Effect of Selected Phytotoxins from *Guanomyces polythrix* on the Calmodulin-Dependent Activity of the Enzymes cAMP Phosphodiesterase and NAD-Kinase[†]

RACHEL MATA,*,‡ ALICIA GAMBOA,*,‖ MARTHA MACIAS,‡ SANDRA SANTILLÁN,‡
MIGUEL ULLOA,§ AND MARÍA DEL CARMÉN GONZÁLEZ§

Departamento de Farmacia, Facultad de Química, Instituto de Ecología, and Instituto de Biología,
Universidad Nacional Autónoma de México, D.F. 04510, México

The effect of a series of phytotoxins isolated from the fungus *Guanomyces polytrix* on calmodulin (CaM)-dependent nicotinamide adenine dinucleotide kinase (NADK) and CaM-dependent cyclic nucleotide phosphodiesterase (PDE) activities was investigated. The results indicated that (2*S*,3*S*)-5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one, (2*S*,3*S*)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4H-2,3-dihydro-naphtho[2,3-b]-pyran-4-one, (2*S*,3*R*)-5-hydroxy-6,8-dimethoxy-2,3-dimethyl-2,3-dihydro-4H-naphtho[2,3-b]-pyran-4-one, (2*S*,3*R*)-5-hydroxy-6,8,10-trime-thoxy-2,3-dimethyl-2,3-dihydro-4H-naphtho[2,3-b]-pyran-4-one, 5-hydro-xy-6,8-dimethoxy-2,3-dimethyl-4H-naphtho[2,3-b]-pyran-4-one, rubrofusarin B, and ergosta-4,6,8(14),22-tetraen-3-one inhibited the activation of both target enzymes in the presence of CaM. On the other hand, (2*S*)-5-hydroxy-6,8-dimethoxy-2-methyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one and (2*S*)-5-hydroxy-6,8,10-trimethoxy-2-methyl-4H-2,3-dihydronaphtho-[2,3-b]-pyran-4-one inhibited the activation of PDE and the basal activity of NADK. Thus, these phytotoxins are CaM inhibitors and may exert their phytotoxic action by inhibiting the CaM-dependent process, although they could also interfere with other cellular metabolic phenomena. This is the first report of the use of the NADK assay to detect or quantify CaM inhibitors, and it could be a valuable tool for studying those CaM isoforms regulating NADK.

KEYWORDS: Spinach calmodulin (CaM); bovine-brain calmodulin; cyclic nucleotide phosphodiesterase (PDE); nicotinamide adenine dinucleotide kinase (NADK); *Guanomyces polythrix*; phytotoxins

INTRODUCTION

Calmodulin (CaM) is an essential protein that serves as a ubiquitous intracellular receptor for Ca^{2+}. The Ca^{2+}/CaM complex initiates a plethora of signaling cascades through the regulation of different enzymes and ion channels that culminate in alteration of the cellular functions. In higher plants, this protein is a fundamental component of the Ca^{2+} signal transduction pathway during germination and plant growth. Unlike animals, higher plants express multiple divergent CaM isoforms exhibiting differential activations/inhibitions of specific target enzymes. Furthermore, some of these isoforms share 90% of structural identity with mammalian CaM, while others show 78% (*1,2*). Thus, if a phytotoxin binds specifically to a CaM isoform that modulates a particular target enzyme and modifies its enzyme regulator properties, this compound could represent

a potential herbicide agent with a selective mode of action, if the target enzyme is only found in plants, or its activity is regulated by CaM only in plants. Some fungal toxins interact with CaM and inhibit the ability to activate CaM-sensitive enzymes. Ophiobolin A, a phytotoxin isolated from several species of the genus *Bipolaris* (*3*), secalonic acid, citreoviridine, and verruculogenone, produced by some *Penicillium* species (*4*), are among the best known examples. It has been suggested that the CaM inhibitory activity exerted by ophobiolin A is involved in its potent phytotoxic activity. Other secondary metabolites that interact with CaM are vinblastine (*5*), quercetin, and some cinnamic acid derivatives (*6*).

