Brief exposures of resting fibroblasts to okadaic acid stimulate DNA synthesis

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Abstract. To study further the factors providing for cellular quiescence, we used okadaic acid (OA) at concentrations (0.1, 1, 10 or 100 nM) inhibiting type 1 and/or type 2A protein phosphatases in mammalian cell cultures. Brief (2 h) exposure of resting (0.2% serum for 72 h) NIH 3T3 mouse fibroblasts to OA with subsequent incubation of cells in a medium with 0.2% serum, stimulated DNA synthesis at all concentrations studied. Maximal stimulation was observed following pre-incubation of resting cells with 10 nM OA. Treatment of cycling cells (10% serum) with OA (2 h pulses at 12 h intervals for 72 h) prevented their exit to the resting state on transfer to a medium with 0.2% serum. Brief exposures of resting cells to OA did not affect the rate of protein synthesis. OA pulses in the late pre-replicative period had no effect on the entry of serum-stimulated cells into the S phase.

Cell fusion experiments with resting (serum-deprived) and proliferating (serum-stimulated) NIH 3T3 cells, using radioautography with a double-labelling technique, revealed that pre-incubation of resting cells with OA for 2 h before and after fusion abrogates their ability to suppress the onset of DNA synthesis in the nuclei of proliferating cells in heterodikaryons. The results indicate that protein phosphatases of type 1 and/or 2A may be involved in the growth-arrest machinery that provides for cellular quiescence.

Negative cell growth control in eukaryotes may be achieved in several ways: either by a short-term metabolic switch whereby rapidly appearing growth inhibitory molecules bring about temporary arrest at critical checkpoints of the cell cycle, or by continuous production of intracellular (endogenous) growth inhibitors resulting in proliferative rest, or both (Epifanova & Brooks 1994). It is well known that catabolic processes prevail in the state of cellular quiescence and that phosphatase activity dominates in resting cells (Epifanova 1977, Clarke & Karsenti 1991, Cossins 1991). In view of this, the possible role of protein phosphatases in negative cell growth control appears to be particularly likely.

Considerable attention has been attributed in this respect to protein phosphatases of type 1 (PP1) and type 2A (PP2A) that dephosphorylate serine and threonine residues (Karsenti *et al.* 1991, Cohen 1994, Shenolikar 1994). One of the common reagents for the study of PP1 and PP2A activities is their potent and specific inhibitor, okadaic acid (OA), a marine-sponge toxin (Cohen *et al.* 1990). Studies with mammalian cells showed that OA inhibits PP2A at

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nanomolar concentrations 10 to 100 times lower than those required to inhibit PP1 (reviewed by Shenolikar 1994). If PP1 and PP2A are involved in cell growth arrest, OA would be expected to stimulate resting cells to enter the cell cycle. Indeed, exposure of cultured mammalian resting cells to OA at nontoxic concentrations (100 nM and less) has been reported to induce certain early cell cycle events, e.g. MAP-kinase phosphorylation (Afshari & Barret 1994), the expression of the proto-oncogenes *c-fos* and *c-jun* (Kim *et al.* 1990, Thévenin *et al.* 1991, Rieckmann *et al.* 1992, Rosenberger & Bowden 1996) and elevation of transcription factor AP-1 activity (Thévenin *et al.* 1991, Rieckmann *et al.* 1992, Rosenberger & Bowden 1996). Other OA-stimulated events, such as the promotion of glucose transport and lipolysis in adipocytes (Haystead *et al.* 1989), increase in collagenase activity in synovio-cytes (Kim *et al.* 1990), or reorganization of vimentin intermediate filaments in brain tumour cells (Lee *et al.* 1992), have been mostly observed under conditions of a prolonged OA treatment and/or with higher concentrations of the drug.

