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Protein Kinase C Is Likely to be Involved in Zoosporogenesis and Maintenance of Flagellar Motility in the Peronosporomycete Zoospores

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The motility of zoospores is critical in the disease cycles of Peronosporomycetes that cause devastating diseases in plants, fishes, vertebrates, and microbes. In the course of screening for secondary metabolites, we found that ethyl acetate extracts of a marine *Streptomyces* sp. strain B5136 rapidly impaired the motility of zoospores of the grapevine downy mildew pathogen *Plasmopara viticola* at 0.1 µg/ml. The active principle in the extracts was identified as staurosporine, a known broad-spectrum inhibitor of protein kinases, including protein kinase C (PKC). In the presence of staurosporine (2 nM), zoospores moved very slowly in their axis or spun in tight circles, instead of displaying straight swimming in a helical fashion. Compounds such as K-252a, K-252b, and K-252c structurally related to staurosporine also impaired the motility of zoospores in a similar manner but at varying doses. Among the 22 known kinase inhibitors tested, the PKC inhibitor chelerythrine was the most potent to arrest the motility of zoospores at concentrations starting from 5 nM. Inhibitors that targeted kinase pathways other than PKC pathways did not practically show any activity in impairing zoospore motility. Interestingly, both staurosporine (5 nM) and chelerythrine (10 nM) also inhibited the release of zoospores from the *P. viticola* sporangia in a dose-dependent manner. In addition, staurosporine completely suppressed downy mildew disease in grapevine leaves at 2 µM, suggesting the potential of small-molecule PKC inhibitors for the control of peronosporomycete phytopathogens. Taken together, these results suggest that PKC is likely to be a key signaling mediator associated with zoosporogenesis and the maintenance of flagellar motility in peronosporomycete zoospores.

The Peronosporomycetes in the kingdom Straminipila are devastating pathogens of plants, animals, fishes, and humans (Baldauf et al. 2000; Dick 2001; Yoon et al. 2002). Several species of this class of microorganisms such as *Phytophthora infestans* and *Plasmopara viticola* are listed among the top 10 economically most important plant pathogens, resulting in multibillion-dollar crop losses worldwide (Agrios 1997). To infect host plants, these pathogens asexually produce characteristic biflagellate (heterokont) motile spores, called zoospores (Judelson and Blanco 2005). Several lines of evidence indicate that these wall-less zoospores locate their hosts guided by the specific chemical signals secreted from the hosts (Horio et al. 1992; Morris and Ward 1992). When zoospores find potential infection sites of the hosts, they stop motility, attach to the host surface using posterior flagella, and rapidly transform to round-shaped cystospores after shedding flagella (Islam et al. 2002a and b). The cystospores then germinate to form germ tubes within 30 to 40 min to invade plant tissues and initiate infection. Some host-specific chemical signals involved in the chemotaxis and subsequent differentiations of zoospores have already been discovered (Horio et al. 1992; Islam et al. 2003; Morris and Ward 1992). It has been hypothesized that zoospores perceive host signals by a G protein-coupled receptor, which are translated into responses via phosphatidic acid and Ca^{2+}-mediated signaling cascades (Islam and von Tiedemann 2008; Islam et al. 2003; Latijnhouwers et al. 2002). The signal transduction pathways of chemotaxis and differentiation of zoospores are poorly understood (Islam et al. 2002a and b, 2003, 2008; Latijnhouwers et al. 2002, 2004).

The phytopathogenic Peronosporomycete *P. viticola*, that has been prevalent on wild species of *Vitis* in North America, was reported for the first time in Europe in 1878 and, since then, has posed a major threat to vineyards all over the world (Müller and Sleumer 1934). *P. viticola* spreads by an extremely efficient cycle of asexual propagation (Kiefer et al. 2002; Riemann et al. 2002). The success of this obligate biotrophic pathogen can be attributed in part to the speed of asexual differentiation to generate biflagellate motile zoospores that target stomata of grapevine leaves through water films. It is believed that zoospores locate the stomata being guided by the host cues followed by encystment and germination to form germ tubes and then penetrate the leaf surface through stomata (Kiefer et al. 2002). Any disruption of zoospore release from sporangia (zoosporogenesis) or zoospore motility remarkably decreases the potential for pathogenesis (Judelson and Blanco 2005; Judelson and Roberts 2002).

Protein kinase C (PKC) is a key regulatory enzyme in signal transduction pathways governing cellular responses (Nishizuka 1988). The presence of PKC in mammalian sperm and spermatozoa of other vertebrates, including sea urchins, has been demonstrated to function as a key regulator of maintaining motility of these specialized cells (Kalina et al. 1995; Rotem et al. 1990; White et al. 2007). Although motility of zoospores is

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considered critical for pathogen cycles and the virulence in Peronosporomycete phytopathogens, the underlying molecular mechanisms of zoospore motility as well as the process of zoospore release from sporangia are poorly understood (Judelson and Blanco 2005; Judelson and Roberts 2002; Walker and van West 2007). Transformants obtained by silencing *Pigpa1* or *Pibzp1* genes in *Phytophthora infestans* produced zoospores with impaired motility and weak virulence, indicating that both the Gα subunit and protein kinases may be involved in the motility of zoospores (Blanco and Judelson 2005; Latijnhouwers et al. 2004). However, the presence of a specific protein kinase and its role in zoosporogenesis and motility of Peronosporomycete zoospores has not been shown.

Marine microorganisms are known to produce diverse secondary metabolites with potentials to inhibit specific enzymes or proteins in many signaling pathways (Karaman et al. 2008; Sebolt-Leopold and English 2006). We evaluated the effect of secondary metabolites from marine *Streptomyces* spp. on the motility behavior of *Plasmopara viticola* zoospores. Interestingly, we found that ethyl acetate (EtOAc) extracts of the *Streptomyces* sp. strain B 5136 exhibited inhibitory activity against *P. viticola* zoospore motility at a concentration as low as 0.1 µg/ml. The active principle in the extract was identified as staurosporine, which is a known broad-spectrum inhibitor of many protein kinases, including PKC (Tamoki et al. 1986). This significant finding prompted us to conduct a detailed study with the objectives to i) test natural compounds structurally related to staurosporine on the motility behavior of zoospores, ii) evaluate the effects of further known PKC and other kinase inhibitors on the motility of zoospores, iii) test staurosporine and some specific kinase inhibitors on zoosporogenesis, and iv) evaluate the performance of staurosporine on suppression of downy mildew disease development in grapevine leaves.

RESULTS

Motility inhibitory activity in the culture filtrate of marine *Streptomyces* sp. strain B 5136.

We tested extracts of 60 strains of marine *Streptomyces* spp. on the motility behavior of *P. viticola* zoospores using the homogeneous solution method (Islam et al. 2004). Although several extracts caused halting of motility followed by rapid lysis of zoospores, the extracts of strain B 5136 exhibited motility inhibitory activities without causing lysis of the spores at concentrations as low as 0.1 µg/ml. In the presence of B 5136 extract, swimming of zoospores was markedly decreased and most of the zoospores concentrated at the bottom of the dish. Microscopic observation revealed that the affected zoospores swam very slowly or spun continuously in tight circles. They moved in a jerky fashion almost straight along their axis with no lateral motion. Finally, these zoospores stopped moving and transformed into round cystospores within several minutes of treatment, depending on the concentration of the extracts. However, *P. viticola* zoospores in nontreated control dishes displayed the characteristic helical swimming almost following a straight line for several hours.