As a part of our systematic search for new herbicidal lead structures from natural sources, we have recently reported the isolation and structure elucidation of several phytotoxins from the mycelium and culture broth of the fungus *Guanomyces polythrix*. The phytotoxins included several naphthopyrone derivatives (**Figure 1**) (*7, 8*). The natural compounds caused noted inhibition of radicle growth of two weed seedlings, *Amaranthus hypochondriacus* L. and *Echinochloa crus-galli* (L.) Beuv. and interacted with both spinach and bovine-brain CaMs (*7, 8*). The interaction was initially demonstrated by a sodium

* To whom correspondence should be addressed. Tel.: (525)-55-622-5289 or (525)-55-622-9013. Fax: (525)-55-622-5329. E-mail: rachel@servidor.unam.mx or agamboa@miranda.ecologia.unam.mx.
† Taken in part from the PhD and Ms theses of M. Macias and S. Santillán.
‡ Facultad de Química.
‖ Instituto de Ecología.
§ Instituto de Biología.

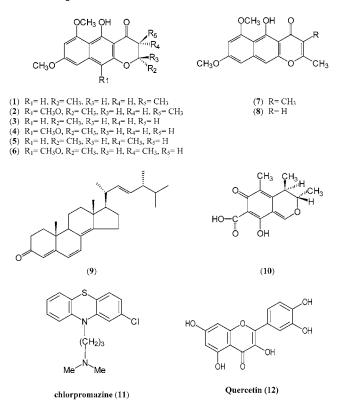**4560** *J. Agric. Food Chem.,* Vol. 51, No. 16, 2003

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Figure 1. Phytotoxins isolated from the coprophilous fungus *Guanomyces polythrix.*

dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) analysis and CaM affinity chromatography. However, these experiments did not show if the binding of the phytotoxins with CaM affected its enzyme regulator properties. Therefore, the main goal of this work was to quantitatively demonstrate that the phytotoxic naphthopyrones derivatives from *G. polythrix* modify the enzyme regulator properties of CaM by evaluating their effect on the CaM-dependent activity of the enzymes bovine-brain phosphodiesterase (PDE) and nicotinamide adenine dinucleotide kinase (NADK) isolated from peas. CaM-sensitive PDE catalyzes the hydrolysis of cyclic nucleotides to nucleotides monophosphates, while NADK catalyzes the conversion of NAD (nicotinamide adenine dinucleotide) into NADP (nicotinamide adenine dinucleotide phosphate), an important coenzyme in many metabolic reactions; thus, the latter enzyme is vital to keep metabolic homeostasis.

MATERIALS AND METHODS

Test Materials. The phytotoxic compounds tested included (2*S*, 3*S*)-5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one (**1**), (2*S*,3*S*)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one (**2**), (2*S*)-5-hydroxy-6,8-dimethoxy-2-methyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one (**3**), (2*S*)-5-hydroxy-6,8,10-trimethoxy-2-methyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one (**4**), (2*S*,3*R*)-5-hydroxy-6,8-dimethoxy-2,3-dimethyl-2,3-dihydro-4H-naphtho[2,3-b]-pyran-4-one (**5**), (2*S*, 3*R*)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-2,3-dihydro-4H-naphtho[2,3-b]-pyran-4-one (**6**), 5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4H-naphtho[2,3-b]-pyran-4-one (**7**), rubrofusarin B (**8**), ergosta-4,6,8(14),22-tetraen-3-one (**9**), and citrinin (**10**) (**Figure 1**). All compounds were isolated from the fungus *G. polythrix* as previously described (*7*, *8*). Chlorpromazine (**11**) and quercetin (**12**), used as positive controls, were purchased from Sigma, St. Louis, MO.

