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Okadaic acid induces dephosphorylation of histone H1 in metaphase-arrested HeLa cells

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SUMMARY

It is shown here that treatment of metaphase-arrested HeLa cells with okadaic acid (0.15-2.5 μM) leads to dephosphorylation of histone H1. This effect is presumably due to the specific ability of okadaic acid to inhibit protein phosphatases 1 and/or 2A, because okadaic acid tetraacetate, which is not a phosphatase inhibitor, has no effect. Dephosphorylation of H1 does not occur if okadaic acid-treated cells are simultaneously treated with 20 nM calyculin A, or if the okadaic acid concentration is 5.0 μM or greater.

The mechanism behind this phenomenon is not known. However, the results suggest that the chain of events leading to histone dephosphorylation may be negatively controlled by a protein phosphatase 2A, while the phosphatase which actually dephosphorylates H1 could be a protein phosphatase 1. It remains to be determined whether the phosphatase involved here is the same enzyme as that which dephosphorylates H1 at the end of normal mitosis.

Key words: mitosis, protein phosphatase, phosphorylation, calyculin A

INTRODUCTION

Histone H1 becomes highly phosphorylated at the beginning of mitosis in mammalian cells, but is dephosphorylated at the end of mitosis (Gurley et al., 1978). Although its precise function is not known, mitosis-specific H1 phosphorylation probably plays an important role. This is suggested by its widespread occurrence among higher eukaryotes, from *Physarum* to vertebrates (see references quoted by Paulson, 1980), and by the observation that the 'mitotic H1 kinase' ($p34^{cdc2}$ kinase) is a component of MPF, which controls the onset of mitosis (Labbe et al., 1988; Arion et al., 1988; Lohka, 1989; Nurse, 1990). H1 phosphorylation could play a role in chromosome condensation (Inglis et al., 1976; Matsumoto et al., 1980; Roberge et al., 1990), repression of transcription (Paulson and Taylor, 1982), or other mitotic events.

Our approach to understanding the function and control of mitotic H1 phosphorylation focuses on the dephosphorylation of H1 at the end of mitosis. Although the H1 kinase has been extensively studied, the 'mitotic H1 phosphatase' has received little attention. It is not known, for example, what type of phosphatase is involved, or whether it is constitutive or regulated.

In order to obtain more information about the process of H1 dephosphorylation in vivo, we have investigated the effects of okadaic acid, a specific inhibitor of protein phosphatases (PPases) 1 and 2A (Bialojan and Takai, 1988; Cohen et al., 1990). Although their precise functions are not known, type 1 and 2A PPases are clearly involved in the completion of mitosis, as demonstrated by a variety of genetic and biochemical evidence (Doonan and Morris, 1989; Ohkura et al., 1989; Booher and Beach, 1989; Cyert and Thorner, 1989; Axton et al., 1990; Dombradi et al., 1990; Kinoshita et al., 1990; Ohkura and Yanagida, 1991; Lee et al., 1991; Doonan et al., 1991; Fernandez et al., 1992; Mayer-Jaeckel et al., 1993).

Since okadaic acid can be used in vivo (Cohen et al., 1990; Hardie et al., 1991), we asked whether it would inhibit dephosphorylation of H1 at the end of mitosis in HeLa cells. If so, it might also inhibit chromosome decondensation and prevent cells from completing mitosis.

In this paper, we show that okadaic acid, at appropriate concentrations, does indeed prevent HeLa cells from completing mitosis. Surprisingly, however, it *induces* H1 dephosphorylation in the metaphase-arrested cells. This dephosphorylation of H1 is blocked by calyculin A, a structurally unrelated inhibitor of PPases 1 and 2A (Ishihara et al., 1989), and by high concentrations of okadaic acid.

MATERIALS AND METHODS

Chemicals and media

Okadaic acid was obtained as a 100 μg/ml (124 μM) solution in dimethylformamide (DMF) from Moana Bioproducts (Honolulu, Hawaii) or Diagnostic Chemicals (Oxford, Connecticut). The tetraacetate derivative of okadaic acid (100 μg/ml in DMF) was obtained from Diagnostic Chemicals and calyculin A (100 μg/ml in methanol) was obtained from Moana Bioproducts. Tissue culture media and supplements were obtained from GIBCO or Sigma.

