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Low concentrations of the phosphatase inhibitor okadaic acid stop tumor cell locomotion

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Abstract

The phosphatase inhibitor okadaic acid exerted a biphasic effect on the shape of spontaneously polarized Walker carcinosarcoma cells. At lower concentrations, the drug suppressed cell polarity ($IC_{50} = 0.14$ μM) and the cells reverted to a spherical shape. At higher concentrations (> 0.25 μM), cells developed large blebs ($IC_{50} = 0.4$ μM). Furthermore, 0.2 μM okadaic acid completely suppressed spontaneous cell locomotion. Two specific inhibitors of protein kinase C did not prevent the actions of okadaic acid on cell shape, showing that this enzyme is very likely not involved. Another phosphatase inhibitor, calyculin A, also suppressed polarity ($IC_{50} = 60$ nM) and produced blebbing cells ($IC_{50} = 70$ nM). 1 μM okadaic acid induced a 40- to 70-fold increase in phosphorylation of the intermediate filament protein vimentin in intact cells. Increased phosphorylation of this major phosphoprotein correlated with the generation of blebbing cells, rather than with inhibition of polarity and may thus be involved in generating the marked shape changes. We conclude that constitutive phosphatase activity is required for motility and control of shape in Walker carcinosarcoma cells.

Keywords: Okadaic acid; Calyculin A; Phosphatase; Tumor cell locomotion; Walker 256 carcinosarcoma cell; Vimentin

1. Introduction

Invasion and metastasis are complex multistep processes. Stimulated cell locomotion together with the proliferation and differentiation characteristics are important features of the neoplastic phenotype (Liotta et al., 1991). Locomotor activity is associated with characteristic shape changes (locomotor morphology, cell polarity). Therefore, it is important to know how shape changes and locomotion of tumor cells are regulated and how locomotion can be stopped. There is substantial evidence that shape changes and locomotion are regulated by selective phosphorylation and dephosphorylation of distinct proteins and that different kinases and phosphatases play a major role. In Walker carcinosarcoma cells, phorbol 12-myristate 13-acetate (PMA) as well as diacylglycerols, activators of protein kinase C, efficiently suppress spontaneous locomotion of Walker carcinosarcoma cells (Keller et al., 1985, 1989; Zimmermann and Keller, 1993). Moreover, highly selective inhibitors of protein kinase C such as *N*-benzoylstaurosporine (CGP 41 251; Meyer et al., 1989) are capable to promote cell polarity and locomotor activity (Zimmermann and Keller, 1992), and another protein kinase C inhibitor, 3-(1-(3-(2-isothioureido)propyl)indol-3-yl)-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione (Ro 31 8220; Twomey et al., 1990), prevents the inhibitory effects of PMA on cell locomotion (Niggli et al., 1996). Activation of protein kinase C appears thus to be a stop signal for locomotion of these tumor cells.

Little is known on the role of phosphatases in tumor cell locomotion. However, it has been shown that the shape of neutrophils and fibroblasts is altered by phosphatase inhibitors such as okadaic acid or calyculin A (Chartier et al., 1991; Kreienbühl et al., 1992; Hirano et al., 1992). This indicates that cell shape and possibly cell locomotion are determined or modulated by steady-state mechanisms such as a kinase-phosphatase couple. The phosphatase inhibitor okadaic acid is also a tumor promotor which can produce a mitotic arrest in human leukemia cells through enhanced phosphorylation (Zheng et al., 1991). Okadaic acid and calyculin A, both isolated from marine sponges, are potent and highly selective inhibitors of two major protein phosphatases that dephosphorylate

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serine and threonine residues, phosphatases 1 and 2A (Cohen et al., 1990). Both drugs inhibit phosphatase 2A in the nM range, but calyculin A has a comparatively higher potency towards phosphatase 1 (Ishihara et al., 1989). Despite their similar action on these two enzymes, they are structurally quite different (see Fig. 1 in Cohen et al., 1990). Okadaic acid is a complex polyether fatty acid, whereas calyculin A is a spiroketal, containing phosphate, oxazole, nitrite and amide functions. Both drugs do not seem to have any effect on other phosphatases or a variety of protein kinases, at least in vitro (Schönthal, 1992; Cohen et al., 1990). They enter intact cells and are thus thought to be valuable tools to assess the in situ functions of phosphatases 1 and 2A.

We have now determined whether okadaic acid or calyculin A can alter shape and locomotion activity of tumor cells, and whether such effects correlate with effects on protein phosphorylation.

2. Materials and methods

2.1. Reagents and suppliers

Glutaraldehyde (Serva Feinbiochemica, Heidelberg, Germany); okadaic acid, sodium salt; calyculin A (LC Services, Woburn, MO, USA); [^{32}P]orthophosphate in aqueous solution, HCl-free (NEN Du Pont, Wilmington, DE, USA). Water-insoluble compounds were dissolved in dimethyl sulfoxide (Fluka, Buchs, Switzerland). Dimethyl sulfoxide alone had no measurable effect in these experiments. The medium was made up as follows: 138 mM NaCl, 6 mM KCl, 0.1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 mM glucose, 20 mM Hepes-NaOH, pH 7.4, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, pH 7.4 and 2% human serum albumin. For assessment of the time course of okadaic acid-induced effects on cell shape, in situ phosphorylation and associated studies, human serum albumin and Na_2HPO_4 were omitted as specified in Section 3. Modification of the medium had only minor effects on the responses to okadaic acid. IC_{50} values from parallel experiments in medium containing no human serum albumin or 2% human serum albumin were 0.16 μM or 0.23 μM okadaic acid for suppression of cell polarity and 0.44 μM or 0.43 μM for generation of blebbing cells.

