogen peroxide, hydroxyl radicals, and secondary reactive intermediates (reviewed in Schreck and Baeuerle (1991) and Schreck et al. (1992a)). Four lines of evidence suggest that ROIs, in particular H$_2$O$_2$, serve as common second messenger-like molecules in the various pathways leading to NF-κB activation. First, many structurally unrelated antioxidants suppress NF-κB activation in response to most inducing conditions (reviewed in Schreck et al. (1992a) and Meyer et al. (1993a)). Dithiocarbamates (Schreck et al., 1992b), NAC (Staal et al., 1990; Schreck et al., 1991), and vitamin E derivatives (Suzuki et al., 1993) are particularly well studied in this respect. Second, in some cell lines NF-κB is directly activated upon addition of micromolar amounts of H$_2$O$_2$ to the culture medium (Schreck et al., 1991; Meyer et al., 1993b). Third, stable overexpression of catalase impairs NF-κB activation while overexpression of Cu/Zn superoxide dismutase superinduces NF-κB activation in response to tumor necrosis factor-α and OA (Schmidt et al., 1995). Last, re-exposure of hypoxic cells to dioxygen is a very potent NF-κB-inducing condition. In view of this requirement for ROIs we wondered whether there are inducers that bypass the ROI step and act more proximal on the NF-κB-IκB pathway. A recent study by Packer and colleagues (Suzuki et al., 1994) suggested that the phosphatase inhibitors OA and calyculin A are such ROI-independent inducers.**

In unstimulated cells, NF-κB is known to reside in a latent form in the cytoplasm (Baeuerle and Baltimore, 1988a, 1988b). This form is stabilized by an inhibitory subunit, called IκB, which is tightly bound to a dimer frequently composed of the DNA-binding subunits p50 and p65 (RelA) (reviewed in Beg et al. (1993)). Upon cell stimulation, IκB is removed and the liberated NF-κB able to translocate into the nucleus and activate target genes by binding to regulatory elements in enhancers and promoters. The removal of IκB is controlled by protein phosphorylation. A yet unidentified kinase rapidly phosphorylates human IκB-α on serines 32 and 36 in response to various inducers (Brown et al., 1995; Traenckner et al., 1995). By phosphorylation, IκB-α which is still bound to NF-κB has apparently turned into a high affinity substrate for an ubiquitin-conjugating enzyme (Traenckner et al., 1994; Traenckner and Baeuerle, 1995). Following this phosphorylation-controlled ubiquitination, IκB-α is rapidly and completely degraded by the 20 S or 26 S proteasome (Palombella et al., 1994; Traenckner et al., 1994). Specific peptide inhibitors of the proteasome which stabilize the phosphoform of IκB have proven as potent inhibitors of NF-κB activation. Antioxidants also prevent the decay of IκB, however, by inhibition of the phosphorylation step and not the proteasome. Hence, antioxidants may act upstream of the kinases/phosphatase system, suggesting that the IκB phosphorylation is redox-controlled.

OA, a C$_{38}$ fatty acid polyether compound, is a toxin which causes diarrhetic shellfish poisoning in humans (reviewed in § R. Rupec and P. Baeuerle, (1995) Eur. J. Biochem., in press.}
Okadaic Acid and Oxidative Stress

Okadaic Acid and Degradation—In HeLa cells, 0.7 μM OA strongly induced an activity which retarded upon native gel electrophoresis a 32P-labeled oligonucleotide with a consensus NF-κB binding site (Fig. 1A, compare lanes 1 and 2). Formation of the newly induced protein-DNA complex was completely prevented in the presence of an antibody specific for the transactivating p65 (RelA) subunit. The activation of NF-κB by OA involved the proteolytic degradation of the inhibitory subunit IκB-α, as shown by Western blotting with an IκB-α-specific polyclonal antibody (Fig. 1B). Hence, the effects of OA on the NF-κB/IκB system were not distinguishable from those of other inducers of the transcription factor.