HeLa cell culture

A mycoplasma-free strain of suspension-adapted HeLa cells, designated H-HeLa (Medappa et al., 1971) was obtained from R. Rueckert at the University of Wisconsin-Madison. Cells were grown in Eagle's minimal essential medium with Earle's salts (MEM) supplemented with 100 i.u./ml penicillin, 100 μg/ml streptomycin, 5% newborn calf serum (heat inactivated), and in addition nonessential amino acids and 1 mM sodium pyruvate. Stock cultures were maintained as spinner cultures in 500 ml bottles with 2 inch teflon-coated stirring bars.

Determination of mitotic index and cell viability

Mitotic indices were determined by a modification of the method of Gomez-Lira and Bode (1981). Typically, a 200 μl sample of a culture was centrifuged and the cells resuspended in 100 μl of 20 mM Tris-HCl, pH 7.4, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, and 1 mM $CaCl_2$ and allowed to swell. After 5 minutes, 10 μl of fixative (3 volumes methanol:1 volume acetic acid) was added. The mitotic index was determined by scanning a slide on a raster under the phase-contrast microscope and scoring as mitotic or interphase each cell that came into view. At least 200 cells were counted for each determination. The use of methanol:acetic acid fixative instead of saponin (Gomez-Lira and Bode, 1981) has the advantage that cells do not change their appearance with time, so that counts can be made hours or days after the sample is taken.

For determination of cell viability, 90 μl of culture was mixed with 10 μl of Trypan Blue solution, prepared as described by Patterson (1979). After 5 minutes, cells were counted using a hemacytometer. Cells stained blue were scored as nonviable.

Cell synchronization and metaphase arrest

Cells were synchronized in S-phase by treatment with 2.5 mM thymidine. After 20-24 hours the cells were pelleted, washed with 0.9% NaCl solution, resuspended in half the original volume of fresh medium, and further incubated at 37°C with magnetic stirring. For arrest in metaphase, 0.25 μg/ml nocodazole was added (from a stock solution 5 mg/ml in dimethyl sulfoxide) 4 hours after removal of thymidine. Using this procedure, cultures with mitotic indices of 80% or more and cell viabilities of 90-95% can be obtained at 16-20 hours after release from the thymidine block (cf. Paulson, 1982).

For reversal of metaphase arrest, nocodazole-blocked cells were taken at 16-17 hours after removal of thymidine, pelleted, washed once with 0.9% NaCl, and resuspended in fresh medium at 5×10^5 to 6×10^5 cells/ml.

Treatment of cell cultures with okadaic acid and calyculin A

Treatments of cells with phosphatase inhibitors, as well as controls, were carried out using small suspension cultures (10-40 ml) in 25 or 50 ml Erlenmeyer flasks with rubber stoppers and 7/8 × 3/16 inch teflon-coated stirring bars. Measured volumes of stock inhibitor solutions were added to the flasks before adding cells and, in some experiments, the solvent was evaporated with a gentle stream of air. Immediately before treatments were to begin, the cell concentration in the parent culture was determined and adjusted, if necessary, to 5×10^5 to 6×10^5 cells/ml by pelleting a portion of culture and resuspending in a smaller volume. Measured volumes of these cells were then added to the small Erlenmeyer flasks. Unless otherwise stated, all treated cultures were incubated for 5 hours at 37°C with magnetic stirring.

Extraction with 5% perchloric acid and analysis of histone H1 phosphorylation

Typically a 10 ml sample of a culture (containing 5×10^6 to 6×10^6 cells) was used. Cells were chilled on ice, pelleted, washed with cold 0.9% NaCl solution, resuspended in 1 ml cold 0.9% NaCl, transferred to a microcentrifuge tube, and pelleted in the Eppendorf 5415C microcentrifuge by depressing the momentary button for 5 seconds. The cells were resuspended with 300 μl 5% (w/v) perchloric acid and extracted for 15 minutes on ice. After removal of insoluble material by centrifugation, proteins were precipitated from the extract in 1.5 ml polypropylene microcentrifuge tubes by adding 100% (w/v) trichloroacetic acid (TCA) to give a final concentration of 25% TCA, incubating at 2°C overnight, and centrifuging (full speed, 10 minutes) in the Eppendorf 5415C. After decanting the supernatant, the pellet was briefly and gently washed with acetone, dried in a vacuum desiccator, and resuspended in 20 μl of 1 mM HCl plus 20 μl of gel sample buffer.

