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Cyanobacterial Nodularin Is a Potent Inhibitor of Type 1 and Type 2A Protein Phosphatases

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SUMMARY

The present study characterizes the inhibitory effects of nodularin, a recently isolated hepatotoxic compound from the cyanobacterium *Nodularia spumigena*, on type 1 (PP1), type 2A, (PP2A), type 2B (PP2B), and type 2C (PP2C) protein phosphatases. Both PP2A and PP1 were potently inhibited (IC_{50} = 0.026 and 1.8 nM, respectively) by nodularin, whereas PP2B was inhibited to a lesser extent (IC_{50} = 8.7 μM). Nodularin had no apparent effect on PP2C, alkaline phosphatase, acid phosphatase, insulin receptor tyrosine kinase, protein kinase A, phosphorylase kinase, or protein kinase C. In a whole-cell extract of T51B liver cells, nodularin inhibited PP1 and PP2A activity with a potency similar to that seen with their purified catalytic subunits. Thus, due to the high specificity of nodularin for PP2A and PP1, this hepatotoxin may prove to be useful as a probe for distinguishing the activity of these protein phosphatases in cell extracts.

Around the world, numerous wildlife and livestock deaths have been attributed to blooms of fresh water cyanobacteria (blue green algae). *Nodularia spumigena* was the first cyanobacterium to be implicated in animals deaths in 1878 (1); however, the hepatotoxin (nodularin) responsible for death was not characterized and its structure elucidated for more than a century (2, 3). Nodularin (Fig. 1) is a cyclic pentapeptide with the structure cyclo-(D-β-methyliso-Asp-L-Arg-Adda-D-iso-Glu-*N*-methyldehydrobutyrine), Adda being a unique β amino acid, (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(*E*),6(*E*)-decadienoic acid (3).

Recently, Fujiki and co-workers (4, 5) in Japan found that, in a cytosolic fraction of mouse liver, both nodularin and the hepatotoxic cyclic heptapeptides known as microcystins caused an apparent increase in protein phosphorylation, a decrease in *in vitro* phosphatase activity, and an inhibition of the specific binding of okadaic acid to its "receptor(s)." Because okadaic acid, a non-phorbol ester tumor promoter (6) produced by dinoflagellates such as *Prorocentrum lima*, is a potent inhibitor of PP1 and PP2A (7-9), the studies in Japan (4, 5) suggested that nodularin and microcystins might also be inhibitors of PP1 and PP2A.

We, and others, have recently demonstrated that, like the polyether fatty acid okadaic acid, microcystin-LR is a potent and specific inhibitor of PP1 and PP2A (10, 11). Here we report that nodularin, which contains the unique β C_{20} amino acid Adda, common to all the hepatotoxic microcystins (3), is also a potent inhibitor of both PP1 and PP2A, is a weak inhibitor of PP2B, and has no apparent effect on PP2C or a variety of other phosphatases and protein kinases.

Experimental Procedures

Materials. Phosphorylase kinase (EC 2.7.1.38), protein kinase A (3′:5′-cyclic AMP-dependent protein kinase), and phosphorylase *b* (EC 2.4.1.1) from rabbit muscle, crude histone (type 2AS), PP2B (calcineurin) and calmodulin (phosphodiesterase 3′:5′-cyclic nucleotide activator) from bovine brain, and PNPP were obtained from Sigma Chemical Co. Nodularin was purified as described by Carmichael *et al.* (2) and can now be obtained from CalBiochem.

Preparation of phosphoprotein substrates. [^{32}P]Phosphorylase *a* from rabbit muscle was prepared essentially according to the methods of Brautigan and Shriner (12), using 30 mg of phosphorylase *b*, 1.36 mCi of [γ-^{32}P]ATP (to give 1 × 10^4 cpm/pmol), and 100 units of phosphorylase kinase. The phosphorylation reaction was carried out for 1.5 hr at pH 8.2 and 30°. After termination of the reaction, phosphorylase *a* was crystallized by adjustment of the pH to 6.8 and placing of the mixture on ice. The crystals were collected by centrifu-

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ABBREVIATIONS: PP1, protein phosphatase type 1; PP2, protein phosphatase type 2; PNPP, *p*-nitrophenyl phosphate; DTT, dithiothreitol; TCA, trichloroacetic acid.