PDE Assay. A PDE assay in the presence of bovine-brain CaM was performed, using a modification of the method described by Sharma and Wang (*9*, *10*). Bovine-brain CaM (0.2 μg) was incubated with 0.015 units of CaM-deficient-CaM-dependent PDE from bovine brain (Sigma) for 3 min in 800 μL of assay solution containing 0.3 units 5′-nucleotidase (from *Crotalus atrox* venom, Sigma), 45 mM Tris-HCl, 5.6 mM Mg(CH₃COO)₂, 45 mM imidazole, and 2.5 mM CaCl₂, pH 7.0. Compounds **1**−**10** were then added to the assay medium at 10, 20, 40, 60, 80, and 100 μM in dimethyl sulfoxide (DMSO), and the samples were incubated for 30 min. Then, 100 μL of 10.8 mM cyclic adenosine monophosphate (cAMP), pH 7.0, were added to start the assay. After 30 min, the assay was stopped by the addition of 100 μL of 55% trichloroacetic acid solution. All of the above steps were carried out at 30 °C. The PDE reaction was coupled to the 5′-nucleotidase reaction, and the amount of inorganic phosphate released represented the activity of the PDE. The phosphate produced in the assay was measured by the method of Sumner (*11*). The wavelength used for the phosphate assay was determined to be 660 nm, by use of a CINTRA 5 spectrophotometer. The means of three independent assays were used. Chlorpromazine and quercetin were used as a positive control (IC₅₀ = 10.2, 20.1 μM, respectively).

NADK Preparation. The aerial portion of 14-day-old pea (*Pisum sativum*) seedlings were harvested, frozen in liquid N₂, and stored frozen at −80 °C until use. NADK was extracted by a modification of the method of Harmon et al. (*12*). All purification steps were performed at 4 °C. Generally, 50 g of frozen seedlings were ground in a chilled mortar. The powered tissue was diluted in the extraction buffer (50 mM TRIZMA (Sigma) pH 7.5, 100 mM KCl, 3 mM MgCl₂, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM dithiothreitol (DTT), and a protease inhibitor cocktail) to a final concentration of 2 g/mL frozen tissue. The homogenate was filtered through four layers of cheesecloth and was centrifuged at 27000*g* for 30 min. NADK was partially purified by successive protamine sulfate precipitation, (NH₄)₂-SO₄ precipitation to 55% saturation, and diethylaminoethyl sephacel (DEAE-Sephacel) column chromatography. The unbound effluent containing NADK activity was collected, and proteins were precipitated, adding solid (NH₄)₂SO₄ to 55% saturation. The precipitated proteins were resuspended in 1 mL of extraction buffer (pH 7.5), brought to 5% (v/v) glycerol, frozen in liquid N₂, and stored at − 80 °C until use.

NADK Assay. NADK assay was performed as previously described (*12*), using 2 μg of spinach CaM as enzyme activator. This CaM amount was found to saturate our enzymatic preparation. Briefly, each of the natural compounds (**1**−**10**) was dissolved in DMSO and tested at 20, 30, 60, 90, and 120 μM. Appropriate concentrations of the tested compounds were added to assay tubes containing the reaction buffer with CaM. The reaction was initiated by adding 10 μL of NADK preparation. The assay tubes were incubated for 20 min at 25 °C, and the reaction was stopped by placing the tubes in a boiling water bath for 2 min. After the tubes were cooled to room temperature, the redox-reporter dyes (phenazine methosulfate and 2,6-dichlorophenolindophenol) in the appropriate buffer were added to the media for the enzymatic determination of NADP. As controls, NADK activators were examined with reaction mixtures either in the presence of 1 mM EGTA or in the absence of exogenous CaMs to verify that the NADK preparation was free of endogenous CaM. NADK activity, in either the presence or absence of CaM, was not affected by addition of DMSO. The activity of the kinase was correlated with the amount of NADP generated. In turn, the rate of formation of NADP was determined by its rapid conversion to reduced nicotinamide adenine dinucleotide phosphate (NADPH) by GPDH in the presence of glucose-6-posphate (GP). The rate of NADPH production was monitored by a spectrocolorimetric analysis, following the decrease in absorbance at 600 nm over the time in the presence of the reporter redox dyes; the rate of NADPH formation is proportional to the amount of NADPH present, which in turn correlates with the amount of NADP initially generated (*12*). NADK assay data were recorded using a CINTRA 5 spectrophotometer.