2.2. Tumor cell culture and assessment of cell shape

Walker 256 carcinosarcoma cells were kindly provided by Dr. B. Sordat (ISREC, Lausanne, Switzerland), and cultured as previously described (Keller et al., 1985). Cells were washed twice in medium. Shape change assays were performed with cells at a final concentration of 10^6 cells/ml. Details of the incubation procedure are given in Section 3. The incubation was terminated by fixation with glutaraldehyde (1% final concentration) at 37°C for 30 min. The shape of at least 100 fixed cells per condition of each experiment was analyzed using Nomarski optics (DIC; Zeiss IM 35 microscope, ×100 objective). Resting cells show a spherical shape with no or only few and small surface projections. Polarized cells are defined as cells with a clearly visible front (ruffles or bleb-like structures), an elongated cell body and a contracted tail. Another category are cells blebbing all over the surface (Keller et al., 1989). These blebs could become very large and determine the overall shape of the cell (see Section 3).

2.3. Locomotion assays

Locomotion of Walker carcinosarcoma cells was studied in narrow paraffin-sealed slide-coverslip preparations (depth 5–12 μm) on a heated stage (37°C) of an inverted microscope (Keller et al., 1985; Keller and Zimmermann, 1986). The path of individual cells was recorded on videotape for 10 min immediately after the preparation had been set up. The entire path of the cells was drawn on a transparency placed on a screen of a TV monitor. Speed (distance travelled against time) was determined by means of morphometry (IBAS, Zeiss, Oberkochen, Germany). The proportion of migrating cell is defined as the percentage of cells which have locomoted within the observation time of 10 min.

2.4. Protein phosphorylation in intact Walker carcinosarcoma cells

2.4.1. Analysis of cell homogenates or subcellular fractions on one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Cells (20×10^6 cells/ml) in medium lacking human serum albumin and phosphate were incubated with 0.3 mCi [^{32}P]orthophosphate/ml for 5–10 min at 37°C, followed by two washes with medium lacking human serum albumin and phosphate. In some experiments, cells were incubated with 0.03 mCi [^{32}P]orthophosphate/ml for 60 min. This procedure yielded essentially the same results as a short incubation (see also Section 3). The cells (3×10^6 cells/ml, 0.45 ml per tube) were subsequently incubated in the absence or presence of phosphatase inhibitors. For analysis of total protein, the reaction was stopped by addition of one volume of an ice-cold solution containing 200 mg/ml trichloroacetic acid, 40 mM NaF and 10 mM Na_2HPO_4. After incubation on ice for 20 min, the precipitates were collected by centrifugation (10 min, 13 000 × g, 4°C). The pellets were washed once with 1 ml of 5% trichloroacetic acid, followed by solubilization in 100 μl sample buffer containing 1% SDS, 50 mM dithiothreitol, 15% glycerol, 62.5 mM Tris/HCl, pH 6.8, 0.001% bromphenol blue (5 min, 95°C). When necessary, the pH of the solubilisates was restored to 7 with a few microliters of 1 M Tris. The proteins were electrophoresed through

5–10 or 15% SDS-polyacrylamide gradient gels (Laemmli, 1970). The gels were dried after staining with Coomassie Blue and exposed to a Trimax XM film (3 M, Rüschlikon, Switzerland) at −70°C with intensifier screens.

In some experiments, subcellular fractions were analyzed. To obtain these fractions, cells, exposed to medium or phosphatase inhibitors (see above), were centrifuged for 5 min at $240 \times g$ (room temperature). The pellets were resuspended in 500 μl of a buffer containing 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, and incubated for 10 min on ice. The cells were subsequently homogenized by ten passages through a fine needle (gauge: 30; length: 13 mm) using a 1 ml plastic syringe, followed by centrifugation for 10 min (4°C, at $1000 \times g$). The supernatants were centrifuged at 4°C and $100\,000 \times g$ for 20 min. The resulting pellets (representing a particulate fraction) were solubilized in 100 μl sample buffer by incubation at 95°C for 5 min. Supernatants (representing the cytosolic fraction) were precipitated and solubilized as described above for total cell homogenates. Both pellets and supernatants were analyzed by SDS-PAGE and autoradiography as described above.

2.4.2. Analysis of cell homogenates or subcellular fractions by two-dimensional PAGE

Total cell homogenates or cytosolic fractions were precipitated with trichloroacetic acid and washed, as described in Section 2.4.1. The pellets were solubilized in 40 μl of a buffer containing 9 M urea, 4% Nonidet P-40, 0.4% ampholytes pH 3.5–10, 1.6% ampholytes pH 3.5–5 and 100 mM dithiothreitol, by incubation for 2 h at room temperature. The samples were stored at −70°C until use. They were centrifuged at $100\,000 \times g$ for 2 h at 4°C prior to isoelectric focusing. The supernatants were applied to first-dimension gels containing 1% ampholytes 3.5–10 and 4% ampholytes pH 3.5–5 (Dunbar et al., 1990). The gels were focused for 17 h at 700 V, and subsequently applied to gradient gels. The gels were analyzed by autoradiography (see above). To evaluate incorporation of phosphate into individual proteins, the autoradiograms were scanned with a TLC scanner II (Camag, Muttenz, Switzerland). To determine the peak area, the troughs next to the peak areas were connected, thereby defining the background.

2.5. Isolation of cytoskeletal and soluble fractions

Cytoskeletal and soluble fractions were isolated by extraction of cells with a buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris/HCl, pH 8, 2 mM phenylmethylsulfonyl fluoride, as described (Lee et al., 1992).

2.6. Immunoblotting

Gels containing total cell homogenates or subcellular fractions (see above) were transblotted to nitrocellulose using a genie blotter from Idea Scientific (Minneapolis, MN, USA). The blot was washed three times with a buffer containing 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20, 4% skim milk powder (TBSTM), followed by incubation for 1 h at room temperature with either a monoclonal anti-vimentin antibody (Sigma, Buchs, Switzerland, clone V9), diluted 1:100, or a monoclonal anti-myosin light chain antibody (Sigma, clone MY-21) diluted 1:200 in 10 mM Tris/HCl, pH 7.4, 0.9% NaCl, 3% bovine serum albumin, 0.02% Na-azide. After several washes with TBSTM, the blot was incubated with a second gold-labeled goat anti-mouse immunoglobulin G/M (Aurion, Wageningen, Netherlands) diluted 1:100 in TBSTM. After 2 h of incubation, the blot was washed twice with TBSTM and twice with H_2O, followed by treatment with Aurion R-gent (Aurion, Wageningen, Netherlands), in order to intensify the gold signal by silver enhancement.