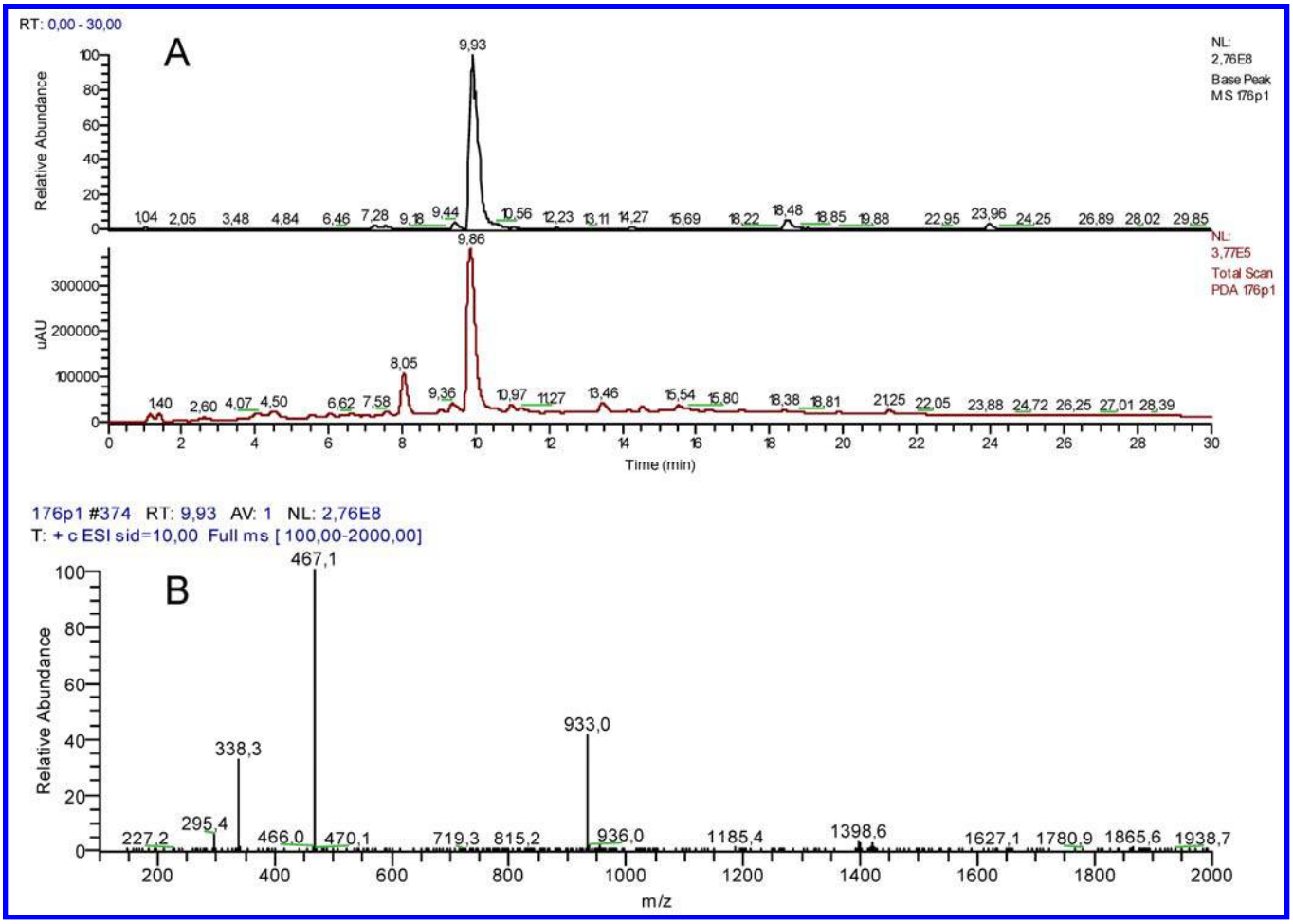

Fig. 1. High-performance liquid chromatography mass spectrometry (HPLC-MS) measurement showing the presence of staurosporine predominantly in the crude extracts of *Streptomyces* sp. B 5136. **A,** HPLC peak at 9.93-min retention time for staurosporine and **B,** MS spectra of staurosporine, $[M + H]^+ = 467$ m/z and $[2M + H]^+ = 933$ m/z. Column: RP-C12 Phenomenex 150 mm; solvent: gradient (MeOH + 0.05% HCOOH and H_2O + 0.05% HCOOH); flow rate : 300 µl/min.

Because some secondary metabolites have previously been isolated from this strain, we first tested the effects of those pure compounds on zoospore motility. Interestingly, only one compound, staurosporine, exhibited an identical motility impairment activity against zoospores as shown by the crude extract. To confirm whether any other compounds in the extract also have similar activities, the crude extracts of strain B5136 were subjected to silica gel thin layer chromatography (TLC) run with a CH_2Cl_2/MeOH, 9:1 solvent, and the plates were then divided into 10 horizontal zones from the origin to the solvent front (13.0 cm). The silica gel from each zone was removed and the compounds therein were tested to determine the effect on zoospore motility through a bioassay. Only the fraction corresponding to the retention value (R_f) of staurosporine (R_f = 0.27) showed motility inhibitory activity similar to the crude EtOAc extract and pure staurosporine. To assess the relative abundance of other compounds, the extract was further analyzed by high-performance liquid chromatography mass spectrometry (HPLC-MS) (Fig. 1). Interestingly, the EtOAc-dissolved compounds of strain B 5136 contained mainly staurosporine with little presence of other compounds. Therefore, staurosporine was considered the principal active ingredient in the extract of *Streptomyces* sp. strain B 5136 that impaired the motility of zoospores without causing lysis (Table 1).

Biological activity of compounds structurally related to staurosporine.

Staurosporine is an alkaloid having an indolocarbazole unit and a sugar unit in its chemical structure. Several compounds structurally related to staurosporine have previously been isolated from different strains of *Streptomyces* spp. To get insight into the structure–activity relationships, we tested some of those compounds, such as *N*-formyl-staurosporine, arcyriaflavin A, staurosporinone or K-252c, *N3*-(α-L-rhamnopyranosyl)-staurosporine or K-252d, 4′-demethylamino-4′,5′-dihydroxy-staurosporine, 7-oxostaurosporine, and *N*-carboxamido-staurosporine structurally related to staurosporine (Fig. 2; Table 1). All of these compounds possessing an indolocarbazole unit in their structures were mostly isolated from different strains of *Streptomyces* spp. and displayed motility-impairing activities in an identical fashion and a dose- and time-dependent manner (Table 1). Among the tested compounds, staurosporine exhibited the highest activity at a minimum inhibitory concentration (MIC) as low as 2 nM followed by its analogues 7-oxostaurosporine (MIC 10 nM) and K-252a (MIC 15 nM). The aglycon of staurosporine, staurosporinone (K-252c), also exhibited motility inhibitory activity in an identical manner but required a much higher dose (approximately 30 μM) compared with staurosporine. This indicates that the indolocarbazole unit is important for the inhibition of zoospore motility. Structural differences in the indolocarbazole unit (e.g., 7-oxostaurosporine) or sugar unit seem responsible for the varying motility inhibitory strengths of these alkaloids (Fig. 2). In most cases, the affected zoospores moved very slowly in tight circles or spun on their own axis with no lateral motion for several minutes, depending on the dose and type of compounds, and then became round cystospores. However, at the concentrations above threshold for motility impairment, the zoospores stopped moving immediately