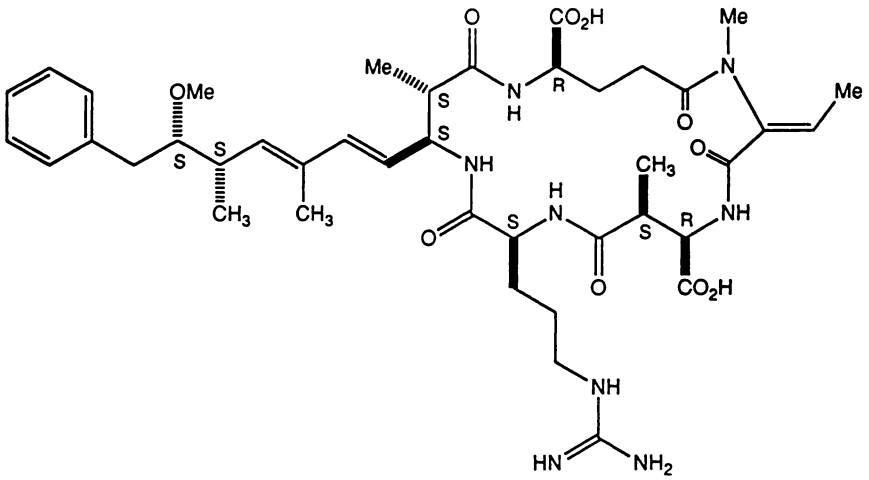

Fig. 1. Structure of nodularin from *N. spumigena*.

gation and washed extensively (more than five times) with 10 ml of 20 mM Tris·HCl, 50 mM 2-mercaptoethanol, pH 6.8 (buffer A). After washing, the crystals were dissolved by the addition of 100 mM NaCl and heating to 30°. This solution was then passed through a column of activated charcoal and Sephadex G-25 fine (12). The collected protein solution was dialyzed overnight against 4 liters of buffer A containing 15 g of activated charcoal. Phosphorylase *a*, which had crystallized overnight, was then redissolved in buffer A containing 100 mM NaCl, passed through a second charcoal-Sephadex G-25 fine column, and recrystallized by dialysis against 2 liters of buffer A. The crystals produced upon dialysis were then redissolved in assay buffer containing 100 mM NaCl (see below) and used immediately (phosphorylase *a* cannot be frozen as crystals or in assay buffer). This results in phosphorylase *a* with a specific activity of ~5.9 × 10^6 cpm/nmol of incorporated phosphate.

Histone (type 2AS from Sigma) was phosphorylated with cAMP-dependent protein kinase (protein kinase A) (13). Briefly, 20 mg of histone were incubated with 1 mg of protein kinase A, in a 20 mM Tris·HCl buffer (pH 7.4) containing 1 mCi of [γ-^{32}P]ATP (150 μM ATP), 100 μM cAMP, 5 mM DTT, and 5 mM $MgCl_2$, in a final volume of 4 ml. The reaction was allowed to continue for 3.5–4 hr at 30° and was terminated by the addition of 1.3 ml of 100% TCA. The precipitated phosphohistone was collected by centrifugation at 3000 × *g* for 5 min. The supernatant was discarded, and the pellet was redissolved in 4 ml of H_2O. TCA (1.3 ml) was added to precipitate the phosphohistone, and this precipitation-resuspension wash was repeated five times. The pellet produced upon the sixth TCA precipitation was washed two times with 4 ml of ethanol/ethyl ether (1:4, v/v) and then two additional times with acidified ethanol/ethyl ether (1:4, 0.1 N HCl) (14). The washed histone was allowed to air dry and was then resuspended in 5 mM Tris·HCl (pH 7.4). This procedure yields phosphohistone with a specific activity of >4.5 × 10^6 cpm/nmol of incorporated phosphate, which can be frozen.

Purification of PP1, PP2A, and inhibitor 2. The catalytic subunit of PP1 was purified to apparent homogeneity, demonstrating a single band upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis and intense silver staining, essentially according to the methods of Brautigan *et al.* (15). The catalytic subunit of PP2A was purified to apparent homogeneity as described by Pallas *et al.* (16), using G-75 Sephadex in the place of Ultrogel-AcA44, as previously reported (15). Inhibitor 2 was purified from rabbit muscle according to the methods of Cohen *et al.* (17).