2.7. Staining of cells for vimentin and F-actin

Walker carcinosarcoma cells were preincubated in medium containing $CaCl_2$, $MgSO_4$ and phosphate at 37°C for 10 min. Then the cells were exposed to okadaic acid at the concentrations indicated in Section 3 at 37°C for 40 min, fixed in 4% paraformaldehyde (final concentration, pH 7.4) at 37°C for 10 min, centrifuged and resuspended in PBS containing 10 mM EDTA. The cells were permeabilized by addition of lysolecithin (0.2 mg/ml) for 10 min, washed in phosphate-buffered saline (PBS) and cytocentrifuged. Cytocentrifuge preparations were allowed to dry for 10 min and then stained as follows. First they were exposed to a mixture containing 10% normal goat serum (Dako, Glostrup, Denmark) and 5% skim milk in PBS containing 10 mM EDTA for 15 min. Afterwards they were exposed to a monoclonal antibody against vimentin (Sigma, St. Louis, MO, USA) at a dilution of 1:40 for 45 min, washed twice for 5 min with PBS, exposed again to 10% normal goat serum in PBS for 15 min and reacted with anti-mouse immunoglobulin G/M (rhodamine conjugated, Bioscience, Emmenbrücke, Switzerland) diluted 1:50, washed twice with PBS for 5 min and imbedded in polyvinyl alcohol. Interference and fluorescence pictures were obtained with a laser scan microscope (LSM 410 Invert, Zeiss, Oberkochen, Germany). Stacks of z sections at 0.25 μm distance were obtained and pictures from the middle of the cell are shown.

Cells were stained for F-actin using rhodamine-phalloidin (Molecular Probes, Junction City, OR, USA) as described previously (Keller et al., 1985).

2.8. Statistical analysis of data

Differences between data were analyzed with the Student's t-test for paired data, with a P value of < 0.05 considered significant.

3. Results

3.1. Biphasic effect on cell shape of increasing concentrations of okadaic acid

Cultures of Walker carcinosarcoma cells showed a substantial proportion of spontaneously polarized cells (Fig. 1A and Fig. 2). Two major phases in the dose-response curve to okadaic acid could be distinguished (Fig. 2). At about 0.2 μM okadaic acid, cell polarity was almost completely suppressed and the cells were mostly spherical (IC_{50} 0.14 μM; Fig. 1B, Fig. 2). At higher concentrations of okadaic acid, Walker carcinosarcoma cells developed first small and then (2 μM okadaic acid) large blebs (IC_{50} 0.41 μM; Fig. 1C, Fig. 2).

Our results suggest that the shape of Walker carcinosarcoma cells is regulated by a kinase-phosphatase couple, and we also addressed the question of the identity of the kinase(s) involved. It is very likely not protein kinase C, as two highly selective inhibitors of this enzyme, Ro 31 8220 and CGP 41 251 (1 μM), did not prevent okadaic acid-induced inhibition of cell polarity and shape changes, although CGP 41 251 shifted the dose-response curve of okadaic acid-induced inhibition of polarity to slightly higher values (Fig. 3). In the presence of 2 μM okadaic acid, no significant difference in cell morphology was observed in the presence and absence of CGP 41 251 or Ro 31-8220. A CGP 41 251-sensitive enzyme may thus contribute to, but is not necessary for the effects induced by inhibition of phosphatases.

3.2. Time-course of the shape changes in response to okadaic acid

The time-course of the morphological responses to 2 μM okadaic acid shows (Fig. 4) that cell polarity was suppressed within 10 min ($t_{1/2}$ about 4.5 min). Up to 5 min, suppression of polarity was essentially associated with an increase in spherical cells. Between 5 and 30 min an increase in non-polar blebbing cells ($t_{1/2}$ about 13 min) could be observed. Thus, the time-course, similar to the dose–response curve, is biphasic. First, polarized cells are transformed into spherical cells. In a second phase spherical and remaining polarized cells are transformed into non-polar blebbing cells.

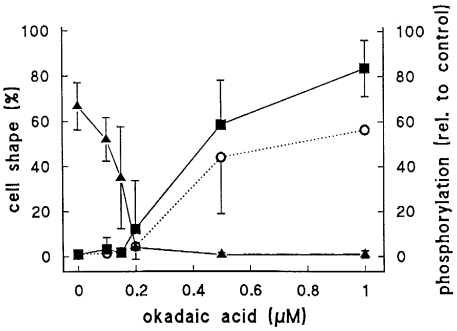

Fig. 2. Concentration-dependent changes in Walker carcinosarcoma cell shape and vimentin phosphorylation induced by incubation with okadaic acid. Cells were prelabeled for 10 min with $^{32}PO_4$ at 37°C, and washed twice. Labeled and unlabeled aliquots of the same cell population (3×10^6 cells/ml) were incubated for 40 min at 37°C in the absence or presence of increasing amounts of okadaic acid in medium without human serum albumin and Na_2HPO_4. Labeled aliquots were precipitated by trichloroacetic acid, followed by analysis on two-dimensional PAGE, the second dimension being a 5–15% gradient gel. Phosphorylation of vimentin was analyzed by scanning of the vimentin spots. Analysis of cell shape was carried out on the unlabeled aliquots after fixation of cells with glutaraldehyde: polarized cells (▲); non-polar cells with blebs (■); phosphorylation of vimentin, relative to controls (○). Background phosphorylation of vimentin in untreated cells was 2 ± 0.4% of maximal values. Mean ± S.D. of 3–4 independent experiments.