The data concerning the effects of OA on cell growth appear to be ambiguous. OA has the characteristics of a tumour promoter in 3T3 cells (Sakai & Fujiki 1991). On the other hand, Rivedal *et al.* (1990) did not find morphological transformation in Syrian hamster embryo (SHE) cells treated with 3.75 to 12.5 nM OA for 4–24 h. Resting SHE cells and human diploid fibroblasts treated with 0.16 mM OA in the presence of 0.5% serum were stimulated to enter the S phase. In addition, senescent cells that were unable to initiate DNA synthesis in response to serum alone, could over-ride the block when treated with both serum and OA (Afshari & Barrett 1994). OA was also shown to induce immortality and acquisition of anchorage-independent growth in senescent SHE cells (Afshari *et al.* 1993). However, OA at concentrations between 10 and 100 nM was found by others to inhibit growth factor-induced H1 kinase expression, pRB phosphorylation and DNA synthesis in different cell types (Metcalf & Milner 1990, Dean *et al.* 1991, Kim *et al.* 1993, Schönthal & Feramisco 1993, Simm *et al.* 1994).

Our earlier cell fusion experiments with resting (serum-deprived) and serum-stimulated NIH 3T3 mouse fibroblasts revealed the ability of resting cells to suppress the entry of stimulated cells into the S period in heterodikaryons (Polunovsky *et al.* 1983). Further studies demonstrated that the observed suppressive effect could be abrogated by brief translational arrest due to the elimination of some negative growth regulator (Setkov *et al.* 1992, Rosenwald *et al.* 1995). The main aim of the present work was to investigate, using the heterokaryon model system and other experimental approaches, how OA would affect the growth-suppressive reactions providing for cellular quiescence.

MATERIALS AND METHODS

Cell culture, growth conditions and materials

NIH 3T3 mouse fibroblasts have been used. Cells were grown as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2.0 mM L-glutamine and 100 µg/ml canamycin in 4% CO₂ humidified air at 37°C, and were maintained by subculturing with 0.05% trypsin solution. For experimental purposes, the cells were plated at 30 × 10³ cells/well in 24-well plates and incubated for 24 h in medium containing 10% FCS. the cells were rendered quiescent by growth to 2/3 confluence, then washed twice with serum-free medium and cultured further in medium supplemented with 0.2% FCS for 3 days. After labelling for 24 h with [³H]-thymidine ([³H]dT), 5–7% of the cells were found to be synthesizing DNA. To obtain proliferating cells, the low serum medium was replaced with fresh medium containing 10% FCS.

OA as an inhibitor of protein phosphatases PP1 and PP2A at concentrations of 0.1, 1, 10 or 100 nM has been used. OA was a kind gift from Dr Philip Cohen (University of Dundee, Dundee, UK). It was dissolved in DMSO to a final concentration of 100 μ M.

Analysis of DNA synthesis

The resting cells, obtained as described in the previous section, were exposed to OA (0.1, 1, 10 and 100 nM) for 2 h and extensively washed with fresh medium. The details of the experiments are indicated in the figure legends. The amount of DNA synthesis was determined following continuous or pulse (1 h) labelling of cells with [3 H]dT (0.37 MBq/ml; 0.92 MBq/mM). The cells were then washed with Hank's solution, harvested using 2.5% trypsin solution, TCA-precipitated, transferred onto glass fibre filters, washed in PBS, ethanol, dried and counted for radioactivity using a Beta-1 counter. Samples from three wells were taken for each fixation point. The results were expressed as mean counts per minute per well.

