To identify phosphoproteins that might play a role in naringin-sensitive hepatocellular cytoskeletal disruption and apoptosis induced by algal toxins, hepatocyte extracts were separated by gel electrophoresis and immunostained with a phosphothreonine-directed antibody. Use of dilute (5%) polyacrylamide gels containing 6 M urea allowed the resolution of one very large (~500-kDa) okadaic acid- and naringin-sensitive phosphoprotein, identified by tryptic fingerprinting, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and immunostaining as the cytolinker protein, plectin. The naringin-sensitive phosphorylation induced by okadaic acid and microcystin-LR probably reflected inhibition of a type 2A protein phosphatase, whereas the naringin-resistant phosphorylation induced by calyculin A, tautomycin, and cantharidin probably involved a type 1 phosphatase. Okadaic acid caused a collapse of the plectin-immunostaining bile canalicus and the general cytoskeletal plectin network into numerous medium-sized plectin aggregates. Inhibitors of protein kinase C, Ca2+/calmodulin-dependent protein kinase, or Ca2+/calmodulin-dependent kinase II had moderate or no protective effects on plectin network disruption, whereas naringin offered 86% protection. Okadaic acid induced a naringin-sensitive phosphorylation of AMP-activated protein kinase (AMPK), the stress-activated protein kinases SEK1 and JNK, and S6 kinase. The AMPK-activating kinase (AMPKK) is likely to be the target of inhibition by naringin, the other kinases serving as downstream components of an AMPKK-initiated signaling pathway.

Many environmental toxins are protein phosphatase inhibitors that exert their toxic effects through overphosphorylation of cellular proteins (1). The diarrhetic shellfish toxin, okadaic acid, is thus an extremely potent inhibitor of type 2A protein phosphatases (PP2A); at higher concentrations, it also inhibits phosphatases of type 1 (PP1)1 (1, 2). Okadaic acid primarily afflicts intestinal cells when ingested orally, but upon intravenous administration it induces liver damage as well (3). Under cell culture conditions, okadaic acid or other algal toxins, like the microcystins, can elicit apoptotic or necrotic cell death in a variety of cell types, including hepatocytes and hematoma cells (4–8).

Inhibition of PP1 and of PP2A have been implicated in okadaic acid-induced cell death (8–10). In rat hepatocytes, inhibition of PP2A by low doses of okadaic acid or microcystin initiates a slow apoptotic process culminating in cell death after 15–24 h (8), whereas the additional inhibition of PP1 by high toxin doses elicits an extremely rapid apoptosis, with characteristic morphological changes observable within a few minutes (11). A number of different protein kinases have been suggested to be involved as mediators of okadaic acid toxicity, including the cAMP-dependent protein kinase (PKA) (12), Ca2+/calmodulin-dependent protein kinase II (CaMK-II) (13), cyclin-dependent protein kinases (14), and mitogen-activated protein kinases (6).

In several cell types, okadaic acid has been shown to induce phosphorylation of the proapoptotic transcription regulator p53 (5, 15), to induce expression of the p53-inducible, proapoptotic protein Bax (10, 16, 17), and to elicit p53-dependent apoptosis (5, 10, 16, 17). In addition, okadaic acid may suppress the effects of antiapoptotic protein factors like Bcl-2 and Mcl-1, both through inactivating phosphorylations (12, 18, 19) and through reduced expression levels (20). Both the rapid and the slow apoptosis induced by algal toxins would appear to involve caspase activation (8, 11), whereas the protective function of Bcl-2 may be exerted only during the slower form of apoptosis (11).

Early cytoskeletal changes are prominent both in slow and rapid toxin-induced apoptosis (4, 8). In rat hepatocytes, the slow apoptosis induced by low doses of okadaic acid was associated with an early phosphorylation of keratin, accompanied by disintegration of the keratin intermediate filament network and of the keratin- and actin-containing bile canalicus sheaths, whereas the general microtubular and microfilamentous networks were unaffected (3, 8, 21). Rapid hepatocyte apoptosis elicited by high concentrations of okadaic acid or microcystin was similarly associated with keratin phosphorylation and with extensive cytoskeletal rearrangements as indicated by surface blebbing and the rounding of monolayer cells (4, 22, 23). In other cell types, intermediate filament networks composed of vimentin, keratin, or neurofilament proteins were terminal kinase; MALDI, matrix-assisted laser desorption/ionization; TOF, time-of-flight; MS, mass spectrometry; PBS, phosphate-buffered saline; PBS-T, PBS containing 0.2% Tween 20; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; S6K, S6 kinase; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase 1.
similarly found to undergo phosphorylation-dependent disintegration after okadaic acid treatment (24–27). Disruption of intermediate filament networks would thus seem to be a characteristic, early feature of apoptosis elicited by algal toxins. Although the molecular mechanism of such network disruption has not been clarified, the ability of okadaic acid to induce phosphorylation and detachment of several cytoskeleton-associated proteins, including the cross-linking protein Tau (28–31), suggests that phosphorylation of structural intermediate filament proteins as well as of their associated cross-linking proteins (32) could be involved.