An Antioxidant Prevents NF-κB Activation by OA But Not OA-induced Protein Phosphorylation—The effect of many stimuli activating NF-κB is suppressed by pretreatment of cells with antioxidants (reviewed in Schreck et al. [1992a]). Vitamin E and derivatives, various thiol reagents, metal chelators, and phenolic scavengers have been reported to prevent NF-κB activation. Particularly potent are dithiocarbamates (Schreck et al., 1990, 1992b). As shown in Fig. 2A, the induction of NF-κB in HeLa cells by 0.7 μM OA was completely suppressed when cells were preincubated for 1 h with 100 μM of the antioxidant PDTC. In contrast to a previous report (Suzuki et al., 1994), we observed also that 30 mM NAC prevented OA-induced NF-κB activation (data not shown). This suggested that OA relied on oxidative stress for the activation of NF-κB. To investigate whether PDTC still allowed for an increased incorporation of phosphate into polypeptides in response to OA, cell cultures were incubated with inorganic [32P]phosphate and 32P-labeled protein by reducing SDS-PAGE and fluorography. Under control conditions, treatment with 0.4 μM OA caused an increased incorporation of radioactive phosphate, which was most pronounced with proteins of apparent molecular masses between 40 and 97 kDa (Fig. 2B, compare lanes 1 and 2). In the presence of 100 μM PDTC, OA still induced an increased protein phosphorylation and the pattern of phosphoproteins was with a rubber policeman, and suspended in 0.5 ml of PBS of 37°C. Fifty μM DCHF (Mobitech) dissolved in dimethylformamide was added and cells incubated for 15 min at 37°C. Thereafter, cells were placed on ice and aliquots of 10,000 cells scanned in a Becton Dickinson FACS SORT according to Boissy et al. (1989) with excitation and emission settings of 495 and 525 nm, respectively. Histograms were analyzed with the software program Lysis II.

Determination of Cellular H2O2 and O2- Production—H2O2 and O2- released from approximately 2 x 10^6 HeLa or F26 cells grown in cuvettes was determined as described previously (McCord and Fridovich, 1969; Loschen et al., 1971; Meier et al., 1989, 1990; Schreck et al., 1992a).

Electrophoretic Mobility Shift Assay—Total cell extracts for EMSA were prepared by resuspending PBS-washed cell pellets in a high salt buffer containing the nonionic detergent Nonidet P-40 (Baeuere and Baltimore, 1988B). After 10 min on ice, debris was removed by centrifugation for 10 min at 15,000 x g and the protein concentration in the remaining supernatant determined by a Coomassie Brilliant Blue assay (Bio-Rad). Equal amounts of protein (10–15 μg) were reacted with 10,000 cpm (Cerenkov counting) of a T4 polynucleotide kinase 32P-end-labeled double-stranded oligonucleotide with a high affinity NF-κB binding motif (Promega) under conditions described previously (Schreiber et al., 1989). For protein-DNA complex typing, 1 μl of p65-specific (Santa Cruz Biotechnology) and IκB-α-specific rabbit antiserum (Zabel et al., 1993) were directly added to the DNA binding reaction. DNA binding reactions were analyzed by electrophoresis on native 4% polyacrylamide gels. Dried gels were exposed to Kodak XR5 films. 32P radioactivity in NF-κB-DNA complexes was quantitated by a β imaging system (Molecular Dynamics).

RESULTS

OA Activates a Prototypic NF-κB Complex by IκB-α Degradation—In HeLa cells, 0.7 μM OA strongly induced an activity which retarded upon native gel electrophoresis a 32P-labeled oligonucleotide with a consensus NF-κB binding site (Fig. 1A, compare lanes 1 and 2). Formation of the newly induced protein-DNA complex was completely prevented in the presence of an antibody specific for the transactivating p65 NF-κB subunit (lane 6, compare to lane 1), while a control antibody against the inhibitory subunit IκB-α was ineffective (lane 4). This shows that okadaic acid induces a prototypic NF-κB complex containing the transactivating p65 (RelA) subunit. The activation of NF-κB by OA involved the proteolytic degradation of the inhibitory subunit IκB-α, as shown by Western blotting with an IκB-α-specific polyclonal antibody (Fig. 1B). Hence, the effects of OA on the NF-κB/IκB system were not distinguishable from those of other inducers of the transcription factor.