Cell fusion procedure

Cells were seeded in 24-well plates at a concentration of 20×10^4 cells/well, cultured in medium with 10% FCS for 48 h and then made quiescent by plating (after three washes) for 3 days in medium with 0.2% FCS. The resting cells were incubated in the presence of OA (0.1, 1 or 10 nM) at various time periods prior to fusion or post-fusion (as indicated in the figure legends) and fused to proliferating cells harvested at the 10th hour of the pre-replicative period, which lasted for approximately 10–12 h. The fusing agent was polyethylene glycol ($M_r \sim 6000$, Merck). To identify the products of fusion, radioautography with a double-labelling technique has been used. For this purpose, cells grown in medium supplemented with 10% FCS, were incubated continuously in the presence of [14 C]-thymidine ([14 C]dT) (9.25 kBq/ml; 1.88×10^3 MBq/mM) for 3 days and then transferred to a medium containing 0.2% FCS. The stimulated cells remained unlabelled. Consequently, in homodikaryons formed by resting cells, both nuclei were labelled with [14 C]dT, whereas in homodikaryons formed by proliferating cells, both nuclei were unlabelled. Heterodikaryons contained a [14 C]dT-labelled and an unlabelled nucleus. To determine the fraction of nuclei synthesizing DNA following fusion, the cells were incubated continuously in the presence of [3 H]dT (0.37 MBq/ml; 0.92 MBq/mM) for 16 h in a conditioned medium containing 0.2% FCS and were then fixed in ethanol–acetic acid (3:1). Radioautographic preparation were routinely obtained using the liquid emulsion, Ilford-K2 (Ilford, Moberley, UK). Five samples were taken for each point. The percentage of [3 H]dT-labelled nuclei of both parental cell types was determined separately in monokaryons, homodikaryons and heterodikaryons from counts on 250–500 cells per coverslip.

To estimate DNA synthesis in stimulated nuclei, we analysed heterodikaryons containing one [14 C]-labelled nucleus with the other one being either unlabelled or labelled only with [3 H] only. To estimate DNA synthesis in resting nuclei, we analysed heterodikaryons containing one unlabelled, or [3 H]-labelled nucleus, and either one [14 C]-labelled or one double-labelled nucleus where the β -tracks of [14 C] label were scattered over and outside the heavy [3 H]-labelled nuclei. For other details concerning radioautographic procedures and identification of cell fusion products see Polunovsky *et al.* (1983).

Protein synthesis evaluation

The cells were grown in 24-well Linbro plates at a concentration of 3×10^4 cells per well in full medium (DMEM+10% FCS) for 6–18 h at 37°C in a humidified atmosphere of 4%

CO₂-96% air, then washed twice with serum-free medium and cultured for another 3 days in medium supplemented with 0.2% FCS to obtain resting cells. The cells were further treated with OA (1 and 10 mM) for 2 h and pulsed with [³H]-leucine (37 kBq/ml; 38 GBq/mM) for 1 h. At the end of the labelling period the cells were TCA-precipitated, harvested, transferred onto glass filters, washed in PBS, ethanol, dried and counted for radioactivity using the Beta-1 counter. Samples from five wells were taken for each point. The results were expressed as mean counts per minute per well.

RESULTS

Okadaic acid stimulated DNA synthesis in resting NIH 3T3 cells

Figure 1 shows the dose-dependent effects of OA on [³H]dT incorporation into resting (serum-deprived) cells after 2 h treatment, with subsequent incubation of cells in medium with 0.2% serum. It can be seen that brief exposures to OA at all concentrations studied (0.1 to 100 mM) stimulated DNA synthesis in resting cells. Maximal stimulation was observed following a 2 h pre-incubation of resting cells with 10 mM OA (1 h and 4 h pre-incubation exerted lesser effects, data not shown). As indicated in Figure 2, 36 h after a 2 h incubation of resting cells with 10 nM OA, the magnitude of the response was about the same as following a 2 h pre-treatment of resting cells with 10% serum, although it was approximately 3.5 times less than under condition of continuous stimulation by 10% serum (data not shown). In the case of continuous presence of 10 nM OA in the medium, the drug failed to stimulate DNA synthesis and even exerted an inhibitory effect (Figures 1 & 2).