In a previous study, we found that the keratin phosphorylation and apoptotic cell death induced by okadaic acid or microcystin in rat hepatocytes could be prevented by the grapefruit flavonoid, naringin (8). Naringin had no such protective effect on hepatoma cells of human or rat origin (8), making the flavonoid interesting both as a potential chemotherapeutic agent (in combination, for example, with a nonspecific algal toxin to allow cancer cell killing while protecting normal cells) and as a tool to investigate the mechanisms of toxin-induced cell death. To identify hepatocellular proteins subject to okadaic acid-induced, naringin-sensitive phosphorylation, and therefore candidates for involvement in the apoptotic process, we have taken a proteomic approach, separating phosphoproteins by one- and two-dimensional gel electrophoresis and identifying them by mass spectrometry. In the present report, we show that one naringin-sensitive phosphoprotein can be identified as the high molecular weight cytolinker protein, plectin, known to be of prime importance for the maintenance of intermediate filament networks (33).

**MATERIALS AND METHODS**

**Reagents**—Okadaic acid and microcystin-LR were from Alexis Biochemicals (Laufelfingen, Switzerland). Calcium A, cantharidin, and tautomycin were from Calbiochem. SDS, acrylamide, and bisacrylamide were obtained from Bio-Rad. Biotinylated anti-mouse antibody, streptavidin-conjugated horseradish peroxidase, fluorescein-conjugated rabbit anti-mouse antibody, Rainbow molecular weight markers (RPN 800), and the ECL Western blotting detection kit were from Amersham Biosciences. Anti-rabbit IgG horseradish peroxidase-linked antibody was obtained from Bio-Rad. Biotinylated anti-mouse antibody (diluted 1:1000 in PBS-T), washed three times and incubated for another 30 min at room temperature with streptavidin-conjugated horseradish peroxidase (diluted 1:3000 in PBS-T). The membranes were washed three times again before they were visualized by chemiluminescence using the ECL Western Detection Kit (Amersham Biosciences). For detection of threonine-phosphorylated proteins, the membranes were incubated for 3 h at room temperature with a rabbit polyclonal anti-phosphothreonine antibody (diluted 1:1000 in PBS-T). After washing three times with PBS-T, the membranes were incubated for 1 h at room temperature with anti-rabbit-horseradish peroxidase (diluted 1:2000 in PBS-T), washed three times, and visualized as described above. The same procedure was used for immunoblotting with other phosphospecific polyclonal rabbit antibodies.

**Sample Preparation for MALDI-TOF MS**—Gels to be used for protein identification by MALDI-TOF MS were stained overnight with 0.25% Coomassie Brilliant Blue R-250 dissolved in 45% methanol, 10% acetic acid, 45% deionized water and then carefully destained with the above solvent before the protein bands of interest were cut out. The bands excised from the stained gel were cut into small pieces (approximately 1 mm in an Eppendorf tube, and washed in 1 ml of water three times for 1 h with shaking. Fifty µl of 100% acetonitrile was then added for 10 min, and the supernatant was discarded. The pieces were subsequently dried in a vacuum centrifuge for 30 min (Automatic Environmental SpeedVac System AES1010 from Savant Instruments, Inc., Holbrook, NY). Enough 10 m dithiothreitol solution in 100 m ammonium bicarbonate to cover the gel (about 50 µl) was added, and the gel was placed at 60°C in order to reduce the disulfide bonds and acrylamidated cysteine moieties (36). The supernatant was discarded, the same volume of 55 m iodacetamide solution in 100 m ammonium bicarbonate was added, and the gel was incubated for 30 min at room temperature in the dark to alkylate the free sulfhydryl groups. The supernatant was discarded, and the gel pieces were washed/rehydrated with 100 µl of 100 m ammonium bicarbonate for 10 min and dehydrated with 50 µl of 100% acetonitrile. This step was repeated once before the liquid phase was removed, and the particles were dried in the vacuum centrifuge for 30 min.

**Protein Digestion and Extraction**—The gel pieces were rehydrated with 0.1 µl µl trypsin in 25 m ammonium bicarbonate, pH 8. Just enough trypsin solution to be absorbed by the gel (about 0.6 µl/mm² of gel) was used. The gel was incubated for 16–24 h at 37°C. The peptides were extracted by washing three times with 20 % m 5% formic acid in 50% acetonitrile for 45 min. The sample was dried in the vacuum centrifuge and stored at −20°C until MALDI-TOF mass spectrometry was performed. The sample was then dissolved in 10 µl of 0.1% trifluoroacetic acid and deionized by repeated passages through a reversed-phase chromatographic column (Ziptip C18) (Millipore Corp., Bedford, MA).