Table 1. Motility inhibitory activity of staurosporine and structurally related compounds against *Plasmopara viticola* zoospores with names of the producing strains of microorganism

Name of compound	Producing strain	Dose (μM)	Motility inhibitory activity (%) on zoospores[a]			
			15 min	30 min	45 min	60 min
Staurosporine	*Streptomyces staurosporeus*	0.002	0 ± 0[b]	12 ± 2	21 ± 7	32 ± 4
	Streptomyces sp. strain B5136	0.01	33 ± 4	67 ± 5	81 ± 9	92 ± 5
		0.02	52 ± 6	88 ± 6	90 ± 4	95 ± 3
N-Formyl-staurosporine	*S. longisporoflavus* R-19	20	0 ± 0	10 ± 1	15 ± 4	41 ± 7
		100	9 ± 2	29 ± 3	42 ± 6	60 ± 6
		200	17 ± 4	32 ± 5	51 ± 7	71 ± 5
		1,000	33 ± 5	45 ± 6	69 ± 5	84 ± 8
Arcyriaflavin-A	*Streptomyces* sp. strain GW11/3535	3	0 ± 0	0 ± 0	22 ± 4	30 ± 3
	Arcyria dematum	15	0 ± 0	19 ± 7	72 ± 6	100 ± 0
		30	30 ± 3	50 ± 9	81 ± 8	100 ± 0
		60	47 ± 7	79 ± 4	98 ± 1	100 ± 0
Staurosporinone	*Streptomyces* sp. strain GW11/3535					
	Nocardiopsis sp. strain K-290	30	0 ± 0	10 ± 3	12 ± 4	15 ± 1
		150	45 ± 7	60 ± 5	69 ± 5	72 ± 7
		300	81 ± 10	88 ± 5	91 ± 8	98 ± 1
N3-(α-L-Rhamnopyranosyl) or K-252d	*Nocardiopsis* sp. K-290	10	0 ± 0[b]	42 ± 7	60 ± 7	100 ± 0
		20	85 ± 6	91 ± 8	100 ± 0	100 ± 0
		40	100 ± 0	100 ± 0	100 ± 0	81 ± 8
4′-Demethylamino-4′,5′-dihydroxy-staurosporine	*Streptomyces* sp. strain GW11/3535	1	0 ± 0	11 ± 2	32 ± 3	38 ± 8
	Streptomyces sp. strain AB1869R-359	5	0 ± 0	52 ± 6	82 ± 4	89 ± 5
		10	72 ± 6	100 ± 0	100 ± 0	100 ± 0
N-Carboxamido-staurosporine	*Streptomyces* sp. strain QD518	2	0 ± 0[b]	0 ± 0[b]	0 ± 0[b]	0 ± 0[b]
		10	0 ± 0	0 ± 0	10 ± 3	18 ± 4
		20	0 ± 0	22 ± 3	32 ± 6	46 ± 7
		40	10 ± 2	31 ± 4	47 ± 9	51 ± 8
		80	19 ± 3	42 ± 8	59 ± 7	63 ± 7
		100	60 ± 9	90 ± 5	100 ± 0	100 ± 0
7-Oxostaurosporine	*S. staurosporeus* ATCC 55006	0.01	0 ± 0[b]	0 ± 0[b]	0 ± 0[b]	0 ± 0[b]
	S. hygroscopicus ATCC 53730	0.05	0 ± 0[b]	0 ± 0[b]	11 ± 3	51 ± 7
		0.1	0 ± 0[b]	0 ± 0[b]	70 ± 6	84 ± 2
		0.2	72 ± 5	85 ± 7	89 ± 9	93 ± 6
		0.4	98 ± 1	100 ± 0	100 ± 0	100 ± 0

[a] Data presented here are the average value ± standard error of at least three replications in each dose of test compound.
[b] Swimming speed drastically decreased and flagella seemed arrested and thus huge turning of zoospores was observed.

after exposure to the compound although no lysis of zoospores occurred even at 10-fold higher than the threshold concentrations of staurosporine, 7-oxostaurosporine, and K-252a.

Motility of zoospores in the presence of various kinase inhibitors.

Staurosporine is a broad-spectrum inhibitor of various kinases, including PKC. The threshold concentration of staurosporine for the inhibition of zoospores motility (2 to 5 nM) is similar to the dose required for PKC inhibitory activity in mammalian cells (50% inhibitory concentration = 5 nM) (Rotem et al. 1990). To better assess whether PKC or any other kinase enzymes are associated with the motility of zoospores, inhibitors of several types of kinases were tested (Table 2; Fig. 2). The specificity of inhibitors is not perfect and various isoforms of protein kinases are prevalent; therefore, we tested a range of inhibitors possessing different mechanisms of actions. Only 7 of 15 potential inhibitors tested had significant effects on the motility of cells over the assessment period of 15 to 60 min. The motility inhibitory effect of PKC inhibitors clearly showed a time- and dose-dependent trend in repeated experiments (Fig. 3). The most dramatic impairment of *P. viticola* zoospore motility was observed with chelerythrine, staurosporine, and K-252a (Fig. 3). The protein tyrosine kinase (PTK) inhibitors tyrphostin A47 and herbimycin A were also effective in inhibiting the *P. viticola* zoospore motility but their effect was not at the level of the other PTK pathway inhibitors (data not shown). However, KN-93, a Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, had very little effect on the motility of zoospores at 2 µg/ml.