Histone H1 phosphorylation was analyzed by cationic disc electrophoresis at neutral pH in polyacrylamide minigels as described by Paulson et al. (1992). Separating gels (5 cm long) contained 10% acrylamide, 8 M urea and 0.375 M Hepes, pH 7.0; stacking gels contained 6% acrylamide, 8 M urea and 0.125 M Ches, pH 9.0; and running buffer contained 11.9 g Hepes free acid and 30 g L-histidine free base per liter. In some cases, piperazine diacrylamide (Bio-Rad) was used in place of *N,N′*-methylenebisacrylamide as crosslinker. All gels were stained with Coomassie Brilliant Blue R-250 as previously described (Paulson et al., 1992).

RESULTS

Okadaic acid (0.5 μM) prevents completion of mitosis by HeLa cells after release from a nocodazole block

To test whether okadaic acid prevents HeLa cells from completing mitosis, we made use of the reversibility of metaphase arrest with the spindle poison nocodazole (Zieve et al., 1980; Hamilton and Snyder, 1982). A suspension culture of HeLa cells was arrested in metaphase (mitotic index 80-85%) with nocodazole and the block was released by pelleting the cells and resuspending them in fresh medium (see Materials and Methods). Samples treated in various ways were then further incubated and at intervals samples were taken for determination of mitotic index and cell viability.

Fig. 1 shows the results of one such experiment. It is clear that the cells treated with 0.5 μM okadaic acid did not complete mitosis; nor did cells that were re-treated with nocodazole (0.25 μg/ml). However, the untreated control culture *did* complete mitosis to a great extent, as shown by the fall in mitotic index to 11% at the last time point. Cultures treated with 0.4% (v/v) DMF (a control for the DMF in the 0.5 μM okadaic acid-treated samples) also completed mitosis, though somewhat more slowly than untreated cultures. In separate experiments, we found that culture samples treated with 0.1 μM okadaic acid or with 0.08% (v/v) DMF were indistinguishable from the untreated control (data not shown).

Completion of mitosis in the control culture was also demonstrated by the observation of many metaphase plates, anaphase and telophase figures, and pairs of G_1 cells. Such figures were never seen in cultures treated with 0.5 μM okadaic acid.

In this experiment, cell viability was approximately 92% at the time of removal from nocodazole. In the samples treated with 0.5 μM okadaic acid or re-treated with nocodazole, cell viabilities fell to about 80%. This is typical for cultures left in metaphase arrest for such a long period of time. In the other cultures, cell viabilities remained at 88 to 92%.

It should be noted that metaphase-arrested cells treated with

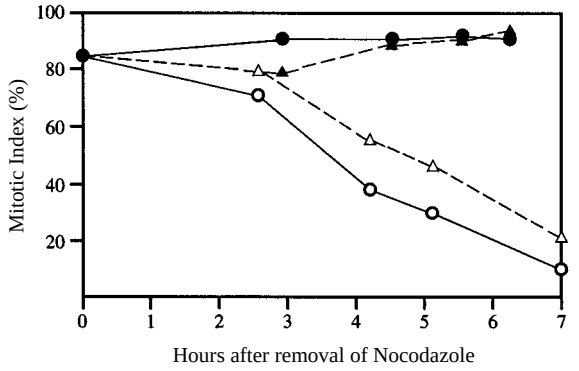

Fig. 1. Okadaic acid (0.5 μM) blocks exit from metaphase arrest following reversal of a nocodazole block. A culture was synchronized with thymidine and blocked with nocodazole (mitotic index, 85%), and at 16 hours after removal of thymidine, nocodazole was also removed. Samples (30 ml suspension cultures) were further incubated and portions were taken at various times for determination of cell viability and mitotic index. One portion was re-treated with 0.25 μg/ml nocodazole (●—●) and others were either given no treatment (○—○); treated with 0.5 μM okadaic acid and 0.4% dimethylformamide (DMF) (▲---▲); or treated with 0.4% DMF alone (△---△).