3.3. Okadaic acid suppresses locomotion of Walker carcinosarcoma cells

A polarized shape is thought to be a prerequisite for efficient locomotion. We therefore studied the effects of okadaic acid on cell locomotion. Indeed, 0.25 μM okadaic acid completely inhibited spontaneous locomotion of Walker carcinosarcoma cells (Table 1). This concentration of okadaic acid also suppressed cell polarity (Fig. 1B, Fig. 2).

3.4. Effect of calyculin A on shape of Walker carcinosarcoma cells

The responses to calyculin A were similar to those observed with okadaic acid with the exception that sup-

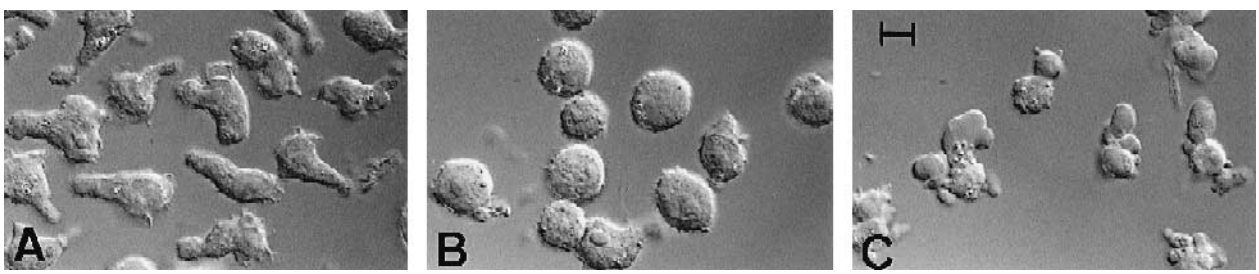

Fig. 1. Shape changes of Walker carcinosarcoma cells induced by okadaic acid. (A) Medium only, (B) medium with 0.25 μM okadaic acid, (C) medium with 2.0 μM okadaic acid. Cells were incubated without or with okadaic acid at 37°C for 40 min. The medium contained 2% human serum albumin. At the end of the incubation period, cells were fixed with glutaraldehyde at 37°C for 30 min and photographed using DIC optics. Scale bar: 10 μm.

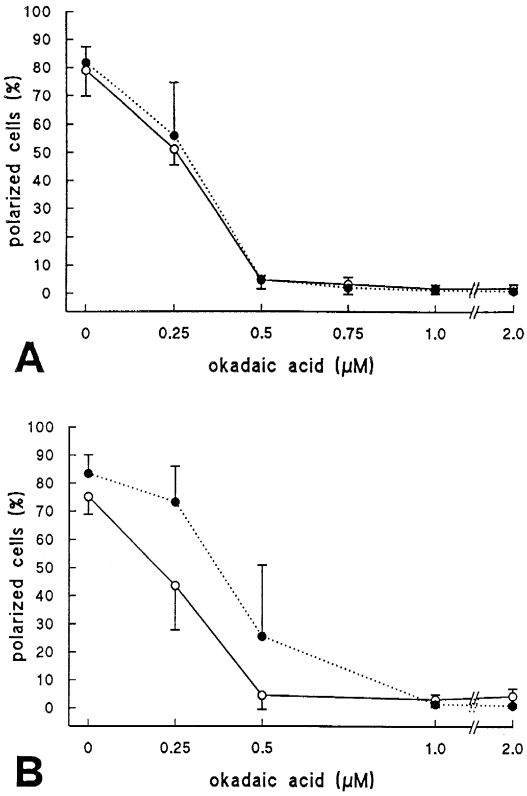

Fig. 3. Effect of preincubation of cells with protein kinase C inhibitors on morphological responses induced by okadaic acid. Cells were incubated either for 35 min in medium without human serum albumin and Na_2HPO_4 at 37°C in the presence of increasing concentrations of okadaic acid (○), as indicated, or they were preincubated with 10^{-6} M of either Ro 31-8220 (A) or CGP 41 251 (B) (●) for 5 min followed by the addition of increasing concentrations of okadaic acid and a further incubation for 35 min. Cells were fixed with glutaraldehyde and shape was determined using DIC optics. Mean ± S.D. of 5–6 experiments.

pression of polarity was associated with formation of non-polar blebbing cells rather than of spherical cells. Using medium without human serum albumin and

Table 1
Inhibition of locomotion by okadaic acid in Walker carcinosarcoma cells

Okadaic acid	% Cells migrated	Speed (μm/min)	
		All cells	Migrating cells
None	18.2 ± 2.8	0.8 ± 0.2	4.3 ± 0.3
0.25 μM	0	0	0
0.5 μM	0	0	0

Cells in suspension were incubated with or without okadaic acid at 37°C for 40 min in medium containing 2% human serum albumin. Then slide-coverslip preparations were made to assess cell locomotion for 10 min using videomicroscopy. Mean ± S.D. of 3 experiments.

Na_2HPO_4 and an incubation time of 15 min, we found a dose-dependent suppression of cell polarity (IC_{50} 64 nM) and, at slightly higher concentrations, a dose-dependent generation of non-polar blebbing cells (IC_{50} 75 nM). The time course of the response was tested using 0.1 μM calyculin A. Cell polarity was almost completely (> 80%) suppressed within 5 min ($t_{1/2}$ about 2 min). $t_{1/2}$ for the development of blebbing cells was about 2 min 15 s (Fig. 5). In contrast to the data obtained with okadaic acid, cells were transformed directly from polarized cells into blebbing cells, without intermediate appearance of spherical cells (compare Figs. 4 and 5). IC_{50} values for the effects of okadaic acid and calyculin A on cell morphology have been summarized in Table 2.