Okadaic acid prevented the entry of cycling cells into the resting state

Exponentially growing cultures (10% serum) were transferred into the medium with 0.2% serum and were given 2 h pulses of OA (10 mM) every 12 h for 72 h. Control cultures were also transferred into the medium with 0.2% serum for 72 h and underwent a medium change every 12 h for 2 h with a fresh medium containing 0.2% serum but no OA. Figure 3 demonstrates that in control cultures there is a drastic reduction (up to 90%) of [³H]dT incorporation into DNA, i.e. the cells stop proliferating and pass into the resting state. In contrast, cycling cells that had been transferred into the medium with low serum but given regular OA pulses, continued to replicate DNA at the same level as they did at the beginning of the experiment, i.e. as in medium with 10% serum. When present continuously for 72 h in medium with 0.2% serum, OA failed to prevent the exit of cells into the resting state.

Brief exposures of cells to okadaic acid in the late pre-replicative period did not affect DNA synthesis

As OA was reported to inhibit growth factor and serum-induced DNA replication at the transition from G₁ to S phase (Kim *et al.* 1993, Schonthal & Feramisco 1993, Simm *et al.* 1994), we investigated DNA synthesis under conditions of brief exposures of cells to OA in the late pre-replicative period (its duration in serum-stimulated NIH 3T3 cells is about 10–12 h). For this purpose, resting cells were stimulated for 8 h with medium containing 10% serum and then transferred to serum-free medium containing 10 nM OA for 2 h. Thereafter, the cells were further incubated in medium with 0.2% serum and fixed as indicated in Figure 4. The results show that brief exposure to OA in the late pre-replicative period did not prevent cells from entering the S phase, as the incorporation rate of [³H]dT into DNA is the same as after 8 h serum stimulation without OA addition.

Okadaic acid abrogated the ability of resting cells to suppress the onset of DNA synthesis in nuclei of proliferating cells in heterodikaryons

Figure 5 demonstrates the incorporation of [^3H]dT into the nuclei of proliferating (serum-stimulated) cells fused to resting (serum-deprived) cells pre-treated with different concentrations of OA for (a) 45 min and (b) 2 h prior to fusion. It can be seen that in control cultures there was a marked inhibition of DNA synthesis in heterodikaryons as compared with monokaryons and homodikaryons. Contrary to this, the percentage of [^3H]dT-labelled nuclei

Figure 1. Incorporation of [^3H]dT into resting cells (72 h incubation in medium with 0.2% serum) treated with OA at concentrations of 0.1 (●), 1 (▲), 10 (△) and 100 (○) nm for 2 h followed by further incubation in medium with 0.2% serum. ▽=control (0.2% serum without OA treatment), ▼=constant presence of 10 nm OA in the medium. Pulse-labelling with [^3H]dT for 1 h before harvesting cells for the determination of incorporated radioactivity at indicated time points. The maximal range of the data is shown by vertical bars.

from proliferating cells in heterodikaryons with resting cells that had been pre-treated with OA, were similar to those in mono- and homokaryons. The effect was particularly well pronounced after a 2 h OA pre-treatment. The inhibitory influence of resting cells towards proliferating cells also disappeared in the case of post-fusion application of OA (Figure 6). The results indicated that OA abrogates the ability of resting cells to suppress the onset of DNA synthesis in nuclei of proliferating cells in heterodikaryons.

Okadaic acid had no effect on total protein synthesis in resting cells

It was found earlier that a 24 h incubation with 5–50 nM OA of C3H 10T1/2 cells caused a partial inhibition of TGFB-induced protein synthesis (Kim *et al.* 1993). We therefore investigated the effects of OA on total protein synthesis in resting cells. The data in Table 1 show that a 2 h exposure to OA at concentrations of 1 and 10 nM did not inhibit the incorporation of [³H]-leucine into resting cells.