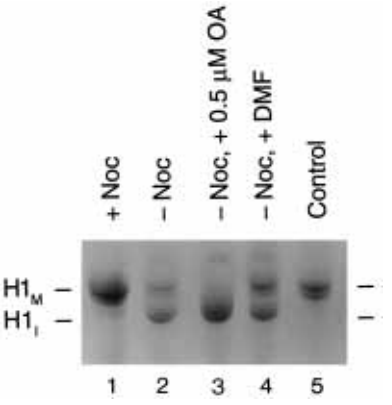

Fig. 2. Treatment with 0.5 μM okadaic acid leads to dephosphorylation of histone H1. At the end of the experiment shown in Fig. 1 (i.e. at 7 hours after removal of nocodazole), cells from each of the culture samples were extracted with 5% perchloric acid and histone H1 phosphorylation analyzed on a Hepes/histidine gel containing 10% acrylamide and 8 M urea (Paulson et al., 1992). Samples (from left to right) were as follows: lane 1, re-treated with 0.25 μg/ml nocodazole; lane 2, released from nocodazole; lane 3, released from nocodazole and treated with 0.5 μM okadaic acid and 0.4% DMF; lane 4, released from nocodazole and treated with 0.4% DMF alone; lane 5, a sample of the culture before removal of nocodazole. In this and subsequent figures, the positions of mitotic histone H1 ($H1_M$) and interphase H1 ($H1_I$) are indicated. Only the portion of the gel containing H1 is shown.

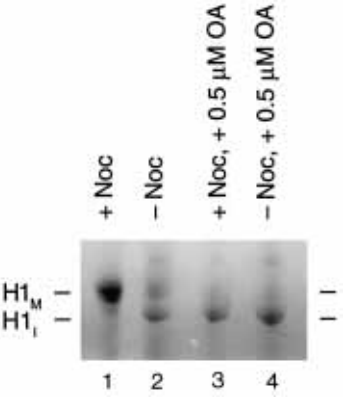

Fig. 3. Okadaic acid (0.5 μM) induces dephosphorylation of histone H1 in metaphase-arrested HeLa cells. At 16 hours after release of the thymidine block, a nocodazole-arrested culture (mitotic index, 77%) was divided and portions were treated in various ways. After 5 hours further incubation, the cells were extracted with 5% perchloric acid and the extracted proteins analyzed on a Hepes/histidine minigel containing 10% acrylamide and 8 M urea (Paulson et al., 1992). Only the part of the gel containing histone H1 is shown. Samples, from left to right, were: lane 1, left in nocodazole; lane 2, released from nocodazole; lane 3, left in nocodazole and treated with 0.5 μM okadaic acid and 0.4% DMF; and lane 4, released from nocodazole and treated with 0.5 μM okadaic acid and 0.4% DMF.

0.5 μM okadaic acid may differ in significant ways from ordinary nocodazole-arrested cells. Indeed, after okadaic acid treatment for 5 hours the chromosomes appear to be hyper-condensed and many cells are highly 'blebbed'.

Okadaic acid treatment leads to dephosphorylation of histone H1

At the final time point of the experiment shown in Fig. 1, histone H1 was extracted from the cells with 5% perchloric acid and its phosphorylation state analyzed on Hepes/histidine gels (Paulson et al., 1992). The results are shown in Fig. 2.

Surprisingly, treatment with 0.5 μM okadaic acid (Fig. 2, lane 3) led to nearly complete dephosphorylation of H1, even though the cells did not complete mitosis. H1 remained phosphorylated in the culture that stayed in nocodazole (lane 1), but was largely dephosphorylated when nocodazole was removed (lane 2), even when cells were treated with 0.4% (v/v) DMF (lane 4). The latter two samples consist of a mixture of $H1_M$ (the highly phosphorylated mitotic form of H1) and $H1_I$ (the interphase form of H1), presumably because some cells have completed mitosis while others have not. Cultures treated with 0.1 μM okadaic acid also contained both $H1_M$ and $H1_I$ (data not shown).

Okadaic acid induces dephosphorylation of H1 in nocodazole-arrested HeLa cells

Fig. 3 shows that 0.5 μM okadaic acid induces dephosphorylation of H1 whether or not the nocodazole block is released. Portions of a metaphase-arrested culture were either left in nocodazole arrest (lane 1), released from nocodazole (lane 2), left in nocodazole and treated with 0.5 μM okadaic acid (lane 3), or released from nocodazole and treated with 0.5 μM okadaic acid (lane 4). In this and all subsequent experiments, treatments were for 5 hours.

