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OKADAIC ACID INDUCES ACTIVATOR PROTEIN 1 ACTIVITY AND IMMEDIATE EARLY GENE TRANSCRIPTION IN RAT PHEOCHROMOCYTOMA CELLS

MECHANISM OF ACTION

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Abstract—The serine/threonine protein phosphatase inhibitor okadaic acid (OA) was found to enhance mRNA transcripts of *c-fos* and of the *jun* family of proto-oncogenes including *c-jun*, *jun B* and *jun D* in cultured pheochromocytoma PC12 cells. This expression remained elevated for more than 8 hr. An increase in the binding of the transcription factor activator protein 1 (AP1) to its DNA consensus sequence that occurred prior to early gene transcription was observed. Enhanced AP1 activity was still observed when OA was added to the cells together with the transcription inhibitor actinomycin D, or with the protein synthesis inhibitor cycloheximide, indicating that it is actually AP1 activation due to posttranslational modifications that triggers transcription of the *fos* and *jun* genes. AP1 was activated through serine/threonine phosphorylation since its activation was abolished when nuclear extracts of OA-treated cells were incubated with protein phosphatase-1 or, to a lesser extent, with protein phosphatase-2A. C-Jun and Jun D proteins are likely candidates for being phosphorylated, since they were shown to constitute the AP1 complex at the time when it was activated (2 hr after OA addition).

Key words: early genes; jun; okadaic acid; PC12 cells; protein phosphatases; transcription factor AP1

OA† is a polyether fatty acid produced by certain dinoflagellates that concentrates in marine sponges and shellfish. It is the major toxic component associated with diarrhetic seafood poisoning [1]. OA has been reported as a potent tumour promoter in mice skin [2]. On the other hand, it has been demonstrated that NIH 3T3 cells, transfected with either *raf* or *ret-II* oncogenes, revert to a normal contact-inhibited phenotype in the presence of OA [3]. OA produces its effects by promoting protein phosphorylation through inhibition of protein phosphatases. It is actually a potent and specific inhibitor of the serine/threonine protein phosphatases type-1, -2A [4] and -3 [5], while -2B is only slightly affected and type-2C is unaffected [4].

The transcription factor AP1 is considered as a regulatory component that converts extra-cellular signals into changes in the program of gene expression (see Ref. 6 for review). This factor is a complex composed of proteins encoded by different members of the immediate early gene *fos* and *jun* families. It binds to the TPA responsive element (TRE) TGACTCA present in certain promoters and activates transcription [7, 8]. The affinity of AP1 binding to the TRE consensus sequence is a function of the type of protein forming the dimer, each containing a leucine zipper region and DNA binding domain [9, 10]. This structure is shared by the members of the fos family (*fosB*, *fosB2* and *fra* genes) and of the jun family (*jun B*, *jun D*) [10]. Heterodimers between different Jun and Fos proteins exhibit enhanced activity compared with Jun/Jun homodimers [11]. Interestingly, an AP1 site is present in the *c-jun* promoter that regulates positively its expression [12], while Jun B functions as a negative regulator of *c-jun* [13].

The early genes are rapidly activated within a few minutes in response to serum growth factors, phorbol ester (TPA), neurotransmitters as well as by depolarizing conditions. The various *jun* genes are differentially expressed in tissues. While *c-jun* and *jun B* transcription is stimulated by phorbol esters and growth factors, similar conditions have minimal effects on *jun D* expression [14, 15], suggesting that the *jun D* gene may have a distinct role in the control of gene activity.

Since c-Jun regulates positively its expression, and since its induction is rapid and unaffected by protein synthesis inhibitors, it is thought that the transcription of the *c-jun* gene is regulated by posttranslational modifications of pre-existing c-Jun protein [16]. Its transcriptional activity has been shown to be differentially regulated by both amino- and carboxy-terminal phosphorylation. Phosphorylation of carboxy-terminal serine/threonine residues by glycogen synthase kinase 3 or casein kinase II inhibits the ability of c-Jun to bind to DNA [17]. On the other hand, a number of protein kinases, including MAP kinases, are able to phosphorylate the NH_2 terminus, and to activate c-Jun. It has been shown that the N-

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† Abbreviations: AP1, activator protein 1; MAP kinase, mitogen-activated protein kinase; OA, okadaic acid; TPA, 12-tetradecanoyl phorbol 13-acetate; TRE, TPA responsive element.

terminal 85-residues region possesses two phosphorylation sites sufficient for transcriptional activation *in vivo* [18].