DISCUSSION

The results of the present work showed that brief (2 h) exposure of resting (serum-deprived) NIH 3T3 mouse fibroblasts to OA (at concentrations of 0.1–100 nM) inhibited the serine–threonine specific protein phosphatases PP1 and/or PP2A, thereby stimulating DNA

Figure 2. Incorporation of [³H]dT into resting cells treated with either 10 nM OA (○) or 10% serum (□ vertical bar) for 2 h and further incubated in medium with 0.2% serum. ■ (vertical bar), constant presence of OA in medium with 0.2% serum; ●, control (0.2% serum without OA treatment). Cumulative labelling of cultures with [³H]dT.

synthesis. This is the first report to demonstrate that pre-treatment of resting cells with OA prior to fusion with proliferating (serum-stimulated) cells abrogates their ability to suppress the onset of DNA synthesis in the nuclei of proliferating cells in heterodikaryons. We have also revealed that repeated 2 h OA pulses of cycling (10% serum) cells transferred to medium with 0.2% serum prevent their exit to the resting state. These findings are in agreement with the data of other investigators who observed stimulation of DNA synthesis (Afshari & Barrett 1994) or induction of early cell cycle events (proto-oncogene and transcription factor expression) following exposures of mammalian cell cultures to OA at a similar range of concentrations (Kim *et al.* 1990, Thévenin *et al.* 1991, Rieckmann *et al.* 1992, Rosenberger & Bowden 1996). The results obtained indicate the probable involvement of PP1 and PP2A in the negative cell growth control that can be overridden by OA.

On the other hand, a number of investigators (Metcalf & Milner 1990, Dean *et al.* 1991, Kim *et al.* 1993, Schönthal & Feramisco 1993, Simm *et al.* 1994) have demonstrated an inhibitory effect of OA on growth factor-induced DNA synthesis in cultured cells. These

Figure 3. Effect of OA on the incorporation of [³H]dT into cells grown in medium with 10% serum for 16 h and transferred to medium with 0.2% serum for 72 h. (○), 2 h pulses of 10 nM OA every 12 h; (●), control (medium change with fresh medium containing 0.2% serum for 2 h, every 12 h); ■ (vertical bar), constant presence of OA in the medium for 72 h. Pulse-labelling with [³H]dT for 2 h before fixation.

differences in response have been attributed to the different concentrations of OA, cell type and other experimental conditions, e.g. the precise periods of application in relation to the cell cycle (Schönthal & Feramisco 1993, Simm *et al.* 1994).

According to our observations, the reason for these discrepancies may also lie in the duration of OA treatment. In the experiments of those who observed an inhibitory effect of OA on cell cycle progression, the contact time of cells with the drug lasted mostly for 24–48 h and more. In our experiments only brief exposures of resting cells to 10 nM OA stimulated the incorporation of [³H]dT into DNA, whereas longer treatment (18–36 h) exerted an inhibitory effect (Figures 1 & 2). Likewise, a constant incubation of cycling cells with OA for 72 h could not prevent their exit into the resting state under condition of low serum, as did brief OA pulses (Figure 3). Metcalfe & Milner (1990) found an inhibition of DNA synthesis in Con A-stimulated lymphocytes following a 48 h treatment with 10 nM OA, while after the first 6–8 h of contact with the drug a rise in [³H]dT incorporation could be observed. Only Afshari & Barrett (1994) reported stimulation of DNA synthesis following a 48 h OA treatment of resting hamster and human fibroblasts with OA concentrations as low as 0.10–0.16 nM. Other investigators did not find any increase in [³H]dT incorporation at similar concentrations of OA during a 48 h treatment of either resting lymphocytes (Rieckmann *et al.* 1992) or fibroblasts (Dean *et al.* 1991). On the other hand, in our study

Figure 4. Incorporation of [³H]dT into resting cells stimulated for 8 h with medium containing 10% serum, thereafter treated for 2 h with 10 nM OA and further incubated in medium with 0.2% serum. ■, OA for 2 h followed by medium with 0.2% serum; □, control (medium with 0.2% serum, without OA).

short pulses of 10 and even of 100 nM OA resulted in stimulation of DNA synthesis in NIH 3T3 cells. We therefore cannot fully share the opinion of Afshari & Barrett (1994) that stimulation of DNA synthesis in arrested cells occurs at OA levels below 1 nM, whereas inhibition of DNA synthesis is brought about at OA levels between 10 and 100 nM. The available data indicate that the range of OA concentrations inducing DNA synthesis may depend on the cell type and on the duration of treatment as well.