It is clear that 0.5 μM okadaic acid induces H1 dephosphorylation, whether or not nocodazole is removed. Determination of mitotic indices verified that only in the sample released from nocodazole (Fig. 3, lane 2) did cells complete mitosis.

Fig. 4 shows that the pattern of H1 dephosphorylation induced by 0.5 μM okadaic acid is quite different from that seen after release from nocodazole. Following removal of nocodazole, the cells in the population complete mitosis asynchronously, over the course of several hours (Fig. 1). However, when any one cell completes mitosis, dephosphorylation of H1 in that cell is very rapid (Gurley et al., 1978). Thus, as time passes and cells complete mitosis (Fig. 4A), increasing amounts of H1 appear in the $H1_I$ (dephosphorylated) position. A significant amount of $H1_M$ remains after 5 hours, because not all cells have completed mitosis, but bands intermediate

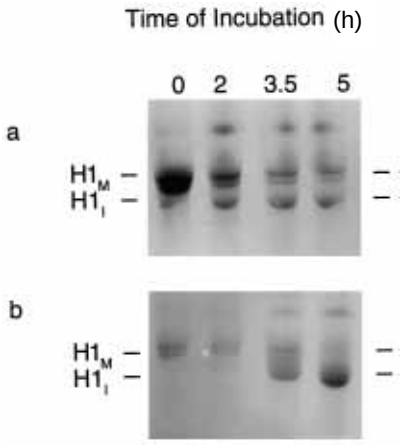

Fig. 4. Time course of histone H1 dephosphorylation in metaphase-arrested HeLa cells treated with 0.5 μM okadaic acid. Portions of a nocodazole-arrested culture (16 hours after release from thymidine) were either (a) released from nocodazole arrest, or (b) left in nocodazole and treated with 0.5 μM okadaic acid. The cultures were further incubated at 37°C and at 0, 2, 3.5 and 5 hours, 10 ml samples were taken for extraction with 5% perchloric acid. Histone H1 phosphorylation was analyzed on Hepes/histidine minigels as previously described (Paulson et al., 1992).

between $H1_M$ and $H1_I$ are never abundant enough to be detected.

By contrast, following treatment with 0.5 μM okadaic acid (Fig. 4B) dephosphorylation is virtually complete at 5 hours and intermediate bands can be clearly seen in the 3.5 hour sample. From these observations we conclude that the dephosphorylation of H1 induced by okadaic acid proceeds synchronously in all the metaphase-arrested cells in the culture.

In other experiments (data not shown), we found that H1 dephosphorylation is *not* induced by treatment of metaphase-arrested cells for 5 hours with 0.5 μM okadaic acid tetraacetate, a derivative that reportedly does not inhibit PPases 1 and 2A (Swain et al., 1991). This result supports the idea that induction of H1 dephosphorylation by okadaic acid is due to the reagent's specific ability to inhibit protein phosphatases (PPases) 1 and/or 2A, rather than a non-specific effect, for example due to the lipophilicity of the molecule.

What concentrations of okadaic acid induce H1 dephosphorylation?

Fig. 5 shows the results of two experiments examining the effects of various okadaic acid concentrations on H1 phosphorylation. We have consistently found that at least 0.15 μM okadaic acid is needed to induce H1 dephosphorylation (Fig. 5A, lane 4). With 0.075 μM okadaic acid, no dephosphorylation is seen after 5 hours (lane 6), and with 0.10 μM okadaic acid, typically only partial dephosphorylation is seen (lane 5).

Two observations suggested to us that the dephosphorylation of H1 induced by 0.15-0.5 μM okadaic acid might be inhibited by higher concentrations. First, the dephosphorylation induced by 0.5 μM okadaic acid (Fig. 4B) appears to be slower than that which occurs at the end of mitosis in vivo (Gurley et al., 1978), suggesting the possibility of a phosphatase partially inhibited by 0.5 μM okadaic acid. Second, dephosphorylation of H1 in vitro following lysis of metaphase-arrested cells (D'Anna et al., 1978; Paulson, 1980) is inhibited approximately 90% by 2.5 μM okadaic acid, and approximately 50% by 0.5 μM okadaic acid (J. R. Paulson, unpublished work).