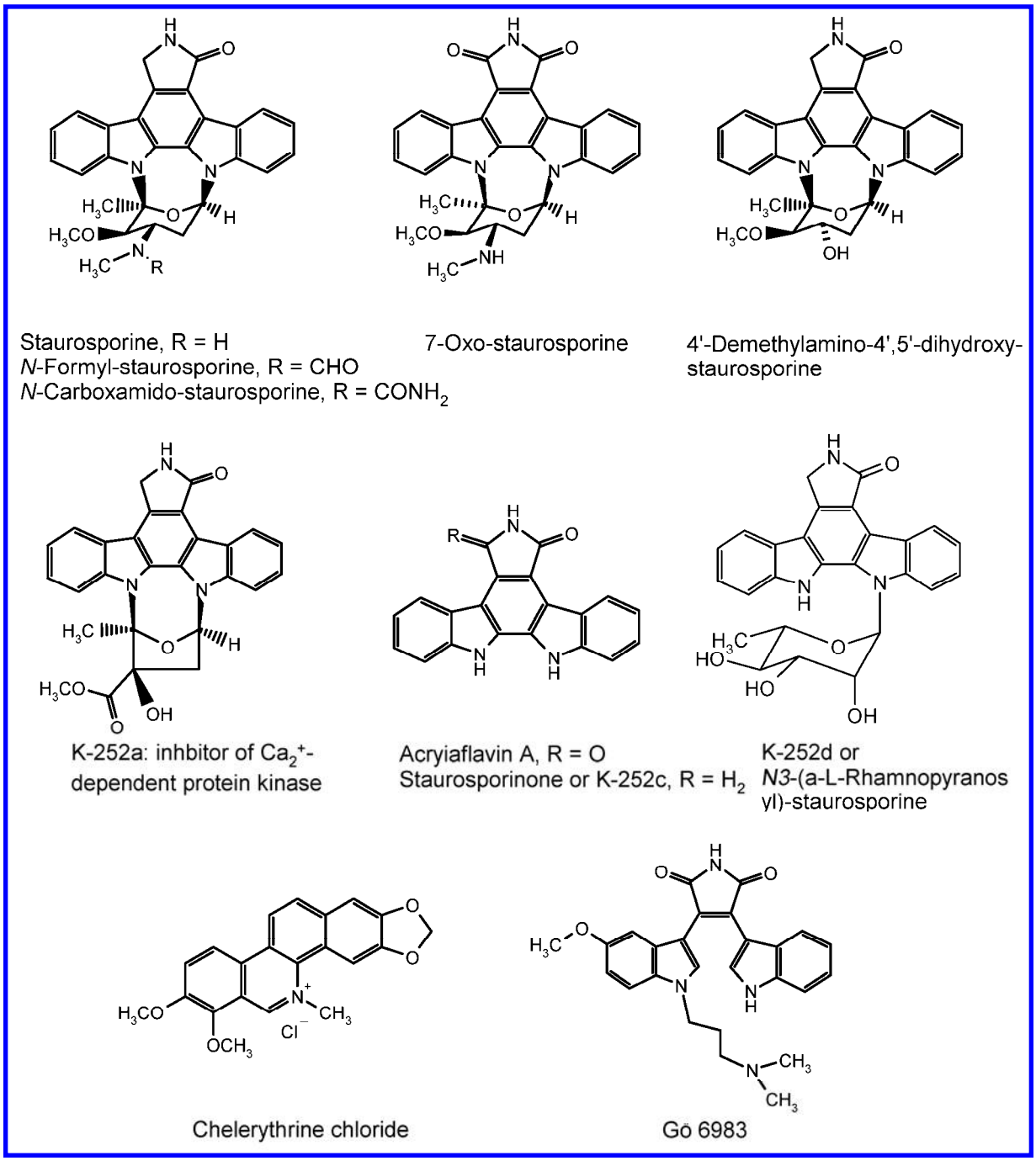

Fig. 2. Structure of staurosporine, some structurally related compounds, and protein kinase C inhibitors tested toward *Plasmopara viticola* zoospores.

Phorbol ester (phorbol-12-myristate-13-acetate [PMA]) is known to stimulate PKC activity in motile cells such as human sperm and spermatozoa of many vertebrates. To get more insight into the mechanisms by which PMA enhances PKC, we tested the effect of PMA on *P. viticola* zoospores. The bioassay revealed that zoospores swam faster in the presence of PMA compared with the control. Addition of the PKC inhibitors chelerythrine or staurosporine in the assay equally blocked the

Table 2. Effect of various kinase inhibitors on the motility of *Plasmopara viticola* zoospores[a]

Kinase or pathway targeted	Compound	IC_{50} (µM)	Type of action or target	Range of concentration tested (µM)	Inhibition of zoospore motility
PKC	Chelerythrine	0.66	Inhibits PKC translocation	0.001–50	Total at 0.01 µM
	Gö6983	0.002–0.006	Diacylglycerol competitor	0.05–50	Total at 5 µM
	Calphostin C	0.05	Ca^{2+}-dependent PKC α_1 and β_1	0.05–50	Total at 30 µM
PKA	KT 5720	0.056	ATP-site competitor	0.01–50	None
CaM kinase II	KN-93	1.00	Ca^{2+}/calmodulin-dependent protein kinase II	0.001–50	None
PKG	KT 5823	…	c-GMP-dependent protein kinases	0.01–50	None
PI3K	LY 294002	1.4	PI3-kinase	0.01–50	None
PTK	Tryphostin A47	2.4	Receptor type PTK	1–50	Total at 12 µM
	Herbimycin A	8–12	Nonreceptor type PTK	1–50	Total at 50 µM
	Genistein	2–6	ATP-site competitor	1–50	None
AKT	Akt1/2 kinase inhibitor	5	Akt1/2 kinase	1–50	None
Broad spectrum	Staurosporine	0.001–0.03	ATP-site competitor	0.001–0.002	Total at 0.05 µM
	K-252a	0.1–0.03	ATP-site competitor	0.001–0.5	Total at 250 nM
	K-252b	…	ATP-site competitor	0.1–50	None
	K-252c	ND	ATP-site competitor	1–50	Partial at ≥ 50 µM

[a] IC_{50}, inhibitory concentration for 50% targeted enzyme/cells; PKC = protein kinase C; PKA = protein kinase A; PKG = protein kinase G; PI3K = phosphoinositide 3-kinase; PTK = phosphotyrosine kinase; AKT = a serine/threonine kinase or protein kinase B; ATP = adenosine tri-phosphate; ND = not determined.