We tested whether formation of large blebs was reversible. Cells were incubated for 40 min in the presence of 2 μM okadaic acid, or for 20 min with 100 nM calyculin A at 37°C, followed by three washes with 10 ml medium containing 2% human serum albumin. The cells were subsequently incubated for 15 min at 37°C, followed by fixation with glutaraldehyde. In these experiments, 84 ± 2% of the cells exposed to calyculin A exhibited large blebs before washing as compared to 20 ± 10% (mean ± S.D., $n = 3$) after washing. Similar results were

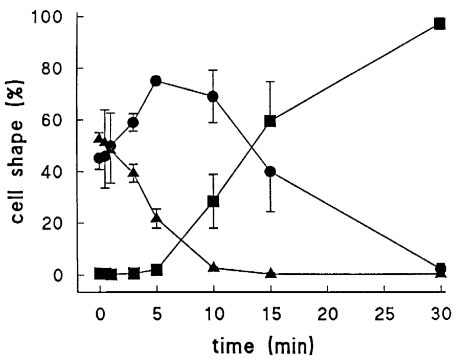

Fig. 4. Time-course of morphological responses induced by okadaic acid. Spherical cells (●); polarized cells (▲); non-polar cells with blebs (■). Walker carcinosarcoma cells were preincubated in medium without human serum albumin and Na_2HPO_4 at 37°C for 10 min. Then 2 μM okadaic acid was added and incubation was continued. Cells were fixed with glutaraldehyde at the time indicated and shape was determined using DIC optics. Mean ± S.D. of 3 experiments.

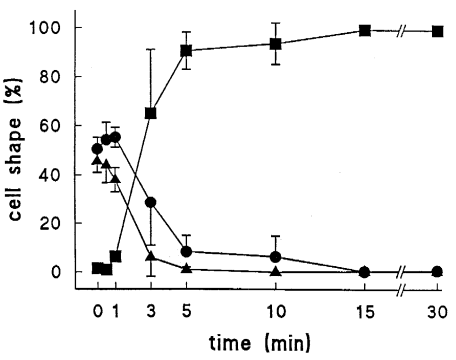

Fig. 5. Time-course of morphological responses induced by calyculin A. Spherical cells (●); polarized cells (▲); non-polar cells with blebs (■). Walker carcinosarcoma cells were preincubated in medium without human serum albumin and Na_2HPO_4 at 37°C for 10 min. Then 0.1 μM calyculin A was added and incubation was continued. Cells were fixed with glutaraldehyde at the time indicated and shape was determined using DIC optics. Mean ± S.D. of 3 experiments.

Table 2
Morphological changes in Walker carcinosarcoma cells induced by calyculin A and okadaic acid (IC_{50} in nM) [a]

	Suppression of polarity	Appearance of blebbing cells
Calyculin A	61 ± 15	75 ± 13
Okadaic acid	140 ± 33	410 ± 100

[a] Mean ± S.D. of 3–4 experiments.

obtained for okadaic acid: 32 ± 3% cells with large blebs before washing; 5 ± 2% ($n = 3$) after washing. Formation of large blebs is thus at least partially reversible. Moreover, 97–98% of the cells treated with calyculin A or okadaic acid excluded trypan blue after washing, showing that the plasma membrane was still intact.

3.5. The effects of okadaic acid on protein phosphorylation in Walker carcinosarcoma cells

Walker carcinosarcoma cells, preincubated with [^{32}P]orthophosphate, were exposed to various concentrations of okadaic acid for 40 min, followed by trichloroacetic acid precipitation of the cellular proteins and analysis by SDS-PAGE and autoradiography. Okadaic acid induced increased phosphorylation of at least three major bands of 57, 16 and 18 kDa (Fig. 6). On immunoblots of total cellular proteins derived from one-dimensional gels, the phosphorylated 57-kDa band comigrated with vimentin, as detected using a monoclonal anti-vimentin antibody. The 18-kDa band migrated slightly slower than myosin light chain as detected using a monoclonal antibody against this protein, whereas the 16-kDa band migrated slightly faster in the gel. On blots of two-dimensional PAGE of total cell extracts derived from cells exposed to 1–2 μM okadaic acid, the monoclonal antimyosin light chain antibody reacted with a protein not corresponding to a major phosphoprotein (results not shown). On these two-dimensional PAGE, two major phosphoproteins of 16 and 18 kDa were observed to remain at the basic end of the gel, suggesting basic proteins. Using non-equilibrium isoelectric focusing in the first dimension under conditions optimal for separation of basic proteins (pH range 7–9; Dunbar et al., 1990), the two phosphoproteins of 16 and 18 kDa entered the gel, forming streaks towards the basic end of the gel. Streaking is typically observed for histones, proteins with a pI of 11–12 (Kuhn and Wilt, 1980). At least part of these bands appearing on one-dimensional gels may thus be identical with low-molecular mass histones. As shown in Fig. 6, and as evaluated using densitometry, phosphorylation of the 16- and 18-kDa bands was not significantly increased or not detectable in cells incubated with up to 0.2 μM okadaic acid (lanes 1–3). Phosphorylation of the 16-kDa band was stimulated 2-fold by 0.5 μM and 3- to 4-fold by 2 μM okadaic acid. Phosphorylation of the 18-kDa band was increased to 17–20% of maximal values at 0.5 μM and to 65–90% at 1 μM okadaic acid. Due to the higher background, it was not possible to evaluate phosphorylation of the 57-kDa band at lower concentrations of okadaic acid. Therefore, phosphorylation of this band was analyzed using two-dimensional PAGE (see below).