Fig. 5B shows that indeed, dephosphorylation of H1 does not occur when metaphase-arrested cells are treated with 5.0 or 10.0 μM okadaic acid (lanes 5 and 6).

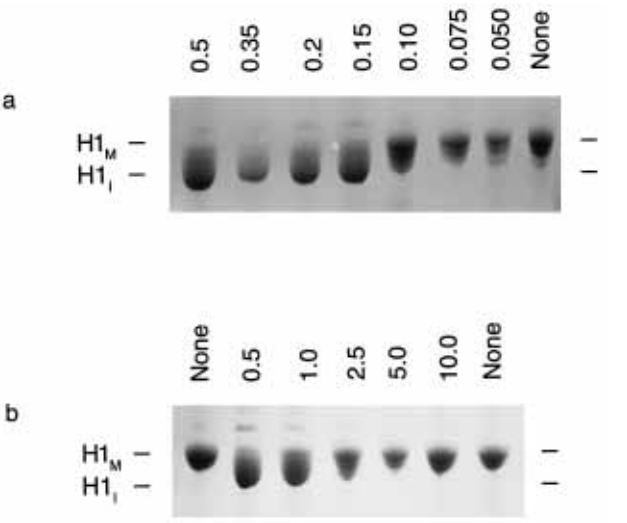

Fig. 5. H1 dephosphorylation is induced by as little as 0.15 μM okadaic acid, but not by concentrations greater than 5.0 μM. In two separate experiments, samples of nocodazole-arrested cultures were treated for 5 hours with various concentrations of okadaic acid as follows (from left to right): (a) 0.5 μM, 0.35 μM, 0.2 μM, 0.15 μM, 0.10 μM, 75 nM, 50 nM, and none; (b) none; 0.5 μM; 1.0 μM; 2.5 μM; 5.0 μM; 10.0 μM; and none. In (b), DMF was evaporated prior to addition of the cells. At the end of the incubation, histone H1 was extracted with 5% perchloric acid and analyzed on Hepes/histidine minigels containing 10% acrylamide and 8 M urea (Paulson et al., 1992). Only the parts of the gels containing histone H1 are shown.

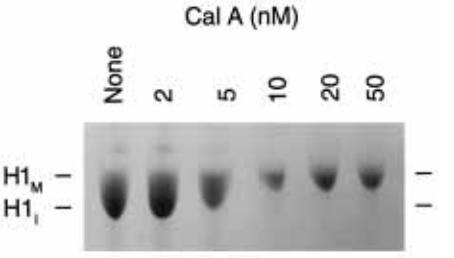

Fig. 6. Calyculin A blocks the dephosphorylation of histone H1 induced by 0.5 μM okadaic acid. Samples of a nocodazole-arrested culture were treated with 0.5 μM okadaic acid and various concentrations of calyculin A, as follows (from left to right): no calyculin A, 2 nM, 5 nM, 10 nM, 20 nM and 50 nM. After further incubation for 5 hours, histone H1 was extracted with 5% perchloric acid and analyzed on Hepes/histidine minigels. Methanol (solvent for calyculin A) was evaporated before addition of the cells, but dimethylformamide (solvent for okadaic acid) was not.

Effects of the protein phosphatase inhibitor calyculin A

Fig. 6 shows that the dephosphorylation of histone H1 induced by okadaic acid is prevented by calyculin A, another specific inhibitor of PPases 1 and 2A (Ishihara et al., 1989). Samples of a metaphase-arrested culture were treated for 5 hours with 0.5 μM okadaic acid and simultaneously with various amounts of calyculin A. Treatment with okadaic acid alone (Fig. 6, lane 1) induces H1 dephosphorylation, as previously shown. However, as little as 10 nM calyculin A blocks this effect (lane

4). In other similar experiments, partial dephosphorylation is sometimes seen with 10 nM calyculin A, but 20 nM is always sufficient to prevent the induction of H1 dephosphorylation.