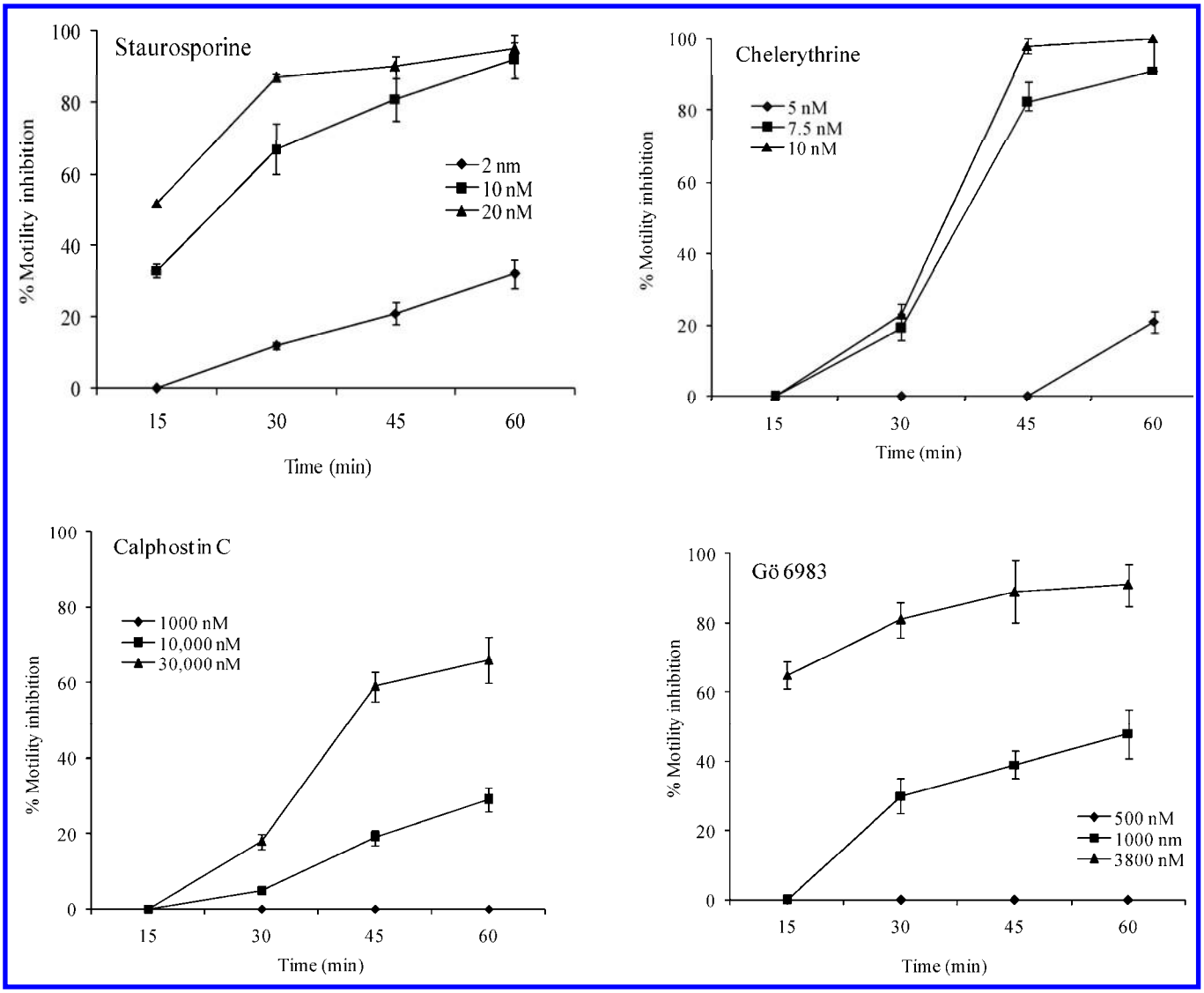

Fig. 3. Motility inhibitory activity of staurosporine and some protein kinase C (PKC) inhibitors against *Plasmopara viticola* zoospores.

swimming speed of zoospores induced by PMA, clearly indicating that PKC is involved in the motility of Peronosporomycete zoospores (data not shown).

Effects of staurosporine and other PKC inhibitors on the motility of zoospores and the germination of cystospores of *Aphanomyces cochlioides*.

To evaluate whether the motility inhibitory effect of staurosporine and PKC inhibitors is a common phenomenon in Peronosporomycete zoospores, we tested staurosporine and other kinase inhibitors against *A. cochlioides* zoospores. An almost identical phenomenon was observed when motile *A. cochlioides* zoospores were exposed to staurosporine or the PKC inhibitor chelerythrine although the required doses for motility inhibition were significantly higher, indicating that the sensitivity of *A. cochlioides* zoospores to inhibitors is lower than in *P. viticola* zoospores (data not shown). Interestingly, zoospores induced to cystospores by staurosporine germinated with excessively branched or curled germ tubes at 0.2 µM and no germination occurred at the dose of 1.0 µM or higher (Fig. 4).

Inhibition of zoosporogenesis by staurosporine and PKC inhibitor chelerythrine.

Freshly harvested and washed *P. viticola* sporangia (3×10^5/ml) typically release zoospores up to 1×10^6/ml in sterile water within 6 h. We tested whether staurosporine and a specific inhibitor of PKC, chelerythrine, have an effect on the process of zoospore release (i.e., zoosporogenesis). Both staurosporine and chelerythrine inhibited zoosporogenesis in a dose-dependent manner (Fig. 5). Zoosporogenesis was completely blocked at 50- and 100-nM doses of staurosporine and chelerythrine, respectively. At the lower doses of these kinase inhibitors, release of zoospores still occurred but most of the zoospores became immobilized soon after their release. Sporangia remained intact (unburst) even at very high concentrations of chelerythrine (10,000 nM). In contrast, staurosporine concentration higher than 1,000 nM caused lysis of the sporangia (data not shown).

Suppression of downy mildew disease on grapevine by staurosporine.

To evaluate the effect of marine natural product staurosporine on downy mildew disease development on grapevine, varying doses (0, 0.002, 0.02, 0.2, 2, 5, and 10 µM) of staurosporine were applied to leaf disks of grapevine 12 h before and after inoculation with freshly harvested *P. viticola* sporangia. At 6 days postinoculation (dpi), heavy sporulation of downy mildew was observed on the abaxial surface of the inoculated leaf disks of the control treatment (0 µM staurosporine). Treatment with staurosporine reduced the sporulation of *P. viticola* on the leaf disks in a dose-dependent manner (Fig. 6). Staurosporine application at 2.0 µM in both pre- and postinoculation of the leaf disks completely suppressed *P. viticola* sporulation (Fig. 6A). However, application of staurosporine at 0.2 µM before inoculating the leaf disks with sporangia reduced the *P. viticola* sporulation significantly more strongly compared with the staurosporine treatment after pathogen inoculation (Fig. 6B). Interestingly, the treated leaf disks remained dark green when staurosporine was applied before inoculation of the leaf disks by the pathogen (Fig. 6A). On the other hand, the natural green color of the heavily infected leaf disks turned dull to light yellow.

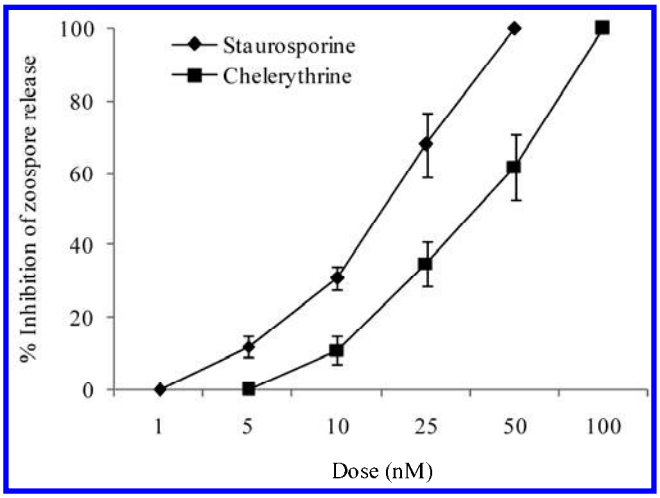

Fig. 5. Effects of staurosporine and chelerythrine on release of zoospores from freshly harvested sporangia of *Plasmopara viticola*. Staurosporine and chelerythrine were first dissolved in small quantities of dimethyl sulfoxide (DMSO) and then diluted with deionized water to prepare a series of concentrations. Varying concentrations of staurosporine and chelerythrine solutions were added to the sporangial suspension in Nunc multidishes to final concentrations of 0, 1, 5, 10, 25, 50, and 100 nM in the total volume of 400 µl and kept in the dark at 23°C for 6 h. The DMSO concentration in the final suspensions was kept to less than 1 µg/ml, which has no effect on zoosporogenesis. The number of motile zoospores released per milliliter of suspension was counted in a hemacytometer as described earlier and the relative percentage of suppression of zoospore release compared with control was calculated (Islam et al. 2004, 2007). The values represented here are mean values of five replications in each treatment ± standard error.