**MALDI-TOF Peptide Mapping**—The matrix used for peptide mapping was a-cyano-4-hydroxycinnamic acid, washed essentially as described by Moore (37). In short, 0.2 m of a-cyano-4-hydroxycinnamic acid powder was suspended in 1.5 ml of acetonitrile, vortexed for 10 s in an Eppendorf tube, and then centrifuged. The supernatant was discarded, and fresh acetonitrile was added; this step was repeated twice. From the
third supernatant, saturated with α-cyano-4-hydroxycinnamic acid, 400 μl were mixed with 200 μl of nitrocellulose (38) dissolved in acetone (20 mg/ml) and 200 μl of isopropyl alcohol, to which was added 1 μl of peptide calibrants (calibration mix 1 from Applied Biosystems). Typically, 2 μl of this matrix solution was mixed with 2 μl of sample in an Eppendorf tube, and 1 μl of the mixture was spotted onto the MALDI sample plate and air-dried. The sample spot was washed once in 5% formic acid and twice in water, for 10 s each time, and air-dried again.

Peptide mass spectra were acquired by MALDI-TOF MS, using a Voyager DE PRO Biospectrometry workstation (Applied Biosystems) operated in linear mode. The accelerating voltage was 20 kV; the guide wire voltage was set to 72%, and the grid voltage was set to 0.002% of the accelerating voltage. The delay time used was 100 ns. For each spectrum, 200 laser shots were averaged. Protein identification was performed by using the Protein Prospector data-mining program (available on the World Wide Web at www.expasy.ch), with a mass accuracy requirement better than 30 ppm.

**Immunofluorescence Microscopy**—Cells incubated for 1 h at 37 °C were washed three times and resuspended in suspension buffer to obtain a cell density of ~1.8 × 10^6 cells/ml. The cells were sedimented (750 rpm for 5 min) onto microscope slides using a Cytospin cytocentrifuge (Shandon Scientific Ltd., Cheshire, UK) before being fixed in 100% methanol for 10 min at –20 °C. The cells were then washed three times with PBS containing 0.3% Triton X-100 (PBS-T; pH 7.5).

For visualization of plectin, the cells were overlaid with monoclonal anti-plectin, diluted 1:40 in PBS-T, for 30 min. The cells were washed three times with PBS-T and overlaid with fluorescein-conjugated antibody, diluted 1:40 in PBS-T, for 30 min in the dark. Finally, the cells were washed three times in PBS-T and twice in H2O and mounted in Mowiol. All of the antibody incubations were performed in a humid chamber at room temperature. The cells were examined in a Nikon Optihot microscope and photographed with Eastman Kodak Co. TMAX 400 film. The percentage of cells with a disrupted plectin organization was quantified by randomly choosing visual fields in the fluorescence microscope and counting 200 cells/microscope slide. The plectin networks were scored as either normal or as disrupted/alttered.

**CaMK-II Assay**—The activity of purified rat brain CaMK-II was measured by means of 32P incorporation into an AutoCam-tide-II substrate, using an assay kit from Upstate Biotechnology, Inc. (Lake Placid, NY).

**RESULTS**

**Okadaic Acid-induced, Naringin-sensitive Phosphorylation of Hepatocellular Proteins**—Protein phosphatase-inhibitory alkaloid toxins like okadaic acid and microcystin have been shown to induce overphosphorylation and disruption of the keratin cytoskeleton in isolated rat hepatocytes and to elicit a slow apoptotic cell death preventable by the grapefruit flavonoid, naringin (8, 21). To identify phosphoproteins that might be involved in mediating these naringin-sensitive toxin effects, freshly isolated rat hepatocytes were treated with various concentrations (0, 15, 30, 60, and 100 nM) of okadaic acid, in the absence or presence of naringin (100 μM). Whole-cell lysates were fractionated by electrophoresis on SDS-10% polyacrylamide gels, and proteins phosphorylated at threonine groups were detected by immunostaining with an antibody directed against phosphothreonine. As shown in Fig. 1A, okadaic acid induced a dose-dependent, naringin-sensitive phosphorylation of a number of hepatocellular proteins in the molecular mass range of 35–200 kDa. The effect of okadaic acid on all of these proteins was detectable at 60 nM; on some, it was detectable even at 15 nM. The efficacy of such low toxin concentrations would seem to implicate inhibition of a type 2A protein phosphatase (1, 2), in agreement with previous studies on okadaic acid effects in isolated rat hepatocytes (8, 21, 39). The antagonistic effect of naringin was most evident at intermediate okadaic acid concentrations (30–60 nM). No effects of okadaic acid (or naringin) were seen on gels stained for total protein with Coomassie Blue (not shown), indicating that the dose-dependent changes in immunostaining intensity reflected protein phosphorylation rather than changes in protein amounts.

Proteins with a molecular mass above 130 kDa were poorly separated on the 10% gels, making it difficult to assess the effects of okadaic acid and naringin in this mass region. In order to obtain better resolution of high molecular weight proteins, the cell lysates were fractionated on more dilute (5%) polyacrylamide gels, and 6 μm urea was included in the gel as well as in the lysate to improve protein solubility. Furthermore, a low running voltage (50–150 V) was used to avoid heating and precipitation of proteins in the gel. By this procedure, we were able to resolve several phosphoprotein bands with molecular masses above 130 kDa (Fig. 1B). One very large (~500-kDa) protein, which exhibited naringin-sensitive phosphorylation even at the lowest okadaic acid dose tested (15 nM), was chosen for further study.