In the present study, we have examined the mechanism of action of the protein phosphatase inhibitor OA on AP1 induction in the neuronal PC12 cell line. It is shown that OA induces posttranslational activation of AP1 through protein phosphorylation, resulting in enhanced transcription of genes from the *fos* and *jun* family and a further sustained increase in AP1 activity.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade and, if not otherwise stated, obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). OA was purchased from LC Services Corp (Woburn, NA, U.S.A.); stock solutions were made up in dimethyl sulphoxide and further diluted in H_2O. The same amount of dimethyl sulphoxide was added in controls. Nu-serum was purchased from Collaborative Research (Bedford, NA, U.S.A.). The anti-Fos and anti-Jun gel supershift antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Radiolabelled nucleotides and Hybond N+ were purchased from Amersham (Amersham, U.K.). Non-labelled nucleotides were from Boehringer (Mannheim, Germany). Protein was measured with the Bradford reagent from Biorad (Hercules, CA, U.S.A.).

Cells. The rat pheochromocytoma PC12 cells were grown at 37° in a humidified 5% CO_2 atmosphere in RPMI 1640 supplemented with 3.5% foetal calf serum and 6.5% Nu-serum. Serum-free medium was added 16 hr before an experiment.

Protein purification. The catalytic subunits of protein phosphatase type 2A and protein phosphatase type-1 were purified according to Ref. 19 from bovine cardiac tissue and rabbit skeletal muscle, respectively. The catalytic subunit of protein phosphatase type-3 was purified from bovine brain, as described previously [5]. The enzymes were stored at −20° in 60% (v/v) glycerol/50 mM Tris–HCl, pH 7.0, 0.1 mM EGTA and 0.1% mercaptoethanol. The proteins were 80–95% pure, as estimated by analysis of silver-stained gels. All phosphatases were assayed with [^{32}P]phosphohistone as substrate and were normalized accordingly.

Northern analysis. Total RNA was isolated from PC12 cells by the guanidinium isothiocyanate method [20] and separated by electrophoresis through 1% agarose-formaldehyde gel followed by capillary transfer to Hybond N+ membrane, that was subsequently stained with 0.01% methylene-blue in 0.5 M acetate buffer, pH 5. Membranes were prehybridized for 2 hr at 42° in 50% formamide, 5× SSPE (20 × SSPE: 3.6 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA), 0.1% Ficoll, 0.1% polyvinylpyrolidone and 0.5% sodium dodecyl sulphate, pH 7.7. Specific mRNAs were detected using DNA probes ^{32}P-radiolabelled by random priming with hybridization performed for 24 hr at 42°. *c-fos* mRNA was detected using a 1.1 kb cDNA probe, *c-jun* mRNA with the plasmid pSV-c-jun [9], *jun B* and *jun D* with respective cDNA fragment [14, 15]. The membranes were washed and exposed to X-ray films at −80° with intensifying screens for a period of 2–5 days. Autoradiographic signals were analysed by comparison with the 28S rRNA revealed by methylene-blue staining of the membrane.

Nuclear extracts and mobility shift assay. Nuclear extracts from PC12 cells were prepared according to Ref. 21, and mobility shift assays were carried out following Ref. 9. A synthetic 18-base pair oligonucleotide containing the human metallothionein IIA TRE was end-labelled with [^{32}P]ATP, using T4 polynucleotide kinase. Nuclear extract (10 μg) was incubated with 2 μg of poly(dI-dC) and the labelled DNA in TM buffer (50 mM Tris–HCl, pH 7.9, 12.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 20% glycerol) for 20 min at room temperature. For gel supershift studies, antibodies were incubated with the nuclear extracts for 6 hr at 4° prior to gel electrophoresis. For dephosphorylation studies, nuclear extracts were incubated with the various protein phosphatases normalized for comparable activity (0.6 pmol/min when assayed towards 2 μM [^{32}P]phosphohistone). Incubation was carried out for 20 min at 30° in a medium containing 50 mM Tris–HCl, pH 7.5, 0.5 mM dithiothreitol and 1 mM EDTA. DNA–protein complexes were resolved on a 4% polyacrylamide gel (38:2 acrylamide to bisacrylamide) in 0.25 × TBE (1 × TBE: 50 mM Tris–Borate, pH 8.3, 1 mM EDTA). The gels were then dried and exposed to X-ray films for 20 hr.