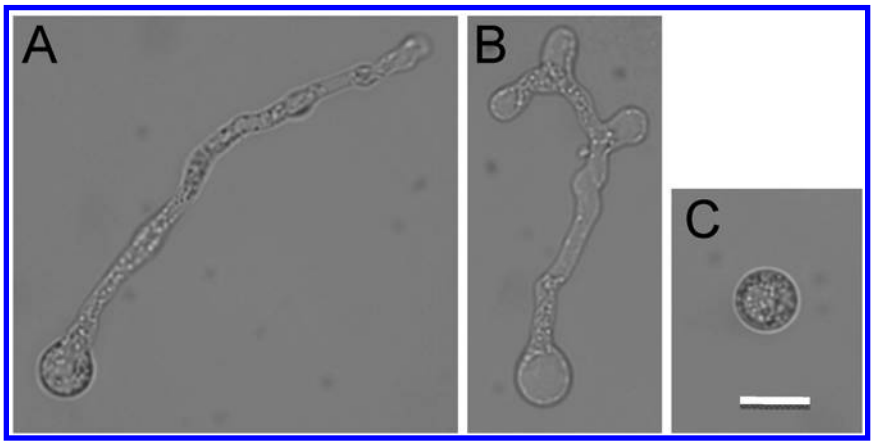

Fig. 4. Effect of staurosporine on germination and germ tube morphology of *Aphanomyces cochlioides* cystospores. **A,** Unbranched germ tube of a germinated cyst; **B,** excessively branched and malformed germ tubes at 0.2 µM; and **C,** ungerminated (germination completely blocked) cysts at 1 µM staurosporine, respectively.

DISCUSSION

In this study, we found that staurosporine and some structurally related carbazoles isolated from marine *Streptomyces* spp. impaired the motility of *P. viticola* zoospores in a dose- and time-dependent manner (Table 1; Fig. 2). In addition, compounds known as selective inhibitors of PKC (chelerythrine, Gö 6983, and calphostin C) also affected the swimming behavior of zoospores (Fig. 3). Although these three PKC inhibitors showed strong motility inhibitory effects, chelerythrine was the most potent compound, arresting motility at concentrations starting from 5 nM. On the other hand, compounds targeting pathways or kinases other than PKC did not significantly affect the motility of zoospores. The PKC activator phorbol ester significantly increased the swimming speed of zoospores, which was completely blocked by the PKC inhibitors staurosporine and chelerythrine, suggesting that the activity of PKC is indispensable for normal swimming of the Peronosporomycete zoospores. Taken together, our results have shown for the first time that PKC plays a key role in the signal transduction mechanisms during maintenance of motility of the Peronosporomycete zoospores of *P. viticola*. Microscopic observation revealed that the motility impairment occurred more rapidly in the presence of chelerythrine, staurosporine, and calphostin C than Gö 6983. These results indicated that motility inhibition by these compounds was most likely consecutive with a decrease in the flagellar beat frequency which, in turn, is directly dependent on the activity of axonemal dynein arms (Narayan et al. 2010).

PKC is known to be involved in many signal transduction pathways leading to a variety of cell responses, including motility, proliferation, differentiation, and gene expression (Tamoki et al. 1986; White et al. 2007). One of the interesting findings of this study is suppression of zoospore release from the sporangia by both staurosporine and the PKC inhibitor chelerythrine at nanomolar doses (Fig. 5). The process of zoospore release from the sporangia of Peronosporomycete microorganisms is known as zoosporogenesis. In the current study, we also showed that staurosporine completely suppressed the development of downy mildew disease on grapevine leaf disks artificially inoculated with freshly harvested sporangia of *P. viticola* (Fig. 6). In earlier work, another staurosporine-producing isolate of *Streptomyces* was also found to inhibit the disease development of *Phytophthora capsici* on peppers (Park et al. 2006). Taken together, these results suggest that PKC might be an interesting target of control of the Peronosporomycete phytopathogens.

Several lines of evidence suggest that zoospores locate hosts and aggregate to infection sites by sensing the gradient of specific chemical signals released from the host (Horio et al. 1992; Islam et al. 2002a and b, 2003; Morris and Ward 1992; Yokosawa et al. 1986). The aggregated zoospores become sluggish, spin in tight circles, and rapidly shed their flagella before becoming round cystospores (encystment) (Islam et al. 2002a and b, 2003; Latijnhowers et al. 2002). It is possible that the gradient of a specific host signal at a lower concentration may attract zoospores by activating a specific isoform of PKC and, when they encounter higher concentrations near the source, the same signal may inhibit another isoform of PKC essential for normal motility and induce encystment of the zoospores. The dual role of platelet PKC isoforms in thrombust formation has recently been demonstrated (Gilio et al. 2010; Strehl et al. 2007). The dual role of PKC in the regulation of large conductance voltage- and Ca^{2+}-activated potassium channels (BK channel) has also been reported (Zhou et al. 2010). The threshold concentrations (nanomolar to micromolar) of PKC inhibitors for the motility inhibition of zoospores shown in this report are comparable with their inhibitory effects on PKC in mammalian cells (Table 2; Fig. 3) (Kalina et al. 1995; Rotem et al. 1990).

Zoospores of *Plasmopara viticola* normally swim in a helical fashion almost in straight lines with less turning. In the presence of kinase inhibitors, zoospores migrated to the bottom of the petri dish, aggregated, and moved very slowly, with remarkably higher turning. At higher doses, they moved very slowly along their own axis with jerky motions or spun in tight circles. Careful microscopic observation revealed that zoospores failed to move flagella, which appeared curled around the spores. The shapes of the affected zoospores changed from reniform-oval to almost round. These phenomena continued for several minutes depending on doses of the inhibitors, and then the zoospores became round cystospores by shedding their flagella. Some of these cystospores germinated within 20 to 30 min of encystment. These observations clearly indicate that PKC inhibitors impaired the normal functions of flagella and made the zoospores disoriented. To investigate whether the aberrant motility of zoospores could be reversed, we diluted the concentration of kinase inhibitors by changing the upper layer of water with fresh sterilized water very carefully. Interestingly, after dilution of the inhibitors to less than the threshold concentrations, the paralyzed zoospores resumed their normal swimming within 30 min (data not shown).

The heterotrimeric G protein is ubiquitous in the signaling pathway that regulates growth and differentiation of many eukaryotic phytopathogens. Latijnhouwers and associates (2004) demonstrated that *Phytophthora infestans* transformants obtained by silencing of the Gα subunit gene *Pigpa1* produced zoospores with severely impaired motility. The authors also observed that those zoospores were less virulent on potato plants compared with the wild type. It is well known that kinases function as intracellular effectors to downstream signal cascades and transduce extracellular signals perceived by G protein-coupled receptors (Wang et al. 2010). PKC plays an indispensable role in the normal motility of mammalian sperm and kinase inhibitors were found to effectively impair their motility (Rotem et al. 1990). In this study, not only motility of zoospores but also the release of zoospores from the sporangia were impaired by both a nonspecific protein kinase inhibitor, staurosporine, and a specific PKC inhibitor, chelerythrine (Herbert et al. 1999), indicating that protein kinase might be involved in both maintenance of the zoospore motility and zoosporogenesis of *Plasmopara viticola* sporangia (Table 2; Fig. 5). In a recent study, Li and associates (2010) demonstrated that silencing of *PsSAK1*, which encodes a stress-activated mitogen-activated protein kinase, severely hindered zoospore development in *Phytophthora sojae*. On the other hand, gene expression profiling during asexual development of the late blight pathogen *Phytophthora infestans* revealed a highly dynamic transcriptome (Judelson et al. 2008).