After cell homogenization, extracts were centrifuged at 100 000 × g, in order to isolate a particulate and a cytosolic fraction. The 57-kDa band was about equally distributed between the two fractions, whereas the two proteins at 16–18 kDa were entirely recovered in the particulate fraction. The latter finding supports their possible identity with nuclear histones (see above). Cytosolic fractions of okadaic acid-treated cells were analyzed by two-dimensional PAGE, transfer to nitrocellulose and autoradiography. A typical result obtained with cells treated for 40 min with 2 μM okadaic acid is shown in Fig. 7. A major phosphoprotein of 57 kDa, consisting of 2–3 adjacent spots, appeared at a position comparable to that of purified rabbit skeletal muscle actin (pI approx. 5.1), analyzed on a separate gel. Vimentin has a pI of 5.3 and a molecular mass of approximately 54 kDa (Osborn, 1993), comparable to that of the okadaic acid-induced phosphoprotein. Vimentin usually appears in several isoforms thought to reflect different phosphorylation states (Osborn,

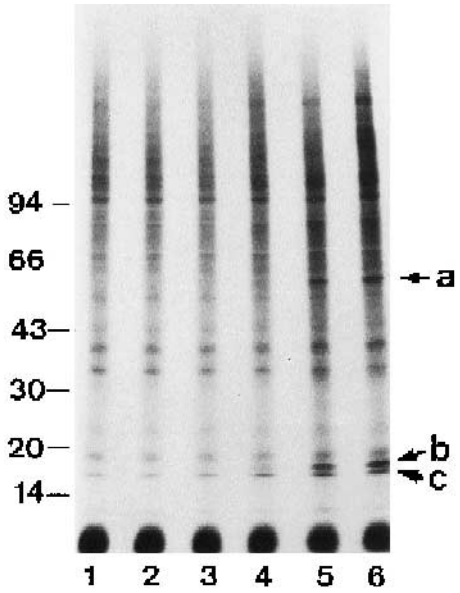

Fig. 6. Effect of increasing concentrations of okadaic acid on protein phosphorylation in intact Walker carcinosarcoma cells. Cells were prelabeled for 5 min with $^{32}PO_4$, at 37°C in medium without human serum albumin and Na_2HPO_4 and washed twice. Cells (3.3×10^6 cells/ml) were incubated for 40 min at 37°C in the absence (lane 1) or presence of the following concentrations of okadaic acid: 0.1 μM (lane 2), 0.2 μM (lane 3), 0.5 μM (lane 4), 1 μM (lane 5), 2 μM (lane 6). The reaction was stopped by the addition of ice-cold trichloroacetic acid (10% final concentration). The precipitated proteins were separated on a 5–15% gradient gel. The corresponding autoradiogram is shown. Molecular masses of standard proteins in kDa are shown on the left side of the autoradiogram. (a) indicates a phosphorylated band of 57 kDa; (b) a band of 18 kDa; (c) a band of 16 kDa.

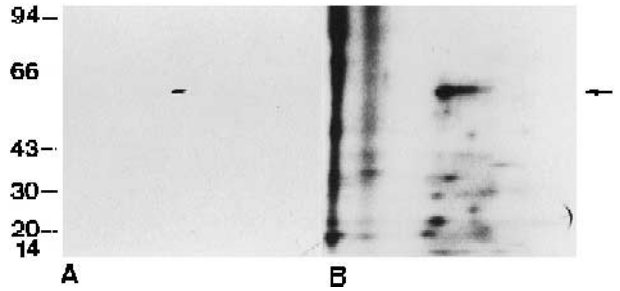

Fig. 7. Identification of vimentin as a major phosphoprotein in a two-dimensional gel of phosphorylated proteins from Walker carcinosarcoma cells treated with 2 μM okadaic acid. Cells were prelabeled for 60 min with $^{32}PO_4$ and washed twice. Cells (3×10^6 cells/ml) were incubated for 40 min at 37°C in the presence of 2 μM okadaic acid in medium without human serum albumin and Na_2HPO_4. A cytosolic fraction was subsequently prepared as described in Section 2. The trichloroacetic acid-precipitated proteins were separated by two-dimensional PAGE, the second dimension being a 5–15% gradient gel. The gel was transferred to nitrocellulose. The resulting blot was first exposed to a film, and subsequently incubated with a specific anti-vimentin antibody, followed by a second gold-labeled goat anti-mouse antibody and silver enhancement. (A) blot; (B) the corresponding autoradiogram. Molecular masses of standard proteins are indicated at the left of the autoradiogram. The arrow indicates the position of vimentin.

1993). Immunodecoration of the blot shown in Fig. 7B with a monoclonal anti-vimentin antibody yielded a major protein exactly comigrating with the major phosphoprotein of 57 kDa (Fig. 7A). This result confirms that this band is identical with vimentin. A quantitative analysis of okadaic acid-induced vimentin phosphorylation was carried out using total cellular proteins separated on two-dimensional gels. As shown in Fig. 2, 1 μM okadaic acid induced a very marked, 40- to 70-fold increase in vimentin phosphorylation. Overphosphorylation of vimentin correlated with the formation of cells with blebs rather than with suppression of cell polarity (Fig. 2). IC_{50} corresponded to 0.41 ± 0.10 μM for the formation of non-polar cells with blebs, as compared to 0.45 ± 0.14 μM ($n = 4$) for vimentin phosphorylation, as determined in the same experiments.

3.6. Effect of okadaic acid on the localization and state of assembly of vimentin and on F-actin localization

In untreated spherical cells vimentin was mainly localized along the cell membrane (Fig. 8A,a). In spontaneously polarized cells vimentin was concentrated in the rear part of the cell (Fig. 8B,b). Okadaic acid produced vimentin aggregates. Cells treated with 0.2 μM okadaic acid showed variable amounts of vimentin along the cell membrane and in addition loose aggregates in the cytoplasm (Fig. 8C,c). Cells treated with 2 μM okadaic acid usually showed just one large aggregate of vimentin in the cytoplasm, but little or no vimentin along the cell membrane or in the blebs (Fig. 8D,d). Overphosphorylation of vimentin thus correlated with the formation of aggregates.