An Antioxidant Prevents NF-κB Activation by OA But Not OA-induced Protein Phosphorylation—The effect of many stimuli activating NF-κB is suppressed by pretreatment of cells with antioxidants (reviewed in Schreck et al. [1992a]). Vitamin E and derivatives, various thiol reagents, metal chelators, and phenolic scavengers have been reported to prevent NF-κB activation. Particularly potent are dithiocarbamates (Schreck et al., 1990, 1992b). As shown in Fig. 2A, the induction of NF-κB in HeLa cells by 0.7 μM OA was completely suppressed when cells were preincubated for 1 h with 100 μM of the antioxidant PDTC. In contrast to a previous report (Suzuki et al., 1994), we observed also that 30 mM NAC prevented OA-induced NF-κB activation (data not shown). This suggested that OA relied on oxidative stress for the activation of NF-κB. To investigate whether PDTC still allowed for an increased incorporation of phosphate into polypeptides in response to OA, cell cultures were incubated with inorganic [32P]phosphate and 32P-labeled protein by reducing SDS-PAGE and fluorography. Under control conditions, treatment with 0.4 μM OA caused an increased incorporation of radioactive phosphate, which was most pronounced with proteins of apparent molecular masses between 40 and 97 kDa (Fig. 2B, compare lanes 1 and 2). In the presence of 100 μM PDTC, OA still induced an increased protein phosphorylation and the pattern of phosphoproteins was
Okadaic Acid and Oxidative Stress

FIG. 1. The effect of OA on NF-κB activation and IκB-α stability in intact cells. HeLa cells were treated for 1 h with the indicated concentrations of OA. A, activation of NF-κB DNA binding activity by OA. Total cell extracts were prepared from control (lanes 1, 3, and 5) and OA-treated cells (lanes 2, 4, and 6) and analyzed by EMSA using a 32P-labeled oligonucleotide probe containing a high-affinity κB motif. Cell extracts were incubated with a control antibody directed against human IκB-α (lanes 3 and 4) or an antibody against the p65 NF-κB subunit (lanes 5 and 6). An OA-inducible protein-DNA complex which is abrogated by anti-p65 but not by anti-IκB-α is indicated by a filled arrowhead. A faster-migrating nongeneric complex was unaffected. A small filled arrowhead indicates the position of an immune complex. The open arrowhead marks the position of the unbound DNA probe. A fluorogram of a native gel is shown. B, OA causes a degradation of IκB-α. Total cell extracts of control (lane 1) and OA-treated HeLa cells (lane 2) were subjected to reducing SDS-PAGE and proteins transferred on filters for Western blotting. IκB-α was visualized by an affinity-purified polyclonal antibody against recombinant human IκB-α and enhanced chemiluminescent staining as described (Henkel et al., 1993). A fluorogram of a section of the filter is shown. An arrowhead indicates the position of a 38-kDa IκB-α-specific signal.

This indicates that PDTC in intact cells did not act by neutralizing the phosphatase inhibitory activity of OA nor by inhibiting major protein kinases responding to OA. In the following we tested the idea that OA is an inducer of oxidative stress.

OA Increases Cellular ROI Production which Is Prevented by PDTC—DCFH is a dye that allows to monitor cellular ROI production in HeLa cells as OA but at a concentration of 0.9 μM OA. At higher OA concentrations, ROI production declined and increasing desintegration of cells was observed microscopically. HeLa and F26 cells showed cell type-specific differences with respect to the OA dose dependence of ROI production but had the same maximum. These data provide direct evidence that the phosphatase inhibitor OA induces oxidative stress in two distinct cell types. The structurally unrelated phosphatase inhibitor calyculin A, which also activates NF-κB (Suzuki et al., 1994), induced equally high levels of H2O2 and O2•- production in HeLa cells as OA but at a higher concentration (Fig. 4C). Maximal induction of oxidative stress was observed at 30 μM calyculin and declined with higher concentrations of the compound.