**Mass Spectrometric and Immunological Identification of the ~500-kDa Protein as the Cytolinker Protein, Plectin**—To identify the okadaic acid- and naringin-sensitive high molecular weight protein, the ~500-kDa protein band was cut from a Coomassie Blue-stained gel and prepared for MALDI-TOF MS as described under “Materials and Methods.” After in-gel tryptic digestion, the peptides were extracted and subjected to MALDI-TOF MS, generating a peptide mass map consisting of 97 peptide ion signals (Fig. 2). The spectrum was calibrated using three peaks of known masses derived from the calibration mixture added to the sample. The peptide mass list was used to search the Swiss-Prot data base with an allowed peptide mass error of 30 ppm. No constraints on species or molecular mass were specified.

The retrieved list of candidate proteins placed rat plectin on top, with 39 matching peptides of 97 (Table I). The matching peptides covered 480 of the 4687 amino acid residues in plectin (i.e. a 10% sequence coverage). The calculated molecular mass
of plectin (533.5 kDa) agreed very well with the molecular mass observed in the gel. The next two entries on the list were also plectins, from Chinese hamster and human, respectively. Since both the molecular mass and the species of origin were correctly predicted, and since the next rat protein on the list had only 24 matching peptide masses, we could conclude that our high molecular weight protein had been unequivocally identified as plectin. It should be noted that when no upper mass detection limit can be applied, very large proteins will tend to show a considerable number of peptide matches by coincidence; it is, therefore, important that there be a considerable difference between the first and second protein candidate.

As a verification of the mass spectrometric identification, a set of 5% polyacrylamide gels were immunoblotted with a monoclonal antibody against plectin (Fig. 1C). The anti-plectin antibody specifically stained the 500-kDa protein band, thus supporting the identification of this protein as plectin. It should be noted that when no upper mass detection limit can be applied, very large proteins will tend to show a considerable number of peptide matches by coincidence; it is, therefore, important that there be a considerable difference between the first and second protein candidate.

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**Time Course of Okadaic Acid-induced Plectin Phosphorylation**—To study the onset and time course of okadaic acid-induced plectin phosphorylation, hepatocytes were incubated with 30 nM okadaic acid for various lengths of time (0–60 min). As shown in Fig. 3A, phosphorylation of plectin could be observed already after 3 min. The extent of phosphorylation then increased gradually with time, to a maximum at 45 min. Naringin (100 μM) effectively antagonized the okadaic acid effect at all time points (Fig. 3A).

To rule out the possibility that the observed changes in phosphorylation intensity were caused by changes in plectin concentration, parallel immunoblots were stained with the antibody against plectin. These blots showed no time- or treatment-dependent variation in plectin staining (Fig. 3B), indicating that the effect of okadaic acid in Fig. 3A reflected plectin phosphorylation rather than plectin accumulation. No accumulation of lower molecular weight, putative plectin degradation products was observed after okadaic acid treatment.

**Induction of Plectin Phosphorylation by Various Protein Phosphatase Inhibitors**—Toxins tend to differ in their relative specificity toward protein phosphatases of type 1 and type 2A. By comparing the effects of different toxins on a given process, it may, therefore, be possible to make some inference about the types of phosphatase likely to be involved (39–41). In the case of plectin phosphorylation, not only okadaic acid (Fig. 1B), but also microcystin-LR (3 μM), calyculin A (500 nM), tautomycin (3 μM), and cantharidin (10 μM)
were able to stimulate the phosphorylation markedly (Fig. 4A), without altering the total cellular plectin content (Fig. 4B). The effect of microcystin, like that of okadaic acid, was effectively antagonized by naringin (100 μM). In contrast, naringin had no effect on plectin phosphorylation induced by calyculin A, tautomycin, or cantharidin (Fig. 4A), suggesting that two different modes of phosphorylation are involved. The naringin-sensitive phosphorylation would most likely reflect inhibition of a type 2A phosphatase, given the ability of the PP2A-selective inhibitor, okadaic acid, to induce maximal phosphorylation at a concentration as low as 30 nM (Fig. 1B) and the propensity of microcystin to preferentially inhibit PP2A in intact hepatocytes (42). Calyculin A and cantharidin, on the other hand, seem to act as preferential PP1 inhibitors in intact cells (1, 43). It is therefore conceivable that inhibition of PP1 by calyculin A or cantharidin induces a naringin-resistant plectin phosphorylation that overrides the naringin-sensitive, PP2A-regulated phosphorylation. Tautomycin inhibits purified PP1 and PP2A with equal potency (44) but has been reported to act more like calyculin A than like okadaic acid in intact cells (45).

**Effects of Protein Phosphatase Inhibitors on Plectin-containing Cytoskeletal Elements**—Treatment of rat hepatocytes with okadaic acid or microcystin-LR has previously been shown to induce a naringin-sensitive fragmentation of the keratin intermediate filament network (8, 21). Since plectin serves both as a cross-linking protein within the intermediate filament network and as a cytolinker between intermediate filaments and actin microfilaments (33), it would be of interest to learn how protein phosphatase inhibitors affect the structural organization of plectin in intact hepatocytes.