We also tested whether okadaic acid affects the sedimentation of cellular vimentin after treatment of cells with non-ionic detergents. The fraction of vimentin sedimenting at low *g* force after cell solubilization is thought to reflect the cytoskeleton-associated or polymerized vimentin. About 50% of total vimentin could be recovered in the cytoskeletal fraction in untreated cells, and in cells incubated for 40 min with 0.2 μM okadaic acid. This value was increased to 75% for cells incubated with 0.5 or 1 μM of the inhibitor, and decreased again to control levels at 2 μM. Total amounts of vimentin remained the same, suggesting that incubation of the cells with okadaic acid may

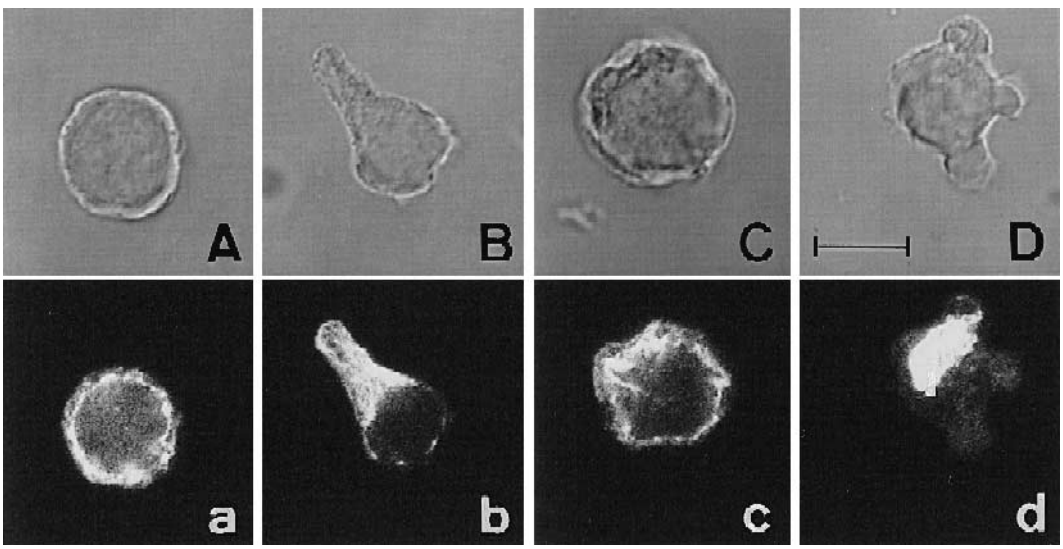

Fig. 8. Effects of okadaic acid on localization of vimentin in Walker carcinosarcoma cells. Pictures of conventional morphology (A–D) and immunofluorescence localization of vimentin using laser scan microscopy through the middle of the cell (a–d) are shown: untreated spherical cell (A,a); untreated polarized cells (B,b); cells treated with 0.2 μM okadaic acid (C,c) and cells treated with 2 μM okadaic acid (D,d). Cells were preincubated in medium for 10 min and then incubated with or without okadaic acid at 37°C for 40 min, fixed and stained for vimentin. Scale bar: 10 μm.

transiently increase vimentin polymerization. Okadaic acid thus induces formation of vimentin aggregates and transiently increased the percentage of cytoskeletal vimentin. The concentrations inducing increases in cytoskeletal vimentin (0.5–1 μM) were higher than those necessary for a substantial repression of the polarized shape (Fig. 2).

We also assessed the localization of F-actin, using rhodamine-phalloidin, in Walker carcinosarcoma cells treated with 2 μM okadaic acid for 40 min, followed by fixation and staining. F-actin was mainly concentrated linearly along the cell membrane of untreated control cells, as well as of cells treated with okadaic acid. The staining intensity of membrane-associated F-actin in the blebs as compared to the rest of the cells was often substantially lower, particularly if the blebs were large, but in other cases similar to the rest of the cell (results not shown).

4. Discussion

The results presented indicate that constitutive phosphatase activity plays a central role in maintaining (1) the spherical shape of resting cells and (2) the polar locomotor morphology of activated Walker carcinosarcoma cells. The present experiments also demonstrate that inhibition of phosphatases is a stop-signal for locomotion of Walker carcinosarcoma cells. Okadaic acid, an inhibitor which acts in vitro with high affinity on phosphatase 2A (IC_{50} 0.5–1 nM), and with lower affinity on phosphatase 1 (IC_{50} 60–500 nM; Ishihara et al., 1989) has a biphasic effect on cell shape: at lower (≤ 0.2 μM) concentrations it suppresses cell polarity in Walker carcinosarcoma cells giving rise to spherical cells. At higher (1–2 μM) concentrations it induces very marked bleb formation (Table 2). Suppression of cell polarization is clearly separated from massive effects on cell shape. One possible explanation of this biphasic effect could be the specific involvement of phosphatase 2A in controlling cell polarity and locomotion, whereas inhibition of phosphatase 1 may lead to marked bleb formation. In line with this hypothesis, calyculin A, which inhibits with the same potency phosphatases 1 and 2A (IC_{50} 0.5–2 nM; Ishihara et al., 1989) converts polarized cells directly into blebbing cells, with no or little intermediate appearance of spherical cells (Table 2). Both phosphatase 1 and 2A are present in Walker carcinosarcoma cells, as found using immunoblotting with specific polyclonal antibodies directed against these enzymes (Niggli and Keller, unpublished observations). It has to be noted that higher concentrations of phosphatase inhibitors may be necessary in intact cells, as the intracellular concentrations of phosphatases 1 and 2A often lie in the range of 0.1–1 μM (Cohen et al., 1990).

In lung carcinoma cells, okadaic acid also induces a spherical morphology and bleb formation. However, in contrast to our findings, okadaic acid, incubated for 18 h with the cells, induces migration of the initially non-motile cells (Maier et al., 1995). The differences between the two studies may be due to differences in the cell types used (initially motile versus non-motile cells) and in the length of incubation, as our results are obtained after short-time incubation with okadaic acid. Stimulation of locomotion by okadaic acid as described by Maier et al. (1995) may therefore be due to events occurring downstream of long-term inhibition of phosphatase activity, such as changes in protein synthesis, rather than pointing to a relatively direct inhibitory role of phosphatases in cell locomotion.