Determination of phosphatase activity. Phosphatase activity against phosphorylase *a* or phosphohistone was determined by the liberation of ^{32}P. Assays (80-μl total volume), containing 50 mM Tris·HCl, pH 7.4, 0.5 mM DTT, 1 mM EDTA (assay buffer), and ^{32}P-phosphoprotein (1–2 μM PO_4), were conducted as described by Zwiller *et al.* (13), using either the purified catalytic subunits of the protein phosphatases (Figs. 2 and 3) or a whole-cell homogenate of T51B liver cells (Fig. 4). Dephosphorylation reactions were routinely conducted for 5–10 min using phosphorylase *a* as a substrate and for 10–20 min using phosphohistone; the longer assays were employed to determine the IC_{50} of nodularin for PP2A, due to the necessity of using such dilute enzyme solutions (see below). In either case, the dephosphorylation of substrate was kept to <10% of the total phosphorylated substrate, and the reaction was linear with respect to enzyme concentration and time. [^{32}P]Phosphate liberated by the enzymes was extracted as a phosphomolybdate complex and measured according to the methods of Killilea *et al.* (18). Inhibition of phosphatase activity by nodularin, okadaic acid, or inhibitor 2 was determined by addition of the inhibitors to the enzyme mixture 10 min before initiation of the reaction with the addition of substrate.

PP2B, alkaline phosphatase, and potato acid phosphatase activities were measured using PNPP as a substrate. PP2B activity was assessed in the presence of Ca^{2+} and calmodulin (7). The assay (180-μl total volume), containing 50 mM Tris base, pH 8.5, 20 mM $MgCl_2$, 0.25 mM DTT, 0.2 μM calmodulin, 0.1 mM $CaCl_2$, and 20 mM PNPP, was conducted at 30° for 15 min. The reaction was terminated by the addition of 900 μl of 1 M Na_2CO_3, pH 10, and change in absorbance, measured at 410 nm, was used to calculate phosphatase activity. Acid and alkaline phosphatase assays were conducted in a similar manner, omitting the calmodulin and $CaCl_2$ at pH of 4.8 and 10.5, respectively.

The activity of PP2C was estimated from the okadaic acid-insensitive, Mg^{2+}-dependent protein phosphatase activity contained in a dilute whole-cell homogenate of T51B liver cells, using phosphorylase *a* or phosphohistone as a substrate, essentially according to the methods of Cohen *et al.* (8, 9).

Protein kinase preparation. Protein kinase C was prepared essentially as described by Zwiller *et al.* (19). Serine/threonine kinase assays were performed as described by Zwiller *et al.* (19). Insulin receptor tyrosine kinase activity was determined according to the methods described by Rosen *et al.* (20), with human placenta insulin receptors being prepared as described by Le Marchand-Brustel *et al.* (21).

Results

The dephosphorylation of phosphohistone and phosphorylase *a* by the purified catalytic subunits of PP1 and PP2A from rabbit muscle is potently inhibited by nodularin (Figs. 2 and 3). However, when the concentration of enzyme in the assay exceeds that of nodularin, the IC_{50} of PP2A, and to a lesser extent that of PP1, appears to shift with dilution (Fig. 2). This "shift in IC_{50}" appears to be caused by the titration of nodularin from the assay by the catalytic subunit of the phosphatases, because it is not observed in assays containing very low concentrations of enzyme and it is also observed with microcystin-