We have attempted to find a concentration of calyculin A alone that would induce H1 dephosphorylation. However, our results suggest that no such concentration exists. In one experiment, for example, samples of a metaphase-arrested culture were treated with both calyculin A and 0.5 μM okadaic acid. At 30 nM calyculin A was clearly sufficient to block the okadaic acid effect, while at 1.8 nM it was not. This indicates that if any concentration of calyculin A exists that will induce H1 dephosphorylation, then some concentration between 1.8 and 30 nM should do so. However, at the same time other samples of the same culture were treated with calyculin A alone at concentrations of 1.8, 2.7, 4.0, 5.9, 8.9, 13.3, 20 and 30 nM. Dephosphorylation of H1 was not observed in any of these samples (data not shown). Thus, if calyculin A induces H1 dephosphorylation at all, it must do so over a very narrow (at most 1.5-fold) range of concentration, while okadaic acid induces H1 dephosphorylation over a 16-fold concentration range (0.15-2.5 μM). These observations are presumably related to the fact that PPase 2A is approximately 100-fold more sensitive to okadaic acid than PPase 1 (Cohen et al., 1990), whereas calyculin A inhibits both PPases 1 and 2A at approximately the same concentration (Ishihara et al., 1989).

DISCUSSION

Okadaic acid blocks cells in mitosis but induces H1 dephosphorylation

Our results show clearly that treatment of metaphase-arrested HeLa cells with okadaic acid (0.15-2.5 μM) leads, over the course of a few hours, to dephosphorylation of histone H1 (Figs 3, 4). This result is surprising because okadaic acid is well known as a specific inhibitor of protein phosphatases (PPases) 1 and 2A (Bialojan et al., 1988; Cohen et al., 1990). It is paradoxical that treatment with a phosphatase inhibitor leads to dephosphorylation. The induction of H1 dephosphorylation by okadaic acid is presumably due to specific inhibition of a PPase 1 or 2A, rather than a non-specific effect, for example due to the lipophilicity of the molecule, because H1 dephosphorylation is not induced by okadaic acid tetraacetate, a derivative that does not inhibit PPases 1 and 2A (Swain et al., 1991).

Okadaic acid does not induce dephosphorylation of histone H1 simply by stimulating cells to complete mitosis, since the dephosphorylation occurs whether or not the cells are released from nocodazole (Fig. 3). Indeed, 0.5 μM okadaic acid alone prevents cells from completing mitosis (Fig. 1). Inhibition of mitosis by okadaic acid has previously been reported (Zheng et al., 1991; Vandre and Wills, 1992; Ishida et al., 1992) and may be due to failure of spindle assembly (Picard et al., 1989; van Dolah and Ramsdell, 1992; Ghosh et al., 1992).

Roles for both PPase 2A and PPase 1

We have shown that okadaic acid induces H1 dephosphorylation in metaphase-arrested HeLa cells over a 16-fold concentration range (0.15-2.5 μM). At concentrations of 5.0 μM okadaic acid or more, however, H1 dephosphorylation is not induced (Fig. 5). The induction of H1 dephosphorylation by 0.5 μM okadaic acid can also be blocked by calyculin A (Fig. 6), another specific inhibitor of PPases 1 and 2A that is structurally unrelated to okadaic acid (Ishihara et al., 1989; Cohen et al., 1990) but, like okadaic acid, is able to enter living cells and act in vivo.

We put forward the hypothesis that the induction of H1 dephosphorylation is due to inhibition of a PPase 2A, while the blocking of this effect by higher concentrations of okadaic acid is due to inhibition of a PPase 1. PPase 2A is known to be approximately 100-fold more sensitive to okadaic acid than PPase 1 (Cohen et al., 1989, 1990). An alternative hypothesis is that induction of H1 dephosphorylation results from partial inhibition of a PPase 1 or 2A, while blocking of this dephosphorylation requires complete inhibition of another phosphatase of the same type.

The first hypothesis predicts that calyculin A alone should not induce H1 dephosphorylation at any concentration, because calyculin A inhibits PPases 1 and 2A at similar concentrations (Ishihara et al., 1989). However, the second hypothesis predicts that calyculin A, like okadaic acid, should induce H1 dephosphorylation over a 16-fold concentration range. In fact, we have been unable to find any concentration of calyculin A that will induce H1 dephosphorylation. Our work indicates that it could do so over at most a 1.5-fold range of concentration. Hence, the second hypothesis can be ruled out.

Which phosphatase dephosphorylates H1?

Since we are using enough okadaic acid to induce H1 dephosphorylation by inhibiting a PPase 2A, it follows that the phosphatase that actually dephosphorylates H1 in this situation is *not* a PPase 2A. This conclusion is significant because some in vitro work (Sola et al., 1991; Agostinis et al., 1992) has suggested that PPase 2A is the best candidate to dephosphorylate H1 at the sites phosphorylated by the $p34^{cdc2}$ kinase.