The Peronosporomycete zoospores are biflagellate, with the anterior flagellum conferring the majority of the motile force and the posterior flagellum primarily steering the motile cell (Islam et al. 2002a and b; Narayan et al. 2010). Therefore, it is likely that the posterior flagellum, which acts as a steering unit during swimming of zoospores, may contain PKC. The observed spinning pattern during motility indicates that the flagellum functioning for steering (posterior flagellum) gets disabled. Detection of PKC in zoospore flagella by immunocytochemistry should provide more insight into the role of PKC in the Peronosporomycete flagellar motility (Kalina et al. 1995; Rotem et al. 1990; White et al. 2007).

Ca^{2+} at millimolar concentrations and the Gα subunit are known to play pivotal roles in the motility and swimming of zoospores (Connolly et al. 1999; Latijnhouwers et al. 2004). Activation of the respective kinase and the phosphorylation of key proteins are involved in the motility of many eukaryotic cells (White et al. 2007). Because most biological systems

controlled by protein phosphorylation are now recognized to be in a complex interaction with Ca^{2+}, cyclic nucleotides, and PKC, it is not surprising that PKC might also play a crucial role in regulating zoosporogenesis and the motility of zoospores in concert with Ca^{2+} by means of phosphorylation of similar or other endogenous substrate proteins. Motility inhibition without lysis of *A. cochlioides* zoospores by nonhost plant secondary metabolites has been shown previously in two reports. The first report indicated that a complex mixture of two chemically different compounds, *N-trans*-feruloyltyramine and 1-linoleoyl-2-lysophosphatidic acid monomethyl ester isolated from the root of *Portulaca oleracea*, displayed motility inhibition of *A. cochlioides* zoospores (Mizutani et al. 1998). Second, a simple metabolite, nicotinamide isolated from the roots of *Amaranthus gangeticus* and pea seedlings, induced sudden motility inhibition followed by encystment of *A. cochlioides* zoospores, which were regenerated zoospores, within 3 h instead of germinating (Islam et al. 2004).

Elucidating the role of PKC in the swimming pattern and motility of zoospores will help to advance our understanding of the biology and pathogenicity of *Plasmopara viticola* and other Peronosporomycete phytopathogens. Kinase inhibitors are a new class of therapeutics with a potential to inhibit multiple targets (Sebolt-Leopold and English 2006). Selectivity of small-molecule kinase inhibitors has been assessed and described recently (Karaman et al. 2008). Staurosporine, a nonspecific kinase inhibitor, not only inhibited zoosporogenesis and motility of *P. viticola* zoospores but also significantly suppressed downy mildew disease development in grapevine leaves (Fig. 6). Therefore, this naturally occurring low-molecu-

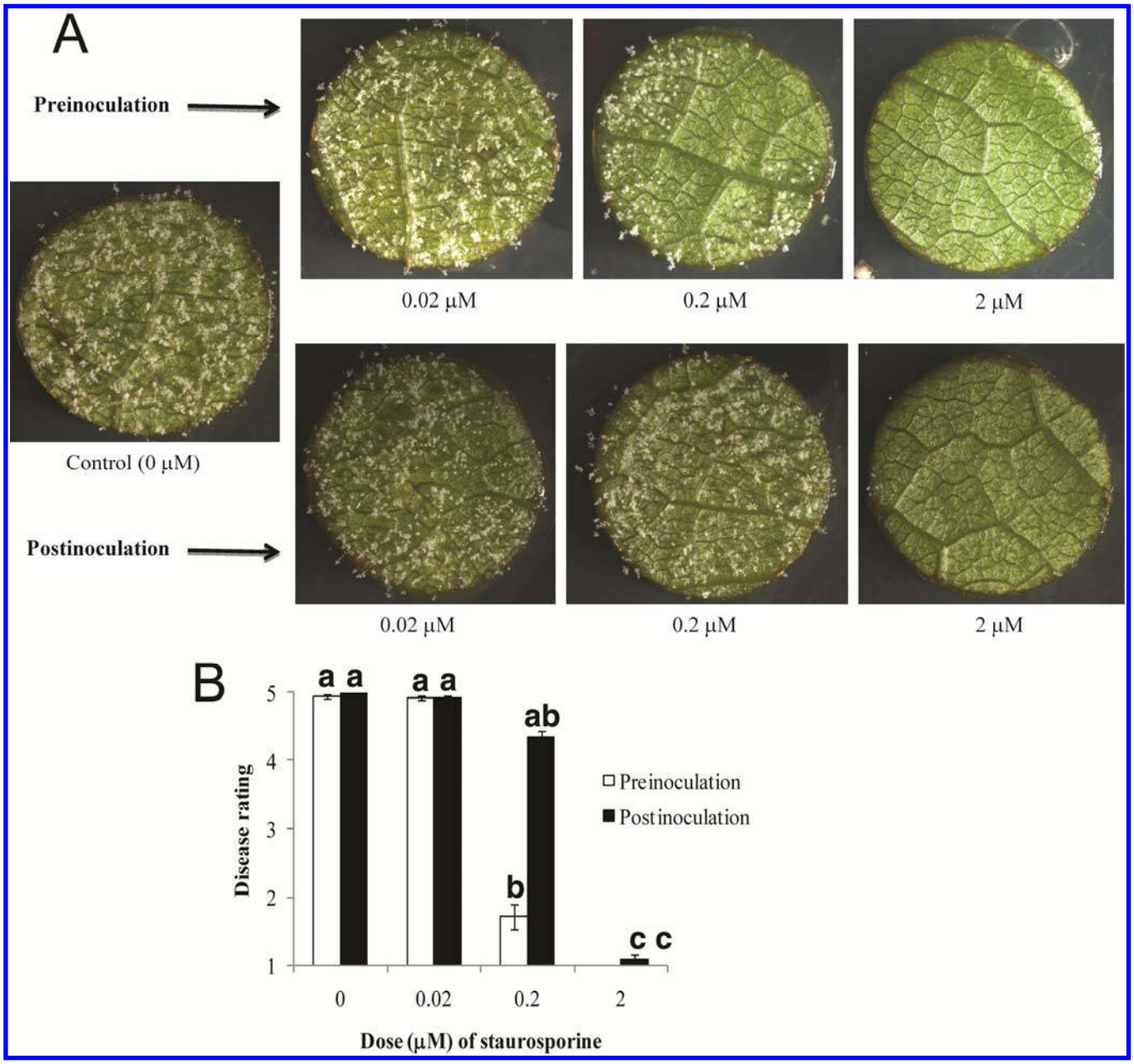

Fig. 6. Pre- and postinoculation effects of marine natural staurosporine on downy mildew caused by *Plasmopara viticola* sporangia on grapevine leaf disks. **A,** Staurosporine was suspended in aqueous dimethyl sulfoxide (1%) and an appropriate dose was sprayed on leaf disks placed on 1.5% water agar 12 h before (pre) or after (post) inoculation with *P. viticola* sporangia (5×10^3/ml). Inoculated leaf disks were incubated at 25°C in 95% relative humidity for 6 days. **B,** Disease intensity was visually assessed using a 1-to-5 scale (Hamiduzzaman et al. 2005), in which 1 = no symptom visible and 2 = up to 25, 3 = 25 to 50, 4 = 50 to 75, and 5 = >75% of the leaf disk area is covered with sporangiophores 6 days postinoculation. Values presented in different graphs are means ± standard errors of means. Different letters (B) denote treatment means significantly different at $P \leq 0.01$ (Duncan's multiple range test).

lar weight protein kinase inhibitor might have high potential as a lead compound for designing effective agrochemicals against the economically important Peronosporomycete phytopathogens. The simple and convenient methods used in this study for the production of sporangia and zoospores and motility and zoospore release bioassays should be useful for isolating similar novel metabolites from other microorganisms.