In untreated hepatocytes, plectin could be visualized by immunostaining as a distinct, uninterrupted outlining of bile canaliculi and as a more diffuse network throughout the cyto-

![Fig. 3](image3.png)

**Fig. 3.** *Time-dependent okadaic acid- and naringin-sensitive phosphorylation of plectin.* Hepatocytes were incubated for up to 60 min at 37 °C with okadaic acid (OA, 30 nM) and/or naringin (Nar, 100 μM) as indicated. The cells were lysed in SDS-containing lysis buffer with 6 M urea, and high molecular weight proteins were separated on a 5% SDS-polyacrylamide gel containing 6 M urea. The gels were blotted, and the membranes were immunostained with a polyclonal phosphothreonine antibody (A) or a monoclonal antibody against plectin (B). Only the part of the gel containing the 500-kDa band is shown.

![Fig. 4](image4.png)

**Fig. 4.** *Phosphorylation of plectin induced by various protein phosphatase inhibitors.* Hepatocytes were incubated for 1 h at 37 °C with microcystin-LR (3 μM), calyculin A (500 nM), tautomycin (3 μM), or cantharidin (10 μM) alone (Inh) or in the presence of naringin (Nar, 100 μM) as indicated. The cells were lysed in SDS-containing lysis buffer with 6 M urea, and high molecular weight proteins were separated on a 5% SDS-polyacrylamide gel containing 6 M urea. The gels were blotted and the membranes immunostained with a polyclonal phosphothreonine antibody (A) or a monoclonal antibody against plectin (B). Only the part of the gel containing the ~500-kDa band is shown.

![Fig. 5](image5.png)

**Fig. 5.** *Effects of protein phosphatase inhibitors on hepatocellular plectin network organization.* Hepatocytes were incubated for 1 h at 37 °C with no addition (control) (A), 30 nM okadaic acid (B), 1 μM microcystin-LR (C), 100 nM calyculin A (D), 1 μM tautomycin (E), or 10 μM cantharidin (F). After incubation, the cells were washed and sedimented onto glass slides, fixed in 100% methanol, and further processed for indirect immunofluorescence staining using a monoclonal antibody against plectin. Bar length, 10 μm.
plasm (Fig. 5A). The staining of the bile canalicular sheath was completely abolished by all protein phosphatase inhibitors tested, and the cytoplasmic plectin network was disrupted (Fig. 5, B–F). Okadaic acid induced the formation of numerous small plectin aggregates (Fig. 5B); the other inhibitors caused the formation of one or a few large aggregates in addition to a diffuse cytoplasmic plectin staining (Fig. 5, C–F).

The fraction of cells with altered plectin organization could be quantified by counting in the fluorescence microscope (Fig. 6). At 30 nM okadaic acid, about 90% of the cells displayed a disrupted plectin cytoskeleton; with other protein phosphatase inhibitors, higher concentrations were needed to induce equivalent changes in plectin organization (100 nM calyculin A or tautomycin, 1 μM microcystin, 10 μM cantharidin). These dose-response characteristics thus provide supporting evidence for an involvement of PP2A in the dephosphorylation of plectin.

The disruption of plectin organization by okadaic acid was effectively antagonized by naringin (Fig. 7, Table II). The effect of microcystin was similarly completely prevented by naringin, in a dose-dependent manner, and tautomycin could be partially antagonized (Table II). However, the network-disruptive effects of calyculin A and cantharidin were unaffected even by the highest naringin concentration (1 mM; Table II).

**Effects of Protein Kinase Inhibitors on the Structural Organization of Plectin**—The stimulation of protein phosphorylation by protein phosphatase inhibitors depends on the protein kinases that actually perform the phosphorylation. Some clues as to the types of kinase involved may be provided by the ability of protein kinase inhibitors to antagonize the effects of the phosphatase inhibitors. Table III shows that the okadaic acid-induced disruption of hepatocytic plectin organization was more or less unaffected by H-7 (100 μM), an inhibitor of protein kinase C (PKC) (46), or by H-89 (20 μM), an inhibitor of cAMP- and cGMP-dependent protein kinases (47). Therefore, none of these kinases would seem to mediate the okadaic acid-induced plectin phosphorylation. KN-62 (10 μM), a specific inhibitor of CaMK-II (48), offered a 25% protection, whereas its inactive analogue, KN-04, was without any significant effect. Olo- moucine (100 μM), an inhibitor of cyclin-dependent protein kinases (CDKs) (49), also offered a 25% protection. Although some CaMK-II or CDK involvement might thus be indicated, the effects of KN-62 and olomoucine could not match that of naringin (86% protection in this series of experiments). Other flavonoids, like the closely related flavanone, prunin (naringenin-7-glucoside) or the isoflavone, genistein, were less effective than naringin (48 and 25% protection, respectively).