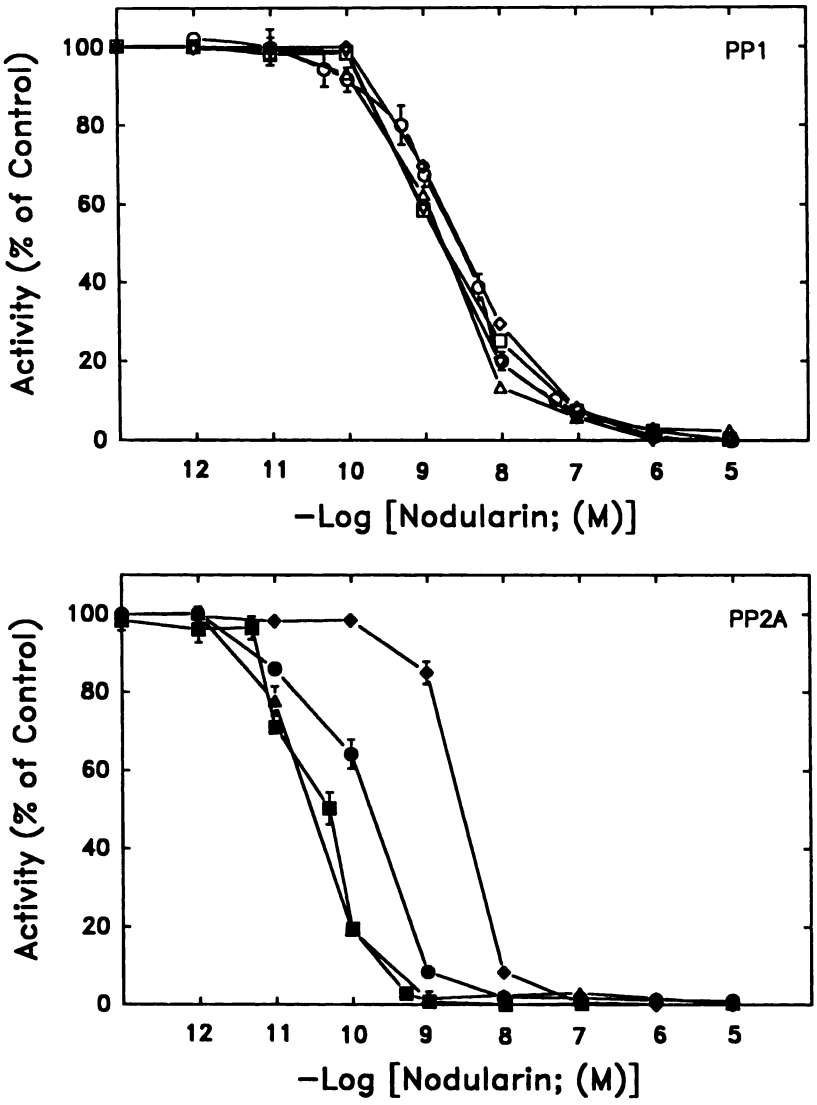

Fig. 2. Effect of enzyme concentration on the inhibition of purified catalytic subunits of PP1 and PP2A by nodularin. Phosphatase activity was determined with phosphohistone as substrate (see Experimental Procedures for details). Phosphohistone phosphatase activity was 250 ± 13 and 1196 ± 54 nmol/min/mg of protein for PP1 and PP2A, respectively. Phosphatase activities of undiluted (◇) and 5- (○), 20- (□), 35- (▽), and 50-fold (△) diluted PP1 and undiluted (◆) and 10- (●), 35- (■), and 50-fold (▲) diluted PP2A are shown. The initial concentration of PP2A and PP1 before dilution was 0.95 and 4.5 nM, respectively. The activity is expressed as percentage of maximal activity ± standard deviation (six experiments).

LR (10) and okadaic acid (9). Once diluted below the titration endpoint, the concentration of enzyme after which further dilution no longer affects the IC_{50}, nodularin inhibits the activity of PP1, PP2A, and PP2B in a dose-dependent manner, being a ~68-fold more potent inhibitor of PP2A than of PP1 and a >4000 fold more potent inhibitor of PP1 than PP2B (Fig. 3; Table 1). Furthermore, under identical conditions, nodularin inhibits both PP1 and PP2A >10 fold more potently than does okadaic acid (IC_{50}, 42 and 0.5 nM, respectively) (Fig. 3).

The activity of PP2C in a crude cell homogenate can be estimated by assessing phosphatase activity in the presence of 5 μM okadaic acid, using either phosphohistone or phosphorylase *a* as a substrate; PP2C activity is that which is Mg^{2+} dependent and not sensitive to 5 μM okadaic acid (8–10). Addition of 20 mM Mg^{2+} to a dilute homogenate of T51B liver epithelial cells causes a ~5% increase in total phosphatase activity, which is not sensitive to okadaic acid (10). This increase is not inhibited by 10 μM nodularin (Fig. 4, *inset*), suggesting that PP2C activity is insensitive to nodularin. Nodularin also has no apparent effect on the activity of acid phosphatase, alkaline phosphatase, phosphorylase kinase, protein kinase A, protein kinase C (Table 2), or insulin receptor tyrosine kinase (data not shown).

To determine whether the potency of nodularin on the native forms of PP2A and PP1 is comparable to that on their respective purified catalytic subunits, we examined the effects of nodularin in a whole-cell homogenate of T51B liver epithelial cells. Fig. 4 shows the inhibitory dose response of nodularin and okadaic acid in a homogenate of T51B cells diluted past the titration endpoint (~59 μg of protein/ml). Nodularin (Fig. 4A) and okadaic acid (Fig. 4B) both potently inhibit the divalent cation-independent (type 1 and 2A) phosphatase activity in the whole-cell homogenate, with 0.1 μM nodularin and 10 μM okadaic acid causing 100% inhibition. Nonlinear regression analysis of the curves (22) indicates that the inhibition of phosphatase activity in the T51B cell homogenate by both nodularin and okadaic acid best fits a two-site model, with 52.7 and 43.4% of the activity associated with a high affinity (PP2A) site (site I) and 47.3 and 56.6% of the activity associated with a low affinity (PP1) site (site II) for nodularin and okadaic acid, respectively. Estimates of the IC_{50} for sites I and II are 8.9 and 950 pM, respectively, for nodularin and 0.1 and 25 nM, respectively, for okadaic acid. These values are slightly lower