Data Analysis. In both enzymatic assays, data are expressed as means ± SEM of at least three experiments. The results are expressed as IC₅₀ values, which were determined from the analysis of the concentration-effect (inhibition of the enzyme activity) curves. The dose response graphics were analyzed using the curve fitting program (Origin Software).

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Table 1. Effect of Phytotoxins **1–10** on CaM-Dependent PDE and NADK Activities (IC$_{50}$)

compounds	CaM-dependent PDE (IC$_{50}$ ± SEM, μM)[b]	CaM-dependent NADK (IC$_{50}$ ± SEM, μM)[c]
1	8.1 ± 0.0426	40.1 ± 0.6589
2	7.2 ± 0.0586	22.0 ± 0.7895
3	6.1 ± 0.0369	ND
4	5.8 ± 0.0354	ND
5	7.6 ± 0.0489	42.2 ± 0.8569
6	6.6 ± 0.0547	24.3 ± 0.8695
7	4.8 ± 0.0685	17.1 ± 0.7589
8	4.7 ± 0.0456	13.3 ± 0.5698
9	5.2 ± 0.0756	90.0 ± 0.6985
10	>100	>120
chlorpromazine[a]	10.6 ± 0.0541	ND
quercetin[a]	20.1 ± 0.0458	ND

[a] Positive standard controls. [b] In the presence of 0.2 μg of bovine-brain CaM. [c] In the presence of 2.0 μg of spinach CaM; ND = not determined.

RESULTS AND DISCUSSION

To quantify the interaction of the phytotoxins **1−10** with CaM, their effect on the activity of two CaM-dependent enzymes, namely PDE and NADK, was investigated. Because CaM-sensitive PDE is widely used as a tool to discover CaM inhibitors, and in general to demonstrate the activity of CaM in biochemical studies (*1*, *3*, *4*), we first assessed the effect of the fungal metabolites on this enzyme using the method of Sharma and Wang (*9*), with the modifications reported by Leung and co-workers (*10*). Bovine-brain CaM was used as activator of the enzyme. All of the tested compounds except citrinin (**10**) showed significant inhibitory effect on PDE activity in a concentration-dependent manner. The inhibitory activity (**Table 1**) of the active compounds was higher or comparable to that of chlorpromazine (IC$_{50}$ = 10.6 μM), a well-known CaM inhibitor (*4*). It is important to point out that none of the tested compounds affected the basal activity of PDE at the concentrations tested.

Next, the effect of the toxins on CaM-dependent NADK was evaluated. This enzyme was selected as the second reporter enzyme, because it is a vegetal enzyme and is only modulated by CaM in plants (*2*). In addition, NADK seems to be regulated by specific plant-CaM isoforms (*1*), which could be specific targets for phytotoxins. The use of this enzyme to detect or quantify CaM inhibitors has not been previously reported. The procedure involved three main steps. The first one was the isolation of a suitable enzymatic preparation free of endogenous CaM from peas, according to the method of Harmon. Because the assessment of the NADK activity was performed by a coupled enzymatic reaction involving GPDH as indicated in Materials and Methods (*12*), the second step of the assay was the evaluation of the effect of the phytotoxins on the basal activity of NADK and GPHD. Finally, in the last step, the ability of the phytotoxins to prevent the stimulation of the NADK preparation in the presence of saturating concentrations of spinach CaM, was assessed. After processing the data of the second step, it was found that compounds (2S)-5-hydroxy-6,8-dimethoxy-2-methyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one (**3**) and (2S)-5-hydroxy-6,8,10-trimethoxy-2-methyl-4H-2,3-dihydronaphtho [2,3-b]-pyran-4-one (**4**) inhibited NADK basal activity with IC$_{50}$ values of 49.1 ± 0.5892 and 26.2 ± 0.4578 μM, respectively. Therefore, these compounds were not further investigated. The remaining compounds did not inhibit NADK or GPDH basal activities at the concentrations tested. As in the case of the experiments with PDE, all the phytotoxins but citrinin