To examine the possibility that naringin might exert its okadaic acid antagonism through a particularly effective inhibition of CaMK-II, its effect on purified CaMK-II was compared with that of KT-5926, a CaMK-II inhibitor more potent than KN-62 (50). As shown in Fig. 8, naringin had little or no direct effect on CaMK-II activity at the concentration (100 μM) that produced maximal okadaic acid antagonism in intact cells. In contrast, KT-5926 inhibited CaMK-II completely at 1 μM. The okadaic acid- and microcystin-antagonistic effect of naringin on plectin phosphorylation is thus unlikely to be due to a direct CaMK-II inhibition.

**Identification of Naringin-sensitive Protein Kinases**—We have undertaken a comprehensive proteomic survey to identify some of the many okadaic acid- and naringin-sensitive proteins visualized by phosphoprotein immunoblotting in Fig. 1. Since a successful mass spectrometric identification from one-dimensional gels is only possible in the case of extremely large, well separating proteins like plectin, our general strategy has been to use narrow range isoelectrofocusing and two-dimensional gel separation in combination with immunoblotting, including phosphospecific antibodies (when available) for final identity verification and further studies. Results pertaining to protein
Okadaic acid was found (as were other protein phosphatase inhibitors) to elicit a naringin-sensitive, activating phosphorylation of the AMP-activated protein kinase (AMPK) at Thr^{172} (Fig. 9A), a site specifically phosphorylated by an upstream kinase, AMPKK (51). AICAR, an adenosine analogue that activates both AMPK and AMPKK through intracellular formation of an AMP analogue (51, 52), similarly caused AMPK to be phosphorylated naringin-sensitively (Fig. 9B). Since AMPKK is not itself a phosphoprotein, and thus represents the top kinase in the AMPKK/AMPK signaling pathway (51), it would seem likely to be identical to the postulated naringin-sensitive protein kinase (8, 39).

Several other protein kinases were also found to be subject to an activating phosphorylation after treatment with okadaic acid or AICAR, including the stress-activated protein kinases SEK1 (Fig. 9, C and D) and its downstream substrate (53), JNK (Fig. 9, E and F). Since the phosphorylations of both SEK1 and JNK were naringin-sensitive, these enzymes would seem likely to be signaling elements downstream of AMPKK/AMPK. It should be noted that stress-activated protein kinases usually act as a scaffolded, triadic signaling module (53), thus leaving room for some yet unidentified kinase kinase kinase between AMPK and SEK1 as well as for additional kinases downstream of JNK.

Okadaic acid and AICAR induced a naringin-sensitive phosphorylation of hepatocytic S6 kinase (S6K) in the tail region (Thr^{421}/Ser^{424}) (Fig. 10, A and B), known to relieve an autophosphorylation effect of this domain (54). Paradoxically, AICAR suppressed S6K phosphorylation (induced by amino acids) at Thr^{388}, the major activating site (54) of the enzyme (Fig. 10C), causing it to become inactive in S6 phosphorylation (Fig. 10D). The possibility should thus be considered that S6K may have functions other than S6 phosphorylation, depending on the pattern of phosphorylation. Whether S6K is a downstream element of SEK1/JNK or resides in a different AMPKK-AMPK-dependent signaling pathway remains to be shown. Furthermore, additional studies will be required to identify the protein kinase ultimately responsible for the naringin-sensitive phosphorylation of plectin.

**DISCUSSION**

Plectin was first identified as a major component of cytoskeletal preparations from C6 rat glioma cells (55) and has since been shown to function as a general cross-linker of the cytoskeleton. Plectin interacts with all three major groups of cytoskeletal proteins (i.e., actin filaments (56–58), microtubules (55, 59), and intermediate filaments (60–62)). Okadaic acid and AICAR induced a naringin-sensitive phosphorylation of plectin in isolated rat hepatocytes (Table II), which could be inhibited by several protein kinase inhibitors (Table III). In addition, the effects of other protein phosphatase inhibitors such as norcantharidin, cantharidin, and Tautomycin (Fig. 9) were also investigated. The results indicate that the effects of naringin on the disruption of plectin network organization induced by protein phosphatase inhibitors are consistent with the findings for the effects of naringin on the disruption of plectin network organization induced by protein phosphatase inhibitors.
phospecific antibodies against activated (phospho-)AMPK (Thr172) (A, C, and E) or 1 mM AICAR (B, D, and F), and with naringin at the concentration indicated. The cells were lysed in SDS-containing lysis buffer; the cellular proteins were separated on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. The membranes were immunostained with phos-specific antibodies against phospho-S6K (Thr421/Ser424) (A and B), activated (phospho-)SEK1 (Thr261) (C and D), or activated (phospho-)JNK (Thr183/Tyr185) (E and F). CT, control (no additions).

FIG. 9. Phosphorylation and activation of protein kinases by okadaic acid and AICAR. Hepatocytes were incubated for 1 h at 37 °C with 100 nM okadaic acid (A, C, and E) or 1 mM AICAR (B, D, and F), and with naringin at the concentration indicated. The cells were lysed in SDS-containing lysis buffer, and the cellular proteins were separated on a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes. The membranes were immunostained with phos-specific antibodies against phospho-S6K (Thr421/Ser424) (A and B), activated (phospho-)SEK1 (Thr261) (C and D), or activated (phospho-)JNK (Thr183/Tyr185) (E and F). CT, control (no additions).