RESULTS

Stimulation of AP1 activity by OA

The effect of the addition of OA to PC12 cells on AP1 binding activity was investigated by mobility shift assay. Figure 1A illustrates the dose-dependent effect of OA on AP1 binding from nuclear extracts of 4 hr OA-treated cells. A very important increase was noted at 0.1 μM OA, that was already noticeable at the concentration of 1 nM. Higher concentration (1 μM) of OA was found to be toxic for the cells, most of them being detached from the dish after 6 hr incubation. We therefore used 0.1 μM OA concentration throughout the present study. The time-course of AP1 induction by OA is presented in Fig. 1B. AP1 binding activity was found to increase considerably 2 hr after OA addition to the cells. The activity was progressively stimulated, reaching a maximum at 6 hr, and declining thereafter.

Immediate early gene induction by OA

We next investigated whether the enhancement in AP1 binding activity was the result of an increased synthesis of the AP1 components. We therefore measured the level of the transcripts of *c-fos* and of the *jun* family in OA-treated PC12 cells. Figure 2 shows the time course of the corresponding mRNA accumulation analysed by northern blot. All four early gene expressions were induced upon 0.1 μM OA addition to the cells. In the case of *c-fos*, the induction was barely visible at 3 hr, but increased dramatically at 4 hr and remained elevated at 10 hr of OA treatment. A very similar pattern was observed for *jun B* expression. The *c-jun* and the