However, the H1 phosphatase whose action we have observed following okadaic acid treatment could be a PPase 1, and this would provide the simplest explanation for the results shown in Figs 5 and 6. Nevertheless, we cannot rule out the possibility that calyculin A and 5.0 μM okadaic acid block the dephosphorylation of H1 indirectly; for example, by blocking steps leading to activation of a phosphatase. If this is the case, then the H1 phosphatase might be neither a PPase 1 nor a PPase 2A.

Is the phosphatase whose activity we have observed in these experiments the same enzyme that dephosphorylates H1 at the end of mitosis in the normal cell cycle? We do not know. It would seem unlikely that okadaic acid should release or activate non-specific phosphatases, in view of the many reports that okadaic acid treatment stimulates protein phosphorylation. But it is possible, for example, that the major 'mitotic H1 phosphatase' in normal mitosis could be a PPase 2A, while the dephosphorylation of H1 that follows okadaic acid treatment is due to a minor, constitutive PPase 1, unmasked by inactivation of the H1 kinase (see below). This alternative would explain the slowness of the dephosphorylation that we have observed, and would also be consistent with the results of Sola et al. (1991) and Agostinis et al. (1992).

How might okadaic acid induce H1 dephosphorylation?

Although the induction of H1 dephosphorylation by okadaic

acid is surprising, it is not difficult to propose mechanisms by which it could come about. At present, however, we have no data that can help to show which of several possibilities is most likely. The simplest explanation for our results is that the histone H1 phosphatase is a PPase 1 that is negatively regulated by a PPase 2A. However, the effect of okadaic acid could be much less direct.

Several groups have reported that okadaic acid causes activation of p34^{cdc2} H1 kinase (MPF) (Felix et al., 1990; Rime and Ozon, 1990; Rime et al., 1990; Picard et al., 1991), but this activation is only transient (Yamashita et al., 1990). In cells where MPF is already active, okadaic acid causes *inactivation* of MPF by triggering the cyclin degradation pathway (Yamashita et al., 1990; Lorca et al., 1991). Lorca et al. (1992) report that low concentrations of okadaic acid (e.g. 0.9 μM) lead to inactivation of MPF because cyclin degradation is negatively regulated by a PPase 2A, but that higher concentrations (e.g. 2.5 μM) prevent inactivation of MPF, even though cyclin is destroyed, because MPF inactivation also requires dephosphorylation of Thr 161 by a PPase 1.

Thus, the okadaic acid-induced dephosphorylation of H1 could be due to a constitutive phosphatase whose activity is masked during metaphase by high H1 kinase activity. Alternatively, a phosphatase not normally active during metaphase might be activated as a result of okadaic acid treatment, possibly by a mechanism that parallels the route by which MPF is inactivated. Further work is needed to distinguish between these two possibilities and to determine the precise mechanism.

Is this phenomenon related to apoptosis?

One possibility that needs to be considered is that the dephosphorylation of H1 that we have observed, and/or the inactivation of MPF observed by others (Yamashita et al., 1990; Lorca et al., 1991), might be effects of apoptosis. It has been reported that 0.5 μM okadaic acid induces morphological changes typical of apoptosis in a variety of cultured cells (Boe et al., 1991), and we have also observed 'blebbing', which is characteristic of apoptosis, in metaphase-arrested HeLa cells treated with okadaic acid or calyculin A (J.R. Paulson and T.L. Betthauser, unpublished work). On the other hand, okadaic acid and calyculin A *inhibit* apoptosis in a Burkitt's lymphoma cell line (Song et al., 1992; Song and Lavin, 1993).

At present we do not know whether the phenomenon we have observed is related to apoptosis, but this possibility can in principle be tested by examining whether other reagents known to induce apoptosis will also induce H1 dephosphorylation and/or MPF inactivation in metaphase-arrested cells. The topoisomerase II inhibitor VM-26 (teniposide) is a possible example of such a reagent, since it induces apoptosis in lymphocytes (Roy et al., 1992), but also induces H1 and H3 dephosphorylation and H1 kinase inactivation in mitotic BHK cells (Roberge et al., 1990).

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