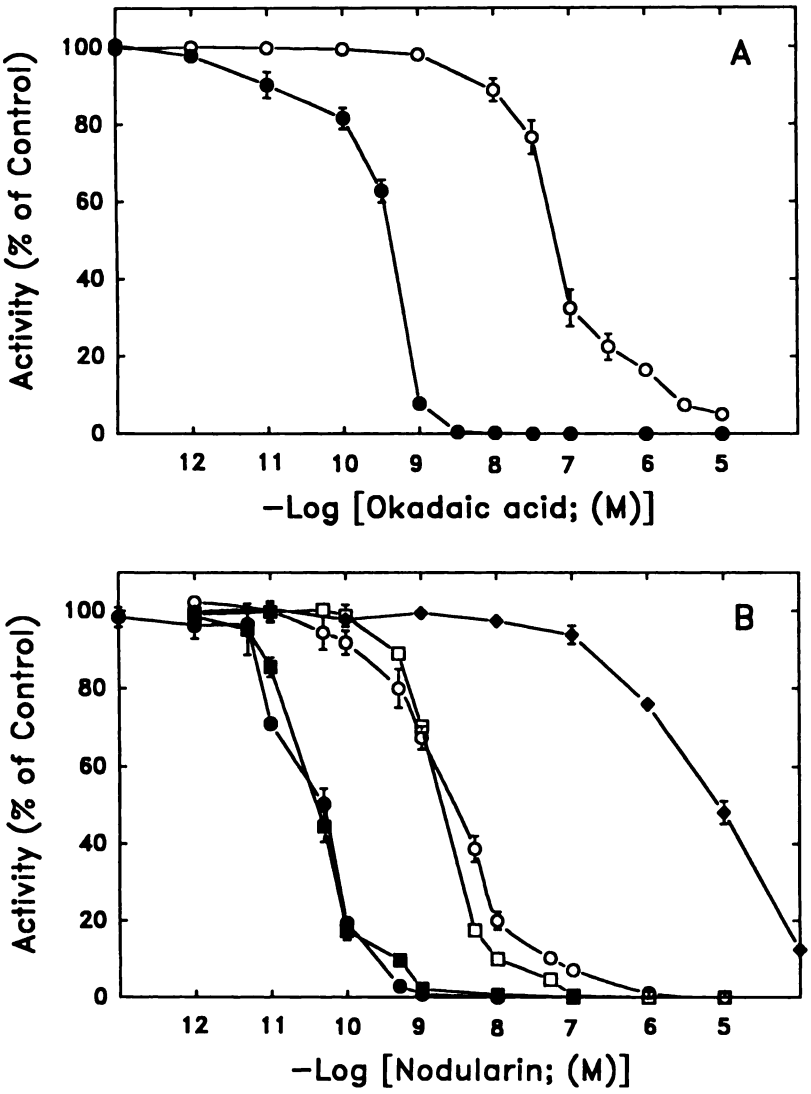

Fig. 3. Effect of okadaic acid (A) and nodularin on the activity of PP1, PP2A, and PP2B. Assays were conducted with enzymes diluted below the titration endpoint (see text; i.e., ~20 pM PP2A and 125 pM PP1) and were conducted as described in Fig. 2, using phosphohistone (○, ●), phosphorylase *a* (□, ■), or PNPP (♦) as substrate. Nodularin was mixed with the enzymes at 23° 5 min before the addition of substrate to start the assay. Phosphatase activity of PP1 (○, □), PP2A (●, ■), and PP2B (♦) is expressed as the mean ± standard deviation (four experiments).

TABLE 1

Relative potency of nodularin for the inhibition of PP1, PP2A, and PP2B activity

Phosphatase activity was assessed with either phosphorylase *a*, PNPP, or phosphohistone as substrate, and assays were conducted as described in Experimental Procedures. Each point represents the mean ± standard deviation (four experiments) from a single phosphatase preparation and is representative of at least three preparations.

Phosphatase	Substrate	Specific activity	IC_{50}
		nmol of P_i/min/mg of protein	*nM*
PP1	Phosphorylase *a*	4200 ± 250	1.6 ± 0.8
PP1	Phosphohistone	250 ± 13	2.1 ± 0.9
PP2A	Phosphorylase *a*	960 ± 44	0.033 ± 0.01
PP2A	Phosphohistone	1930 ± 41	0.021 ± 0.005
PP2B	PNPP	290 ± 38	8700 ± 200

than the IC_{50} values obtained with nodularin or okadaic acid using the purified catalytic subunits of PP2A and PP1 (Fig. 3; Table 2).