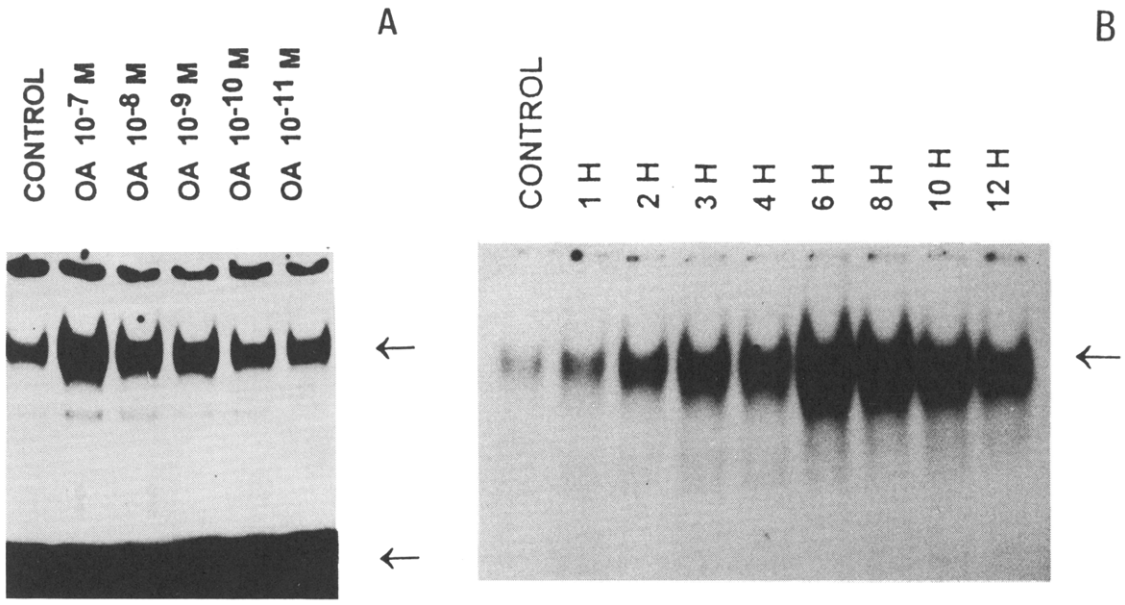

Fig. 1. Induction of AP1 by OA analysed by mobility shift assay. Nuclear extracts were isolated from PC12 cells stimulated with OA and incubated with ^{32}P-labelled synthetic DNA probe containing the AP1 consensus site TRE. The lower complex is a non-specific complex which is variably present and competes poorly with the specific non-labelled AP1 probe. Upper arrow indicates AP1 complex; lower arrow indicates the free probe. (A) Concentration-dependent effects of cells incubated with OA for 4 hr. (B) Time-dependent effects of cells incubated with 0.1 μM OA. The figure is representative of five experiments.

jun D genes showed basal mRNA expression; they were nonetheless strongly induced after 3 hr of OA treatment and stayed also elevated up to 10 hr treatment.

Effect of actinomycin D and cycloheximide on the OA-induced AP1 induction

OA produced an increase in the transcription factor AP1 after 2 hr treatment, that is prior to any discernible induction of the corresponding mRNAs. This may suggest that the OA-induced AP1 activation is triggered independently of gene transcription. In order to test this hypothesis, we examined the AP1 activation in the presence of the transcription inhibitor actinomycin D (Fig. 3A). Treatment of PC12 cells with actinomycin D alone produced a progressive decay in AP1 activity with time. When OA was added together with actinomycin D, a peak of AP1 activity emerged together with the decay. The AP1 activation was clearly observable at 2 and 2.5 hr after OA addition. A similar AP1 activation was observed when cells were treated with the protein synthesis inhibitor cycloheximide (Fig. 3B). This observation further indicates that OA-induced AP1 activation was not the result of protein neo-synthesis, but was clearly due to posttranslational modifications.

Effect of anti-Fos and anti-Jun antibodies

We investigated the presence of the heterodimer elements present after 2 hr OA treatment by the use of specific antibodies raised against each Fos and Jun protein (Fig. 4). The various antibodies were added to a nuclear extract from PC12 cells treated with OA, prior to the mobility shift assay. Only the addition of the anti-Jun D antibody, and to a lesser extent, the addition of anti-c-Jun antibody resulted in a supershift (Fig. 4). The supershift is characterized by the appearance of an electrophoretic band of higher molecular mass than AP1 corresponding to the complex DNA–protein–antibody the migration of which is further retarded. This finding suggests that the products of the *c-jun* and *jun D* genes were part of the AP1 complex present 2 hr after OA addition to the cells.

AP1 is activated through protein phosphorylation

To investigate whether the AP1 activation induced by OA at 2 hr was achieved through phosphorylation, nuclear extracts from OA-treated PC12 cells were incubated with purified catalytic subunits from several serine/threonine protein phosphatases, prior to the mobility shift assay. Figure 5 shows that treatment of extracts with protein phosphatase-1 or, to a lesser extent, with protein phosphatase-2A, resulted in a strong decrease in AP1 activity. Protein phosphatase-3 had no effect. This result demonstrates that some components of the AP1 complex were indeed phosphorylated on serine or threonine residues 2 hr after OA addition to the cells.

DISCUSSION

In PC12 cells, the serine/threonine protein phosphatase inhibitor OA was found to activate