H2O2 Production and NF-κB Activation Show an Overlapping OA Dose-Response—We investigated whether the production of ROIs was related to the activation of transcription factor NF-κB in a dose-response to OA. In HeLa cells, weak activation of NF-κB was observed after treatment of cells with 0.1 μM OA (Fig. 5A, lane 2). The activation increased weakly up to 0.6 μM OA. Optimal activation occurred between 0.7 and 1 μM OA, as shown quantitated in Fig. 4B. The OA dose for optimal NF-κB activation was the same as that for optimal H2O2 production in HeLa cells (compare Figs. 5B and 3A). At higher OA concent-
trations, NF-κB activation declined, as was seen with the \( \text{H}_2\text{O}_2 \) production. In F26 cells, a similar correlation between \( \text{H}_2\text{O}_2 \) production and NF-κB activation in response to OA treatment was observed (Fig. 6, A and B). A strong NF-κB activation was seen at OA concentrations >0.4 \( \mu \)M (Fig. 6A, lane 5), which again corresponded to the OA concentrations that yielded an increased production of \( \text{H}_2\text{O}_2 \) (see Fig. 4B). These data support a causal relationship between the extent of ROI production and NF-κB activation in response to OA treatment of HeLa and F26 cells.

Temporal Coincidence of \( \text{H}_2\text{O}_2 \) Production and NF-κB Activation by OA—A second criterion for a causal relationship between NF-κB activation and \( \text{H}_2\text{O}_2 \) production in response to OA is a kinetic coincidence of the two events. HeLa cells showed an approximately 15-min delay before 0.7 \( \mu \)M OA could induce \( \text{H}_2\text{O}_2 \) production (Fig. 7A). After 40–50 min, \( \text{H}_2\text{O}_2 \) production started to plateau. The activation of NF-κB showed a very similar kinetic profile (Fig. 7B). There was no significant activation of NF-κB before a 20-min OA treatment and a plateau was reached after 60 min. F26 cells required approximately 30 min before there was a significant increase in \( \text{H}_2\text{O}_2 \) production (Fig. 8A). In this cell line, NF-κB activity did not strongly increase before 40 min after addition of 0.7 \( \mu \)M OA (Fig. 8B). These data show that NF-κB activation follows the production of \( \text{H}_2\text{O}_2 \) in OA-treated cells which is consistent with the idea that \( \text{H}_2\text{O}_2 \) serves as a messenger of NF-κB activation (Schmidt et al., 1995).

A Calcium Chelator Prevents Both \( \text{H}_2\text{O}_2 \) Production and NF-κB Activation by OA in a Similar Concentration Range—A third criterion for a causal relationship between NF-κB activation and \( \text{H}_2\text{O}_2 \) production in response to OA is that both events are inhibited by a drug within a similar concentration range. Here, we tested TMB-8, a chelator of intracellularly released calcium. TMB-8 prevented the production of \( \text{H}_2\text{O}_2 \) in HeLa cells treated with 0.7 \( \mu \)M OA in a dose-dependent manner (Fig. 9A), suggesting that intracellularly released Ca\(^{2+}\) was a cofactor in the induction of oxidative stress. Half-maximal inhibition was obtained with 300 \( \mu \)M of the compound. TMB-8 had a similar effect on NF-κB activation (Fig. 9B). Two hundred \( \mu \)M TMB-8 were sufficient to completely suppress NF-κB activation in response to 0.7 \( \mu \)M OA. This corresponded to a 30% inhibition of \( \text{H}_2\text{O}_2 \) production. The nonlinear dose-response behavior of NF-κB activation (see Figs. 4A and 5) makes it likely that such a reduction of \( \text{H}_2\text{O}_2 \) production is sufficient to prevent NF-κB activation.
NF-κB (5 μM) require a ROI step for signaling. However, the antioxidant OA was set to 100%.

of H₂O₂ or 5 mM of the glutathione synthesis inhibitor L-buthionine-(S,R)-sulfoximine in several cell lines. We have observed that 30 mM NAC suppresses NF-κB inhibition in HeLa cells.