If site I truly represents the activity of PP2A and site II that of PP1, then the addition of inhibitor 2 (a heat-stable cytosolic protein that completely inhibits the activity of PP1 at a dose of 100 nM) (23) should cause a decrease in activity comparable

TABLE 2

Effect of nodularin on the activity of various protein kinases and phosphatases

Phosphatase and kinase reactions were performed as described in Experimental Procedures. Protein kinase C and cAMP-dependent protein kinase (protein kinase A) activities were determined with histone, phosphorylase kinase with phosphorylase *b*, and acid phosphatase and alkaline phosphatase with PNPP as substrate. Assays were conducted at 30° in the absence or presence of the indicated concentration of nodularin. Activity is expressed as the mean ± standard deviation (four experiments).

Enzyme	Additions	Specific activity
		nmol/min/mg of protein
Alkaline phosphatase	None	6242 ± 223
Alkaline phosphatase	Nodularin (1 μM)	6239 ± 334
Acid phosphatase	None	3725 ± 198
Acid phosphatase	Nodularin (1 μM)	3889 ± 321
Protein kinase C	None	7.24 ± 1.34
Protein kinase C	Nodularin (1 μM)	6.66 ± 1.24
Protein kinase A	None	1.61 ± 0.14
Protein kinase A	Nodularin (1 μM)	1.73 ± 0.12
Phosphorylase kinase	None	0.48 ± 0.01
Phosphorylase kinase	Nodularin (1 μM)	0.47 ± 0.02