Figure 5. Incorporation of $[^3\text{H}]\text{dT}$ into the nuclei of proliferating cells fused to resting cells pretreated with 0.1, 1 and 10 nM OA for (a) 45 min and (b) 2 h. The resting cells were obtained as described in Materials and Methods. Proliferating cells were taken for fusion at the 10th h of the pre-replicative period. Control=fusion of proliferating cells to resting cells without OA pre-treatment. □, monokaryons; ▨, homodikaryons; ■, heterodikaryons. Vertical bars represent the 95% confidence limits.

These discrepancies are hardly surprising, as multiple phosphorylation/dephosphorylation reactions play a central role in both negative and positive intracellular regulatory circuits, governing the functions of the main protein families (cyclins and cyclin-dependent kinases) controlling the critical checkpoints in the cell cycle (Clarke & Karsenti 1991, Karsenti *et al.* 1991). The results of our studies suggest the involvement of PP1 and/or PP2A also in the transition of cycling cells to proliferative rest and its maintenance. In our experiments OA, even at concentrations as low as 0.1 and 1 nM, could still override the influence of

Figure 6. Incorporation of $[^3\text{H}]\text{dT}$ into the nuclei of proliferating cells following fusion with resting cells. The cells were treated with 0.1, 1 and 10 nM OA for (a) 45 min and (b) 2 h post-fusion and further incubated for 16 h in conditioned medium containing 0.2% serum. Control=fusion of proliferating cells to resting cells without subsequent OA treatment.

Table 1. Incorporation of [^3H]-leucine (pulse-labelling for 1 h) into resting cells pre-treated for 2 h with 10 nM OA and further incubated in medium containing 0.2% serum. Control cells were incubated in medium with 0.2% serum ($P < 0.1$)

Time after pre-treatment with OA (h)	Control (cpm)	OA (cpm)	
		1 nM	10 nM
1	2276 $\pm$ 22	2673 $\pm$ 88	2409 $\pm$ 17
2	2562 $\pm$ 308	3172 $\pm$ 517	2483 $\pm$ 206
4	2743 $\pm$ 260	2762 $\pm$ 166	2415 $\pm$ 224
8	2124 $\pm$ 210	2354 $\pm$ 229	2084 $\pm$ 29

Data represent the means of 3–5 determinations $\pm$ SE.

endogenous growth inhibitors produced by resting cells (Figures 1, 5 & 6). This finding implies that PP1 and PP2A may be considered as participants of negative growth control in mammalian cells.

We have previously found that brief pre-treatment of resting NIH 3T3 cells with protein synthesis inhibitors (cycloheximide or puromycin) stimulates the onset of DNA synthesis and early cell cycle events, presumably due to elimination of some short-lived repressor proteins (Setkov *et al.* 1992, Rosenwald *et al.* 1995). In the present experiments OA, under the conditions used, did not affect total protein synthesis in resting cells. This does not exclude, however, that its stimulating effect on DNA synthesis might be due to a direct interference with dephosphorylation and inhibition of particular proteins necessary for the pre-replicative events. A selective induction by OA of cell cycle regulatory genes in transformed cells has been reported by You & Bird (1995).

Altogether, the data allow us to place tentatively the functions of PP1 and/or PP2A into the general context of the growth-arrest machinery responsible for cellular quiescence in eukaryotes. This multifunctional negative complex includes the already mentioned short-lived repressor proteins, the products of growth arrest-specific genes (Schneider *et al.* 1988), proteolytic enzymes (Rosenwald 1990, Setkov & Kazakov 1993), the inhibitors of cyclin-dependent kinases (see Peter & Herskowitz 1994, Sherr & Roberts 1995, for reviews) and probably other, not yet identified components with antiproliferative activity.

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