DISCUSSION

The mechanism by which the PP inhibitor OA activates NF-κB is a matter of debate. Packer and colleagues (Suzuki et al., 1994) reported that the activation of NF-κB by OA or calycin A cannot be prevented by the antioxidants NAC (20 mM) or dihydrolipoate (1 mM), suggesting that OA does not require a ROI step for signaling. However, the antioxidant rotenone (5 μM), which inhibits tumor necrosis factor α signaling (Schulze-Osthoff et al., 1993), prevented OA-induced NF-κB activation. It was speculated that the inhibitory effect of rotenone on OA was not caused by its antioxidative potential but by the reduction of cellular ATP levels by approximately 50% (Suzuki et al., 1994). However, it is questionable whether this degree of ATP depletion was sufficient to inhibit protein kinases in the cell, most of which have Kₘ values for ATP in the micromolar range. Here we show that the antioxidant PDTC is a potent inhibitor of OA activity and ROI production without having an apparent influence on overall protein phosphorylation. PDTC was also shown to prevent H₂O₂ production and NF-κB activation in response to Fcγ2a receptor stimulation in J 774 cells (Muroi et al., 1994). It seems that antioxidants profoundly differ with respect to their effect on OA-induced NF-κB activation. These differences may come from a distinct uptake, subcellular distribution, or metabolism of the antioxidants in the cell. Non-thiol antioxidants, such as PDTC and rotenone, are presumably less rapidly metabolized or taken up better than the more physiological compounds NAC and lipoic acid. Menon et al. (1993) have observed that 30 mM L-cysteine prevents both OA- and tumor necrosis factor α-induced NF-κB activation in primary and transformed MRC-5 cells. It was also observed by these authors that the NF-κB activation by OA was enhanced in the presence of either micromolar concentrations of lipoic acid. Menon et al. (1993) have observed that 30 mM NAC suppresses NF-κB inhibition in HeLa cells.

Fig. 5. Dose dependence of NF-κB activation by OA in HeLa tumor cells. A, EMSA analysis. HeLa cell cultures were treated with the indicated concentrations of OA and cell extracts analyzed for κB-specific DNA binding activity using EMSA. For details see legend to Fig. 1. A section of a fluorogram from a native gel is shown. A filled arrowhead indicates the position of the OA-induced NF-κB-DNA complex. A faster-migrating nonspecific complex was unaffected. B, quantitation of NF-κB activity. The 3²P radioactivity in the NF-κB-DNA complexes was quantitated by a β imager and is shown plotted against the OA concentration. The maximal NF-κB activation seen at 0.9 μM OA was set to 100%.

Fig. 6. Dose dependence of NF-κB activation by OA in F26 primary fibroblasts. A, EMSA analysis. F26 cell cultures were treated with the indicated concentrations of OA and cell extracts analyzed for κB-specific DNA binding activity using EMSA. For details, see the legend to Fig. 1. A section of a fluorogram from a native gel is shown. A filled arrowhead indicates the position of the OA-induced NF-κB-DNA complex. A faster-migrating nonspecific complex was unaffected. B, quantitation of NF-κB activity. The 3²P radioactivity in the NF-κB-DNA complexes was quantitated by a β imager and is shown plotted against the OA concentration. The maximal NF-κB activation seen at 1 μM OA was set to 100%.

Fig. 7. Time dependence of H₂O₂ production and NF-κB activation in response to OA in HeLa tumor cells. A, H₂O₂ production. The H₂O₂ released by HeLa cells in response to a treatment with 0.7 μM OA was determined and is shown plotted against time. B, NF-κB activity. Total cell extracts from HeLa cells treated for the indicated periods of time with 0.7 μM OA were prepared and analyzed by EMSA as described in the legend to Fig. 1. A section of a fluorogram is shown. A filled arrowhead indicates the position of the OA-inducible NF-κB-DNA complex. (data not shown). These data would also be consistent with a requirement of a pro-oxidant condition for OA signaling.