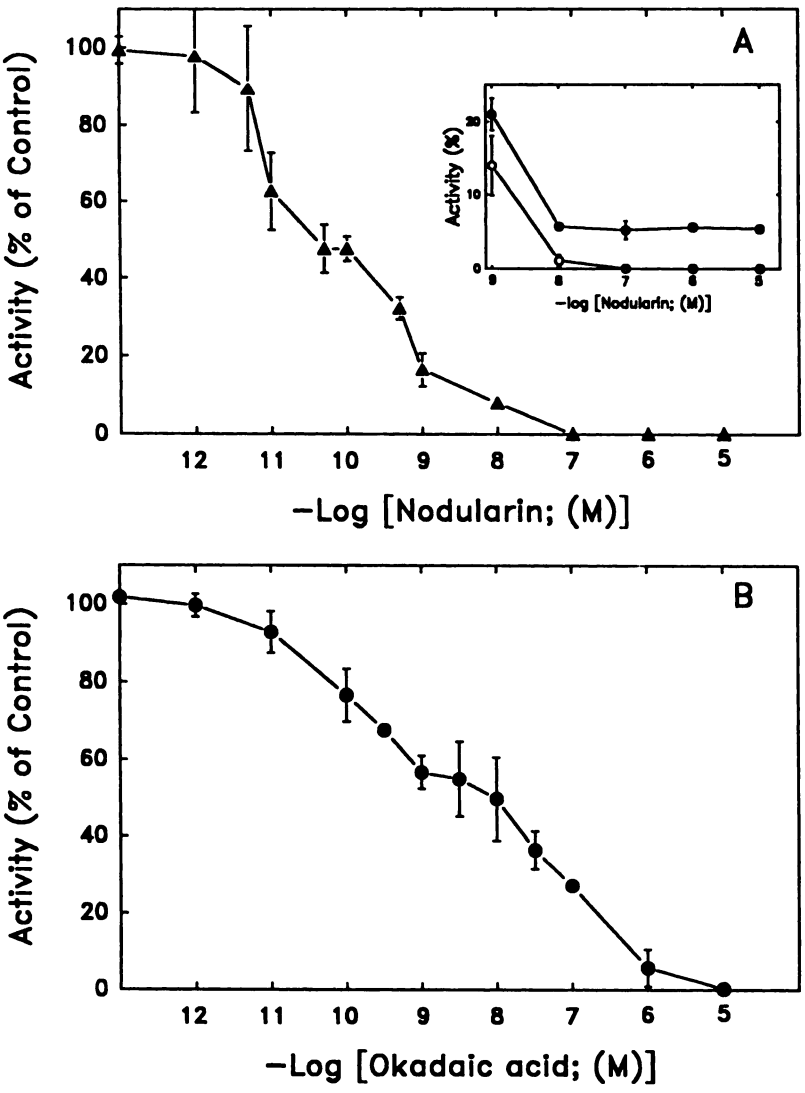

Fig. 4. Effect of nodularin on protein phosphatase activity in a whole-cell homogenate of T51B rat liver epithelial cells. One-day post-confluent cells from a single 60-mm dish were rinsed with phosphate-buffered saline at 4°, scraped, and homogenized in 1 ml of Tris buffer (20 mM Tris·HCl, 1 mM EDTA, 2 mM DTT, pH 7.4). This homogenate was then diluted until the titration endpoint was reached (~59 μg of protein/ml), and the inhibitory effect of nodularin (A) or okadaic acid (B) was assessed using phosphohistone as substrate, as described in Fig. 2. Each point represents the mean ± standard deviation of four experiments, each conducted in triplicate. A, *Inset*, effect of nodularin on phosphatase activity in the absence (○) or the presence (●) of 20 mM $MgCl_2$.

to that contained in site II. As shown in Table 3, this was not the case. The addition of 100–300 nM inhibitor 2 causes a decrease in total divalent cation-independent protein phosphatase activity of ~25%. This compares with an estimate of ~53% and 44% for site II obtained with nodularin and okadaic acid, respectively. Further, the addition of 100 nM inhibitor 2 and either 0.1 nM nodularin or 1.0 nM okadaic acid (doses that maximize the inhibition of the purified catalytic subunit of PP2A while causing minimal inhibition of the activity of the purified catalytic subunit of PP1) causes a decrease in activity of 70.6 and 79.6%, respectively (Table 3). Thus, the phosphatase activity associated with site II appears to be due to the actions of PP1, some PP2A, and possibly some other divalent cation-independent protein phosphatase(s) that is insensitive to inhibitor 2 but sensitive to nodularin and okadaic acid. Alternatively, there may be interaction between inhibitor 2, nodularin, and the native form of PP1 or PP2A that affects the concentrations of nodularin and okadaic acid that inhibit protein phosphatase activity.

Discussion

This study describes and characterizes nodularin, a hepatotoxic cyclic pentapeptide produced by *N. spumigena*, as a potent

TABLE 3

Comparison of the effects of nodularin, okadaic acid, and inhibitor 2 on the activity of the purified catalytic subunits of PP1 and PP2A and on the divalent cation-independent protein phosphatases contained in a homogenate of T51B liver cells

Phosphatase activity was assessed in the absence of divalent cations, as described in Experimental Procedures, with phosphohistone as a substrate. Each point represents the mean ± standard deviation (six experiments).

Inhibitor	Phosphatase activity		
	PP1	PP2A	T51B homogenate
	% of control		
Nodularin, 0.1 nM	91.8 ± 8.6	19.4 ± 3.4	47.5 ± 3.2
Okadaic acid, 1.0 nM	98.0 ± 2.0	7.9 ± 1.0	56.0 ± 4.3
Inhibitor 2			
100 nM	2.0 ± 1.1	99.1 ± 0.9	76.5 ± 5.7
300 nM	ND[a]	ND	74.7 ± 7.8
Inhibitor 2, 100 nM, + nodularin, 0.1 nM	1.9 ± 0.9	17.6 ± 5.2	29.4 ± 4.1
Inhibitor 2, 100 nM, + okadaic acid, 1.0 nM	2.1 ± 0.9	5.7 ± 2.2	20.4 ± 4.7

[a] ND, not determined.

inhibitor of PP1 and PP2A and a weak inhibitor of the Ca^{2+}/calmodulin-dependent phosphatase PP2B (Fig. 3; Table 1). In contrast, up to 10 μM nodularin has no apparent effect on the Mg^{2+}-dependent protein phosphatase PP2C, acid phosphatase, alkaline phosphatase, cAMP-dependent protein kinase, protein kinase C, phosphorylase kinase, or the insulin receptor tyrosine kinase (Fig. 4; Table 2).

The effects of nodularin appear to be enzyme and not substrate directed, in that with the three substrates tested, phosphohistone, phosphorylase *a* (Fig. 3), and PNPP (data not shown), the type of substrate has no apparent effect on the inhibitory actions of nodularin. With PP1 and PP2A, essentially identical concentration-inhibition curves were obtained using phosphohistone or phosphorylase *a* as substrates (Fig. 3; Table 1).

The inhibitory effects of nodularin differ substantially among the nodularin-sensitive phosphatases. The catalytic subunit of PP2A from rabbit muscle is inhibited ~68 times more strongly than the catalytic subunit of PP1, even though the catalytic subunits of PP1 and PP2A have almost 41% sequence identity (24). Estimates of the amount of phosphatase present in these assays, 19 and 90 pM for PP2A and PP1, respectively, indicate that the inhibition of PP2A activity occurs at a concentration of nodularin that is only slightly higher than the concentration of PP2A in the assay. This suggests there is a very strong, perhaps stoichiometric, interaction between nodularin and the catalytic subunit of PP2A. The inhibitory effect of nodularin on PP2B is much lower than that on either PP1 or PP2A (Fig. 3; Table 1).