There is considerable evidence to indicate that the interactions between plectin and other cytoskeletal proteins are regulated by protein phosphorylation. For example, plectin can be phosphorylated at Thr4542 by the cyclin-dependent protein kinase CDK1/Cdc2 during mitosis, causing it to dissociate from vimentin filaments (64, 65). Since plectin phosphorylation in the present study was detected by an anti-phosphothreonine antibody, it is clear that the toxin-sensitive phosphorylation site must likewise be a threonine. However, the mass spectrometric method used (MALDI-TOF) is not suitable for the identification of phosphorylation sites; furthermore, the sequence coverage of this very large protein was, at 10%, insufficient for site identification.

Other protein kinases can also phosphorylate plectin. When phosphorylated by PKC in interphase cells, plectin dissociates from vimentin filaments, whereas PKA would seem to enhance plectin binding to vimentin (66). The association of plectin with lamin B is broken upon phosphorylation of either binding partner by PKA or PKC (66). Under cell-free conditions, plectin can be phosphorylated by CaMK-II as well as by PKA (67). There is thus no lack of candidate kinases for mediation of toxin-induced plectin phosphorylation. However, in the present study, neither PKA nor PKC inhibitors prevented okadaic acid-induced plectin phosphorylation, and inhibitors of CaMK-II or CDKs were only partially preventive.

The flavonoid, naringin, which is known to antagonize several effects of algal toxins in isolated hepatocytes (8, 21, 39, 68), was a very effective suppressant of okadaic acid- and microcystin-induced plectin phosphorylation. Although naringin’s mechanism of action is not known, inhibition of some protein kinase has been considered likely. Many flavonoids are potent and specific protein kinase inhibitors (cf. for example the inhibition of the mitogen-activated protein kinase kinases MEK1 and MEK2 by PD-98059 (5’-methoxy-6’-aminoflavone) (69), and of cyclin-dependent protein kinases by flavopiridol (70)). The okadaic acid-antagonistic effect of naringin on hepatocytic autophagy can be mimicked by several inhibitors of CaMK-II (71, 72), but as shown in the present study, naringin had no direct inhibitory effect on CaMK-II; nor did it affect PKA or PKC activity in in vitro assays.2

However, by using a proteomic approach in combination with phosphospecific immunoblotting, we were able to identify a number of naringin-sensitive protein phosphorylations in intact hepatocytes. One of these, the activating phosphorylation of AMPK at Thr172, is uniquely performed by the upstream kinase, AMPKK, which is not itself a phosphoprotein and which must, therefore, be the top kinase of its phosphorylation cascade (51). AMPKK is, accordingly, very likely to be the putative naringin-sensitive protein kinase, an assumption strengthened by the ability of the AMPKK/AMPK activator, AICAR, to mimic the effects of okadaic acid as well as to be antagonized by naringin.

The AMPKK/AMPK duo is a sensitive sensor of energy charge and metabolic stress (73) and could well be involved in a toxic stress response. It has been suggested that AMPK may signal through the stress-activated kinase, p38 (74); it would thus not seem unreasonable to assume that SEK1 and JNK, which are both phosphorylated naringin-sensitively, could be downstream mediators of AMPPK/AMPK signaling in hepato-

2 A.-K. R. Larsen, M. T. N. Møller, H. Blankson, H. R. Samari, L. Holden, and P. O. Seglen, unpublished results.
cytes. It is too early to tell whether S6K, phosphorylated naringin-sensitively at Thr423/Ser424 in its tail region, belongs in the same signaling pathway, but its ability to be phosphorylated in rat cardiomyocytes by a stress-activated pathway involving p38 (75) is clearly compatible with a position downstream of JNK in hepatocytes. The fact that AICAR stimulated tail phosphorylation while inhibiting the catalytic activity of S6K might suggest a dual function of the latter but would more likely indicate an AMPKK/AMPK-independent inhibitory effect of AICAR on S6K.

Interestingly, naringin and okadaic acid (39), AICAR/AMPK (52), stress-activated protein kinases (76), and S6 (77) have all been implicated in the regulation of hepatocytic autophagy, perhaps suggesting a regulatory or even causal connection between the putative AMPKK/AMPK/SEK1/JNK/S6K signaling pathway, autophagy, and cytoskeletal elements like plectin and keratin (21). Any of the kinases in this pathway could, in principle, be the effector of plectin phosphorylation. S6K has been shown to be capable of keratin phosphorylation, but only under cell-free conditions (78), whereas stress-activated protein kinases have been implicated in cytoskeletal phosphorylations (p38-induced actin rearrangements) in intact cells (79, 80). Keratin phosphorylation can be stimulated both by AMPK under cell-free conditions and by AICAR in intact hepatocytes (81), suggesting that intracellular cytoskeleton phosphorylations may in fact be carried out directly by AMPK.