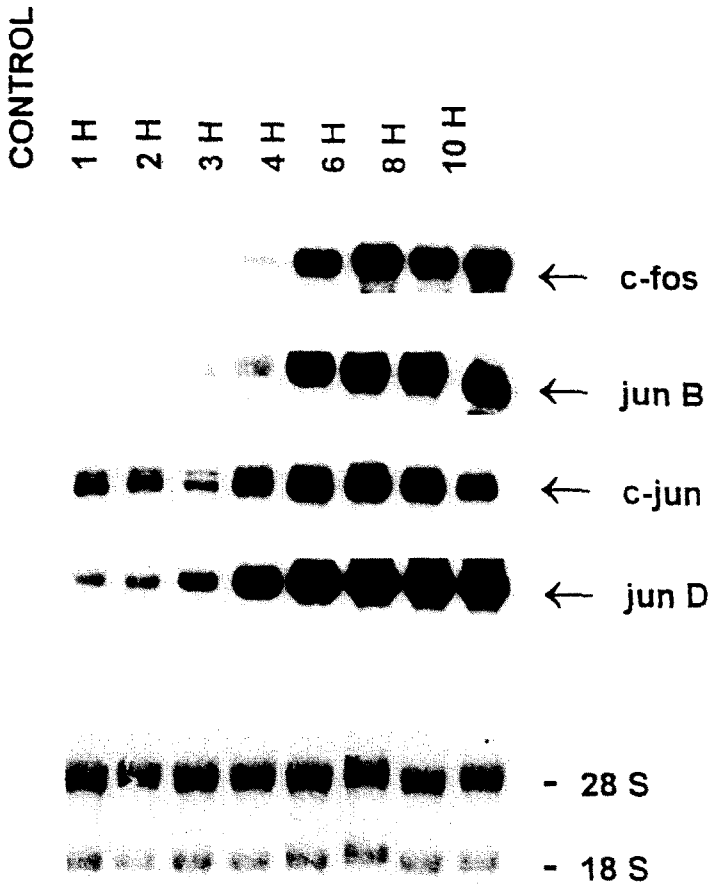

Fig. 2. Effect of OA on the induction of mRNA transcripts for c-fos and members of the jun family. PC12 cells were incubated with 0.1 μM OA for the indicated time, and total RNA was isolated. Northern blot analyses were performed using various cDNA probes ^{32}P-radiolabelled by random priming. The lower panel presents the rRNA revealed by methylene-blue staining of the blot, showing equal loading of RNA. This experiment is representative of two experiments performed in duplicate.

transcription of *c-fos* and all three *jun* genes, as well as the binding of the transcription factor AP1 to its DNA consensus sequence. The kinetics of gene induction, however, were found to be very different from those elicited by growth factors, TPA or second messengers. *C-fos* mRNA synthesis is usually initiated at 20 min, peaks in less than an hour and is back to basal expression after 2 hr [6]. In the present study, OA-induced *c-fos* expression began only at 3 hr of treatment and stayed elevated for incubation times up to 10 hr. Similarly, the expression of *c-jun* and *jun B* peaks around 1 hr when stimulated by growth factors or TPA, whereas we noticed that the OA-induced transcription of these *jun* genes began also at 3 hr and stayed elevated for more than 8 hr. The *jun D* gene expression was also substantially induced by OA, which is somewhat surprising since TPA and serum have only minimal effects on *jun D* mRNA levels [14, 15].

Since the pattern and kinetics of immediate early gene expression upon OA addition were very different from those elicited by growth factors, TPA or second messengers, it might be possible that OA triggers gene expression by a mechanism distinct from that used by these compounds. OA has been shown to induce early gene transcription in T cells [22] and in fibroblasts [23, 24]. In T cells, the induction of early genes by OA was very similar to what we observed in PC12 cells. The transcription of the fos and jun genes, including the *jun D* gene, was stimulated, the effect being visible 3 hr after OA addition [22]. In fibroblasts, the induction of *c-fos* [23] and of the genes from the jun family [24] begins somewhat earlier and stays up after 8 hr of treatment, but higher doses of OA were used. It has been shown that the sustained increase in mRNAs induced by OA is due to at least two effects, increased transcription of the genes and increased half-life of the mRNAs [24].

When the binding of AP1 factor to its DNA consensus sequence (TRE) upon OA addition was examined, we found that it increased 2 hr after OA addition, that is prior to early gene transcription, suggesting that it is actually the AP1 activation that leads to increased gene transcription. Enhanced AP1 activity was still observed when OA was added to