In addition to nodularin, to our knowledge only four other compounds that specifically inhibit serine/threonine phosphatases have been reported in the literature, 1) okadaic acid, a polyether carboxylic acid produced by *P. lima* and other marine dinoflagelates (8), 2) acanthifolicin, which has a structure identical to that of okadaic acid, except that acanthifolicin contains a episulfide in the place of the double bond at carbons 9–10 in okadaic acid (25, 26), 3) calyculin A, a novel spiro ketal obtained from the marine sponge *Discodermia calyx* (27, 28), and 4) microcystin-LR, a cyclic heptapeptide produced by most strains of *Microcystis aeruginosa* (10, 11); two endogenous proteins, inhibitor 1 and inhibitor 2, are also specific inhibitors of PP1 (23). The inhibitory profile of nodularin is similar to that of okadaic acid and microcystin-LR, with all displaying a greater potency against PP2A than PP1 and all being very weak inhibitors of PP2B. Calyculin A appears to have an equal potency against PP2A and PP1 (28), and its effects on PP2B and PP2C have not been reported, to our knowledge.

In our hands, okadaic acid has an IC_{50} of 0.51 and 42 nM for PP2A and PP1, respectively (Fig. 3) (10). Thus, under the same conditions, nodularin is a ~20-fold more potent inhibitor of both PP1 and PP2A than is okadaic acid (Fig. 3; Table 1). The relative potencies of microcystin-LR for PP2A and PP1 (IC_{50} of 0.04 and 1.7 nM, respectively) (10) are very similar to those of nodularin (Table 1), and the reported IC_{50} for calyculin A for both PP1 and PP2A ranges from 0.3 to 0.7 nM (28), suggesting that both nodularin and microcystin-LR (10) are slightly less potent inhibitors of PP1 and ~10-fold more potent inhibitors of PP2A than is calyculin A.

The level of protein phosphorylation in cells is dependent on the relative activities of both protein kinases and protein phosphatases. In comparison with protein kinases, however, there have been considerably fewer studies on the functions of serine/threonine protein phosphatases. This is partly due to the lack of specific protein phosphatase inhibitors that can be used as probes. In cell homogenates and crude tissue extracts, calyculin A, microcystin-LR, okadaic acid, and nodularin all appear to inhibit the activity of PP1 and PP2A with potencies similar to those observed in assays using the purified catalytic subunits (Fig. 4) (9–11, 28). However, a simple concentration-inhibition relationship by itself does not appear to be sufficient to attribute the activity contained in an extract to PP2A or PP1 specifically.

As shown in Fig. 4, the divalent cation-independent protein phosphatase activity in a crude homogenate of T51B liver cells is completely inhibited by nodularin or okadaic acid at a concentration well below that necessary to affect PP2B [i.e., 0.1 and 10 μM for nodularin (Fig. 3) and okadaic acid (7), respectively]. In addition, nonlinear regression analysis of the inhibition curves obtained with both nodularin and okadaic acid seems to suggest that the activity in the extract could be divided into two components, one with an estimated IC_{50} close to that obtained with the purified catalytic subunit of PP2A and the other with an estimated IC_{50} close to that obtained with the purified catalytic subunit of PP1 (see Results). Nonetheless, the addition of 100–300 nM inhibitor 2, a dose that totally inhibits the activity of purified PP1 (Table 3) (23), inhibits only about 50% of the activity predicted to be due to PP1 by nonlinear regression analysis of the curves. A similar observation was made previously by Takai *et al.* (29), using okadaic acid and ileal smooth muscle extracts. Therefore, the "PP1" component identified by the inhibition curves may be composed of more than one cation-independent protein phosphatase. One possibility is that this activity is due to one of the recently cloned serine/threonine protein phosphatases that are similar to PP1 and PP2A but that have not yet been purified and characterized biochemically (i.e., PPX, PPY, and PPZ) (30). Alternatively, nodularin and okadaic acid may interact with the native enzymes or the enzyme-inhibitor 2 complex in a manner that alters the sensitivity of the enzyme to these inhibitors. Considering the structural similarity of nodularin and microcystin-LR, the latter idea may be supported by recent studies of MacKintosh *et al.* (11), which demonstrate that the binding of inhibitors 1 or 2 to PP1 prevents the subsequent binding of microcystin-LR.

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