Finally, one of the interesting aspects of our study is that it parallels earlier work on the role of PKC in flagellar motility of mammalian sperm and spermatozoa of aquatic vertebrates (Rotem et al. 1990; White et al. 2007). Our findings suggest that the requirement of PKC activity may be a common mechanism for maintaining motility of all these cross-kingdom specialized cells. Because motility is critical for the life cycles and pathogenicity of pathogens, elucidation of the details of signal transduction pathways might help us to design strategies for biorational management of the notorious Peronosporomycete phytopathogens.

MATERIALS AND METHODS

Materials.

Inhibitors of protein kinases such as chelerythrine chloride, Gö 6983, calphostin C, tyrphostin A47, herbimycin A, K-252a, K-252b, K-252c, KN-93, KT 5720, KT 5823, LY 294002, genistein, and akt1/2 kinase inhibitor and an activator of PKC, PMA, were purchased from Sigma-Aldrich or Biomol (Fig. 1). Staurosporine and structurally related compounds available in the laboratory were either previously isolated from marine *Streptomyces* spp. or synthesized (Fig. 2). All other chemicals were of at least reagent grade. Whenever possible, stock solutions of chemicals were prepared in distilled water. Stock solutions of other kinase inhibitors were prepared in dimethyl sulfoxide (DMSO). The concentration of DMSO in the incubation medium never exceeded 1%, a condition that does not affect zoospore motility.

Cultivation of marine *Streptomyces* spp., extraction, and chromatographic procedure.

The strains of marine *Streptomyces* spp. used in this research were obtained from the collection of the Institute of Organic and Biomolecular Chemistry, University of Göttingen, Germany. Each strain was cultured in 1-liter flasks in M2 medium (10 g malt extract, 4 g glucose, and 4 g yeast extract) with 50% (vol/vol) artificial sea water for 3 days at 28°C and shaking at 95 rpm. The pH of the M2 medium was adjusted to 7.8 using 2N NaOH and the medium was sterilized for 33 min at 121°C. The culture suspension was freeze dried and the residue was suspended in 200 ml of water, then repeatedly (3 times) extracted with equal volumes of EtOAc. The EtOAc extract was concentrated in vacuo and used in the zoospore bioassays and chromatographic analyses.

The crude EtOAc extracts of strain B 5136 were subjected to preparative TLC and HPLC-MS analyses to detect and identify zoospore motility inhibitors, respectively. Merck silica gel 60 F254 0.25-mm-thick TLC plates were used for preparative TLC. The HPLC-MS was done using an RP-C12 Phenomenex 150-mm column using a gradient solvent system (MeOH + 0.05% HCOOH and H_2O + 0.05% HCOOH) with a flow rate of 300 µl/min and an ESI 0-2000 MS detector under a UV/VIS-diode-array at 200 to 800 nm (step width 5 nm).

P. viticola strain, production of zoospores, and motility bioassay.

Sporangia of *P. viticola* were isolated from infected leaves of grapevine (*Vitis vinifera* 'Müller-Thurgau') received from B. Berkelmann, Geisenheim Research Center, Germany. This strain was originally gained from infected leaf materials of 'Riesling' grapevine in 1996 and, since then, propagated on fresh leaves of Müller-Thurgau.

We maintained this strain by regular culturing on the lower surface of young grapevine leaves in petri dishes containing 1.5% agar at 25°C and 95% relative humidity (Islam and von Tiedemann 2008). At day 6 of cultivation, the sporangiophores bearing lemon-shaped sporangia were harvested into an Eppendorf vial by a microvacuum cleaner. The freshly harvested sporangia were separated from sporangiophores by filtration through 50-µm nylon mesh, washed twice, and then incubated in sterilized water (sporangia at approximately 3×10^5 ml^{-1}) in the dark for 6 h at room temperature (23°C) to release zoospores. These zoospores remained motile for 10 to 12 h in sterilized water and were used for the motility bioassay.

The bioassays were carried out by homogeneous solution methods as described earlier (Islam et al. 2002a and b, 2005, 2007). Briefly, pure compounds were first dissolved in small quantities of DMSO and then diluted with distilled water to prepare a series of solutions for each compound. Exactly 40 µl of the appropriate sample solution concentration was directly added to a 360-µl suspension of sporangia (approximately 3×10^5 ml^{-1}) or zoospores (approximately 1×10^6 ml^{-1}) in Nunc Multidish (Nunc) to obtain a final volume of 400 µl and gently mixed with a glass rod. The final concentration of DMSO in the sporangia or zoospore suspension was less than 1% in all treatments; 1% DMSO alone was completely inactive in assays for zoosporogenesis and motility of zoospores. A sporangia or zoospore suspension in separate dishes (400 µl) was used as control. The number of released zoospores from sporangia or settled or stopped zoospores was determined microscopically (×20 magnification) (Islam et al. 2002a and b, 2004, 2007). The percentage of halted zoospores or number of released zoospores per milliliter was calculated as described previously (Islam et al. 2004, 2007). The data are averages ± standard errors of at least three replications for each dose of tested compounds.

Leaf disk assay and assessment of disease severity.

The third and fourth leaves from the top of *V. vinifera* Müller Thurgau plants were collected from the greenhouse and then surface sterilized for 1 min with 70% ethanol followed by repeated washing with sterilized tap water. Leaf disks (1 cm in diameter) were punched from excised and surface-sterilized leaves from different plants and then mixed. Each leaf disk was placed on 1.5% solidified water agar in each well of Nunc tissue culture multidishes. The leaf disks were inoculated by spraying with a suspension of freshly harvested *P. viticola* sporangia (5×10^3 sporangia ml^{-1}). Appropriate concentrations of staurosporine suspended in 1% DMSO were sprayed on leaf disks 12 h before or after the inoculation of leaf disks with *P. viticola* sporangia. The multidishes were placed in a growth chamber (16 h of light) at 25°C in 95% relative humidity. A control treatment using sterilized water instead of staurosporine was also applied. Each treatment was replicated five times. The whole experiment was repeated three times with similar results.

Disease intensity was visually assessed 6 dpi using a 1-to-5 scale (Hamiduzzaman et al. 2005), in which 1 = no symptoms visible and 2 = up to 25, 3 = 25 to 50, 4 = 50 to 75, and 5 = >75% of leaf disk area covered by sporangiophores. Data were statistically analyzed using the statistical package SPSS (version 12). The mean values ± standard errors are presented in the graph and tables.

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