Some information about the protein phosphatase(s) involved in plectin dephosphorylation can be obtained by comparing the effects of different phosphatase-inhibitory toxins. The toxins examined in the present study differ in their absolute as well as their relative potencies toward PP1 and PP2A, yet all of them induced plectin phosphorylation as well as fragmentation of the plectin network. Okadaic acid is readily taken up by isolated hepatocytes (72), and its high absolute potency with regard to plectin phosphorylation and network disruption would be strongly indicative of a PP2A involvement, its affinity for PP1 being 2 orders of magnitude lower (1, 2). Microcystin also behaves as a selective intracellular PP2A inhibitor by virtue of its specific binding to PP2A (42), although under cell-free conditions it is an equally potent inhibitor of PP1 (42, 82). Unfortunately, the slow cellular uptake of microcystin (72), being dependent on the bile acid transport system (83, 84), renders its absolute in-cell potency low and nondistinctive.

Calycin A is a more potent inhibitor of PP1 than of PP2A under cell-free conditions (40), and in intact hepatocytes it inhibits PP1 10 times as potently as does okadaic acid (85). The fact that calycin A was no more potent than okadaic acid as a plectin network disruptant therefore supports an involvement of PP2A in plectin dephosphorylation. Okadaic acid has previously been shown to be 10 times as potent as calycin A in suppressing hepatocytic autophagy (72) and endocytosis (86), despite the similar uptake (72) and PP2A affinity (40, 85) of the two toxins. It is, therefore, possible that calycin A acts as a preferential PP1 inhibitor in intact cells (e.g. due to PP1 binding, as appears to be the case with cantharidin and its derivatives (43)). Tautomycyn has similar affinities for PP1 and PP2A (44) but seems to have calycin-like effects in intact cells (45). The plectin phosphorylation and network disruption induced by calycin A, cantharidin, and tautomycyn could, therefore, reflect the engagement of PP1 as well as PP2A in plectin dephosphorylation.

The various protein phosphatase-inhibitory toxins differed strikingly in their ability to be antagonized by naringin. Whereas naringin suppressed okadaic acid- and microcystin-induced plectin phosphorylation and offered virtually complete protection against their network-disrupting effects, it did not antagonize calycin A or cantharidin detectably and antagonized tautomycyn only moderately. The inhibitory effects of calycin A could be reversed by toxin washout, ruling out the possibility that naringin resistance was caused by irreversible phosphatase inhibition. Differential naringin sensitivity would rather seem to correlate with the relative protein phosphatase-inhibitory effects of the toxins: naringin antagonized the PP2A inhibitors but not the putative PP1 inhibitors. As a working hypothesis, it would thus seem reasonable to assume that plectin phosphorylation/dephosphorylation is controlled by two different mechanisms: a naringin-sensitive, PP2A-regulated mechanism, and a naringin-resistant, PP1-regulated mechanism. How these two putative phosphorylation pathways might be organized relative to each other and relative to plectin can only be a matter of conjecture. The toxin-induced phosphorylation of keratins (8) and of various other hepatocellular proteins displays a similar differential naringin sensitivity, suggesting a pathway duality upstream of plectin itself.

A naringin-sensitive, PP2A-regulated mechanism has been shown to be involved in a slow apoptotic process elicited by low concentrations of okadaic acid or microcystin and culminating in hepatocytic death 15–24 h later (8). Higher toxin doses, which may cause the additional inhibition of PP1, induce a more rapid apoptosis, with characteristic morphological changes observable within a few minutes (11). A number of different protein kinases have been suggested to be involved as mediators of okadaic acid toxicity, including PKA (12), CaMK-II (13), CDKs (14), and mitogen-activated protein kinases (6). As discussed above, PKA, CaMK-II, and CDKs have also been implicated in plectin phosphorylation. Plectin phosphorylation and plectin network disintegration could thus well be early, possibly causative, aspects of the slow, toxin-induced apoptosis. In colon carcinoma cells, caspase-mediated keratin cleavage during apoptosis was preceded by, but apparently not dependent on, keratin hyperphosphorylation (87). In rat hepatocytes, toxin-induced apoptosis was similarly preceded by keratin hyperphosphorylation and network disruption, in parallel with the plectin changes (3, 8, 21). Plectin is sensitive to endoproteolytic cleavage by calpain (88) or caspases (89) and was found to be cleaved at an early stage of receptor-mediated apoptosis in mammary carcinoma cells (89) and rat pancreatic acini (90). In the present study, however, no plectin cleavage fragments were seen on immunostained gels during the first hours after toxin treatment of hepatocytes, using an antibody capable of detecting such fragments (90). The plectin network disintegration that occurred during the treatment period would thus be more likely to be due to the rapid plectin phosphorylation than to caspase-induced cleavage. Whether the toxin-induced plectin phosphorylation demonstrated in the present study is also contributing causatively to the disintegration of the hepatocellular keratin cytoskeleton (21) and the bile canalicular sheaths (3), and eventually to hepatocyte apoptosis (8), will have to be the subject of future research.

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