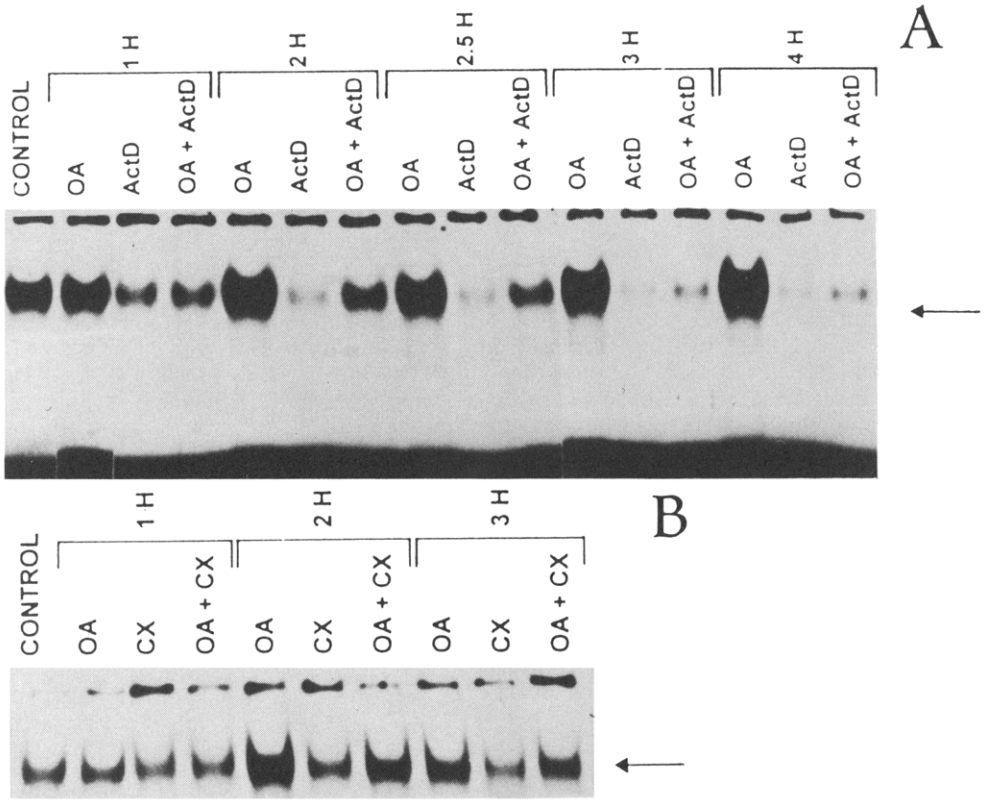

Fig. 3. Effect of actinomycin D (A) and cycloheximide (B) on the OA-stimulated AP1 activity, analysed by mobility shift assay. PC12 cells were incubated with 20 μg/mL actinomycin D (ActD), 10 μg/mL cycloheximide (CX) and 0.1 μM OA, as indicated. ActD was added 20 min before OA. Nuclear extracts were prepared from cells incubated for the indicated times after treatment. The arrow indicates AP1 complex. The figures are representative of two experiments.

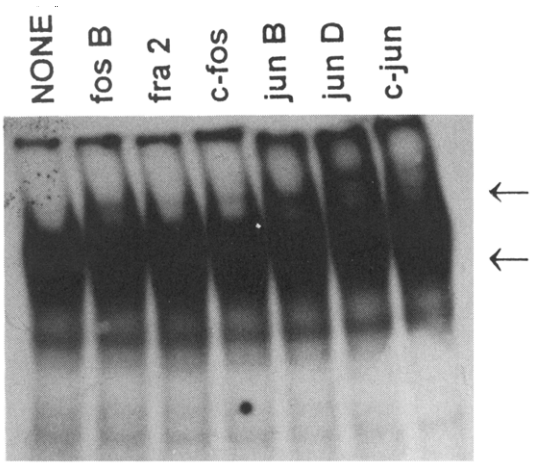

Fig. 4. Effect of anti-oncoprotein antibodies on OA-stimulated AP1 activity. Nuclear extract from PC12 cells (14 μg) treated with 0.1 μM OA for 2 hr was incubated in the absence or in the presence of 1 μg antibodies against various Fos and Jun proteins, as indicated, for 6 hr prior to analysis by mobility shift assay. The gel was exposed to the film for 4 days. The upper arrow indicates the supershift band; lower arrow indicates AP1 complex. The figure is representative of three experiments.