All published studies relied on a pharmacological approach to test for an involvement of ROIs in the signaling of OA. The conflicting results prompted us to directly investigate whether...
OA leads to an enhanced cellular production of ROIs. We found that OA is a strong inducer of cellular ROI production by two distinct approaches. First, OA was shown to increase the cellular oxidation of the dye DCFH; second, an increased H$_2$O$_2$ and O$_2^-$ production was directly measured in culture supernatants of OA-stimulated HeLa tumor cells and F26 primary fibroblasts. ROI production and the activation of NF-$\kappa$B correlated both with respect to kinetics and dose dependence. We could not observe that only the tumor cell line would respond to OA treatment; F26 primary fibroblasts showed a similar responsiveness as HeLa tumor cells. NF-$\kappa$B activation by OA was also reported in human B lymphocytes, another type of primary cells (Rieckmann et al., 1992). Hence, the finding that primary cells are less or unresponsive toward OA cannot be generalized.

The slow kinetic of ROI production in response to OA suggests that a number of reactions have to occur before NF-$\kappa$B is activated. Alternatively, it may reflect a very slow uptake of the PP inhibitor into the cell. Inhibition of PP1 and PP2A may allow enhanced phosphorylation of a substrate $X$. Phosphorylated $X$ may then, for instance, cause directly or indirectly a rise in intracellular calcium which is where TMB-8 might interfere in the signaling pathway. Intracellular calcium is also required for activation of NF-$\kappa$B by endoplasmic reticulum stress-inducing conditions and two drugs inhibiting the Ca$^{2+}$-ATPase in the endoplasmic reticulum membrane turned out to be potent NF-$\kappa$B inducers (Pahl and Baueerle, 1995). There is one report that calyculin A induces an increase in intracellular calcium (Ishihara et al., 1989). Whether PKC is activated and required in a subsequent step needs future studies. Thelenin et al. (1989) provided evidence that PKC is not involved in OA signaling. Very clearly, ROIs are produced in response to OA stimulation of cells. It is likely that the ROI scavenger PDTC as well as the costimuli H$_2$O$_2$ and L-buthionine-(S,R)-sulfoximine act at this late stage of OA signaling. This is also where signals

from other NF-$\kappa$B inducers, such as tumor necrosis factor $\alpha$, interleukin-1, and UV light may converge into the pathway. As described in the Introduction, an as yet unidentified kinase/phosphatase system appears to be controlled by ROIs and ultimately activates NF-$\kappa$B through a phosphorylation-controlled proteolytic degradation of I-$\kappa$B-$\alpha$. The very slow action of OA and the apparent involvement of ROIs make it very unlikely that an I-$\kappa$B-$\alpha$ phosphatase is a direct target for OA inhibition. In conclusion, this study cannot support the previous notion that OA activates NF-$\kappa$B independently of ROIs. On the contrary, OA was found to be a strong inducer of oxidative stress which may provide a further explanation for its broad biological effects.

Our observation that both OA and calyculin A are potent inducers of oxidative stress may well explain their tumor promoting potential. Tumor promotion by OA inhibitors could not be correlated to events controlling the cell cycle. On the contrary, in myeloid leukemic cells OA induced cell cycle arrest and apoptosis (Ishida et al., 1992) and in raf and ret-II-transformed fibroblasts the drug reversed the transformed phenotype to that of normal contact inhibited cells (Sakai et al., 1989; reviewed in Mackintosh and Mackintosh, 1994). Based on our present finding that both OA and calyculin A potently induce oxidative stress we would like to propose that PP1 and PP2A inhibition has a tumor promoting effect by virtue of deregulating a phosphorylation-controlled ROI-producing cellular process. A similar mechanism was proposed for the tumor-promoting activity of phorbol esters (Cerutti, 1985), which are activators of several PKC isozymes. A number of observations are consistent with this hypothesis. OA has been reported to cause mutagenesis of eukaryotic but not prokaryotic DNA (Aonuma et al., 1991). DNA damage is a well documented effect of oxidative stress (reviewed in Halliwell and Gutteridge,

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3 H. L. Pahl and P. A. Baueerle, manuscript submitted.
Aonuma, S., and the activation of NF-κB by OA and calyculin A showed a lag phase of 15–30 min, as was observed in our study for the production of ROIs and the activation of NF-κB. This raises the possibility that PKC is involved in tumor promotion, ROI production, and NF-κB activation by the non-phorbol tumor promoter OA. An involvement of PKC would also be consistent with the inhibitory effect of the Ca²⁺ chelator TMB-8. Ca²⁺ is required for 1

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