Previous experiments suggest that protein kinase C plays an important role in regulating shape and locomotion of Walker carcinosarcoma cells. Activators of protein kinase C such as PMA or diacylglycerols revert polarized cells into spherical cells (Keller et al., 1985, 1989, Niggli et al., 1996). In contrast to relatively unspecific inhibitors of protein kinase C (Zimmermann and Keller, 1993) highly selective inhibitors of protein kinase C such as Ro 31-8220 prevent the effect of PMA (Niggli et al., 1996). This indicates that activation of protein kinase C may be a stop-signal and inhibition of this enzyme a go-signal for Walker carcinosarcoma cells. Now we find that inhibition of phosphatases 1 and/or 2A has the same effect as activation of protein kinase C. The effect of okadaic acid is however very likely independent of protein kinase C activity, as CGP 41 251 and Ro 31-8220 cannot prevent its actions (Fig. 3). Similarly, the effects of okadaic acid on the cytokeratin network in hepatocytes are not prevented by inhibitors of protein kinase C (Blankson et al., 1995). Our results thus provide evidence for two independent pathways, induced by either increasing protein kinase activity or decreasing phosphatase activity, leading to suppression of tumor cell locomotion. Interestingly, okadaic acid acts not only as a tumor promotor, but, for some cell types, has been shown to revert the phenotype of oncogene-transformed cells to that of normal cells, and to inhibit neoplastic transformation of fibroblasts (Schönthal, 1992).

We have addressed the question, how these phosphatase inhibitors exert their effects on cell shape. A major phosphoprotein induced by okadaic acid in Walker carcinosarcoma cells is the intermediate filament protein vimentin, as identified in this work. Vimentin phosphorylation appears to be regulated by a kinase-phosphatase couple, and inhibition of phosphatases reveals activity of kinases on vimentin. Marked overphosphorylation of vimentin correlates with bleb formation (Fig. 2). Vimentin, and the related protein desmin, have been shown to be a substrate of a number of kinases in vitro and in situ, such as protein kinases A, C, G, the calmodulin-dependent kinase and the cdc2 kinase, as summarized by Inagaki et al. (1996). Little is known on phosphatases specifically dephosphorylating vimentin, except that a calcium-stimulated phosphatase could play a role in mouse L-929 cells (Evans, 1989).

Our observations on the effect of 1–2 μM okadaic acid or 0.1 μM calyculin A on Walker carcinosarcoma cells

agree well with previous work on different cell types, where treatment with these phosphatase inhibitors induces overphosphorylation of vimentin or cytokeratin (Chartier et al., 1991; Hirano et al., 1992; Lee et al., 1992). Increased phosphorylation has several effects on vimentin organization. Visualization of vimentin in Walker carcinosarcoma cells using immunofluorescence shows that overphosphorylation of the protein correlates with formation of vimentin aggregates (Fig. 6). Interestingly, phosphorylation in the head domain of vimentin leads to filament disassembly, whereas tail domain phosphorylation results in filament rearrangements, e.g., in formation of bundles or aggregates (Inagaki et al., 1996). In 3T3 fibroblasts, exposure to calyculin A has been shown to induce phosphorylation of multiple sites of the C-terminal tail domain of vimentin (Hirano and Hartshorne, 1993). This agrees with our findings on okadaic acid-induced formation of vimentin aggregates. Moreover, we found that increasing amounts of okadaic acid induce transient increases in the amount of cytoskeletal vimentin, optimal at 0.5–1 μM of the inhibitor. However, at concentrations that completely suppress polarized cells (Fig. 2), no effect on the amount of cytoskeletal vimentin and only very minor effects on vimentin phosphorylation are observed. Suppression of cell polarization is thus very likely not due to reorganization of vimentin induced by okadaic acid.

Formation of vimentin aggregates may contribute to the major shape changes observed at 1–2 μM okadaic acid. Local weakening of the F-actin cortical network may also contribute, as at least in part of the blebs membrane-associated F-actin was decreased. Cell lines lacking the actin-crosslinking protein ABP-280 show prolonged blebbing (Cunningham, 1995). Okadaic acid-induced formation of large blebs was partially reversible, suggesting that the cytoskeletal changes possibly causing bleb formation may also be reversible, and that blebbing is not immediately followed by cell death, although okadaic acid seems to be cytotoxic in long-term experiments (Schönthal, 1992).

We observed increased phosphorylation of two bands of 16 and 18 kDa induced by 0.5–2 μM okadaic acid. Increased phosphorylation of these bands correlates with formation of blebs, rather than with suppression of polarity. These bands correspond very likely to low-molecular mass histones, as they showed typical streaking in the region of the basic end of non-equilibrium isoelectric focusing gels optimal for separation of basic proteins. Indeed, increased phosphorylation of a 15-kDa histone in mouse fibroblasts treated with 1 μM okadaic acid for 30 min has been reported (Mahadevan et al., 1991). Myosin light chain, identified on two-dimensional PAGE with immunoblotting, does not correspond to a major phosphoprotein upon stimulation of cells with 1–2 μM okadaic acid. We observed only a small increase in myosin light chain phosphorylation under these conditions (results not shown). In macrophages, high concentrations of okadaic acid suppress cell motility, correlated to increased phosphorylation of myosin light chain (Wilson et al., 1991). In the latter study, in contrast to our findings on Walker carcinosarcoma cells, 3 μM okadaic acid is required for half-maximal effects.

In summary, the results show that phosphorylation as well as dephosphorylation reactions play a role in determining cell shape and locomotor activity of Walker carcinoma cells, involving different kinases and phosphatases. Activation of protein kinase C (Niggli et al., 1996) or inhibition of a constitutively active phosphatase (this work) results in a stop signal for locomotion. A biphasic effect of okadaic acid on cell shape is described. Marked shape changes and bleb formation observed at higher concentrations of okadaic acid correlate with the appearance of three major phosphoproteins of 16, 18 and 57 kDa, the latter being identical with the cytoskeletal protein vimentin. The possibly minor phosphoproteins involved in suppression of cell polarity have not yet been identified and are the subject of current investigations.

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