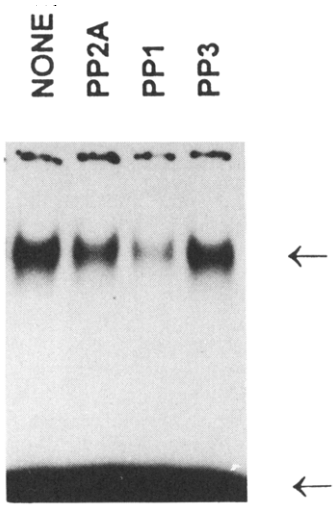

Fig. 5. Effect of protein phosphatases on OA-stimulated AP1 activity. Nuclear extract from PC12 cells (10 μg) treated with 0.1 μM OA for 2 hr was incubated in the absence or in the presence of comparable activity (see Materials and Methods) of catalytic subunits of protein phosphatase type-1 (PP1), type-2A (PP2A) or type -3 (PP3). Incubation was carried out for 20 min at 30° prior to analysis by mobility shift assay. Upper arrow indicates AP1 complex; lower arrow indicates the free probe. The figure is representative of three experiments.

the cells together with the transcription inhibitor actinomycin D, or with the protein synthesis inhibitor cycloheximide, further assessing that the primary events triggered by OA were posttranscriptional. That AP1 activation actually triggers gene transcription further extends the previous demonstration that TRE is the promoter element most likely responsible for the transcriptional activation of early genes by OA[24].

At this point, a picture emerges concerning the mode of action of OA. Through protein phosphorylation, this compound triggers AP1 activation (2–3 hr after OA addition), that turns on early gene transcription (at 3–4 hr), accumulating Fos and Jun proteins which combine to produce greater AP1 binding activity. This explains the surge in AP1 activity observed at around 6 hr after OA addition. This later increase was found to be transcription dependent since it was not detected when cells were treated with OA and actinomycin D. Using gel mobility supershift assays, we found that the products of the *c-jun* and the *jun D* genes were present in the AP1 complex at the time of its activation (2 hr after OA addition). These two Jun proteins can therefore be considered as putative targets of the OA-induced regulation, although other targets cannot be excluded.

Since OA inhibits several protein phosphatases, the identification of the proteins, the phosphorylation level of which is influenced by OA, is fairly difficult. Nevertheless, we found that OA induced AP1 phosphorylation, since AP1 activity was inhibited when nuclear extracts were incubated with the OA-sensitive protein phosphatase-1 or -2A. The Jun proteins themselves and/or MAP kinases appear as possible candidates for being phosphorylated on sites the dephosphorylation of which is inhibited by OA. Dephosphorylation of c-Jun by protein phosphatase-2A has been reported [25, 26]. The regulation of c-Jun by phosphorylation is well documented [17, 18, 27]. It contains C-terminal glycogen synthase kinase-3 and casein kinase II phosphorylation sites that regulate negatively its ability to bind to DNA, whereas the activation of c-Jun is mainly due to MAP kinases that phosphorylate serine residues in the amino-terminal region of the protein [27, 28]. Much less is known about Jun D phosphorylation, although it has recently been shown that it is also regulated by glycogen synthase kinase-3 phosphorylation [29]. On the other hand, MAP kinases are serine/threonine kinases known to play a pivotal role in signal transduction, that require phosphorylation on tyrosine and threonine residues for activity [30]. They undergo autophosphorylation on tyrosine and it has been shown that threonine dephosphorylation by protein phosphatase type-2A inactivates them [30], which makes OA a likely effector of their activity. Thus, the combination of two events achieved by a 2 hr OA treatment (basal MAP kinase activation and inhibition of Jun dephosphorylation) probably induces sufficient accumulation of c-Jun phosphorylation to reach a level of AP1 activity capable to trigger early gene transcription.

In conclusion, this study provides evidence that inhibition of serine/threonine protein phosphatases type-1, -2A and/or -3 by OA results in a potent activation of AP1. This activation leads to transcription of immediate early genes from the *fos* and *jun* families, and to sustained elevation of AP1 activity for many hours. It remains to be elucidated whether the sustained AP1 elevation is responsible for some of the known biological effects of OA, like tumour promotion or cell death. The study also suggests that the protein phosphatases are potentially important inhibitors of cell growth.

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