(**10**) inhibited the activity of the kinase promoted by spinach CaM. Rubrofusarin B (**8**), 5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4H-naphtho[2,3-b]-pyran-4-one (**7**), (2S,3S)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one (**2**), and (2S, 3R)-5-hydroxy-6,8,10-trimethoxy-2,3-dimethyl-2,3-dihydro-4H-naphtho[2,3-b]-pyran-4-one (**6**) (**Table 1**) were the strongest inhibitors of the complex spinach−CaM−NADK, with IC$_{50}$ values of 17.1, 13.3, 22.0, and 24.3 μM, respectively. In our previous report, compounds **2** and **6**, both possessing a methoxyl group at C-10, induced the major changes in the electrophoretic mobility of spinach CaM (*6*). On the other hand, the related compounds (2S, 3S)-5-hydroxy-6,8-dimethoxy-2,3-dimethyl-4H-2,3-dihydronaphtho[2,3-b]-pyran-4-one (**1**) and (2S,3R)-5-hydroxy-6,8-dimethoxy-2,3-dimethyl-2,3-dihydro-4H-naphtho[2,3-b]-pyran-4-one (**5**) (**Table 1**) were less active in the enzymatic assays. The overall results indicate that among the naphthopyrones series those phytotoxins possessing a double bond between C-2 and C-3 are the most active CaM inhibitors (i.e., compounds **7** and **8**). Finally the sterol whose antioxidant and cyclooxygenase inhibitory properties were recently reported (*13*) showed the least inhibitory effect on the complex Ca^{2+}−CaM−NADK.

In summary, the phytotoxins of *G. polythrix* interact with both Ca^{2+}−CaM−NADK and Ca^{2+}−CaM−PDE. Thus, these compounds may act as CaM antagonists in vivo inhibiting CaM-dependent phenomena during plant growth, although they could also interfere with other cellular metabolic phenomena. The inhibitory effect of these compounds is comparable or higher to that of other CaM antagonists (*3−6*, *14*). Because the relative order of CaM inhibition by the phytotoxins was similar for the PDE and NADK assays, the latter could be also useful to discover CaM inhibitors. Finally, we proposed the employment of this bioassay for monitoring CaM isoforms that regulates the activity of NADK.

ABBREVIATIONS USED

CaM, calmodulin; cAMP, cyclic adenosine monophosphate; DEAE-Sephacel, diethylaminoethyl Sephacel; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; GPDH, glucose-6P dehydrogenase; GP, glucose-6-posphate; PDE, cyclic nucleotide phosphodiesterase; NAD, nicotinamide adenine dinucleotide; NADK, nicotinamide adenine dinucleotide kinase; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced nicotinamide adenine dinucleotide phosphate; SDS−PAGE, dodecyl sulfate−polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride.

LITERATURE CITED

(1) Lee, S. H.; Johnson, J. D.; Walsh, M. P.; Van Lierop, J. E.; Sutherland, C.; Xu, A.; Snedden, W. A.; Kosk-Kosicka, D.; Fromm, H.; Narayanan, N.; Cho, M. J. Differential regulation of Ca^{2+}/calmodulin-dependent enzymes by plant calmodulin isoforms and free Ca^{2+}concentration. *Biochem. J.* **2000**, *350*, 299−306.

(2) Zielinski, R. E. Calmodulin and calmodulin-binding proteins in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 697−725.

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(3) Au, T. K.; Wallace, S. H.; Chick, W. S.; Leung, P. C. The biology of ophiobolins. *Life Sci.* **2000,** *67,* 733−742.

(4) Pala, I.; Srinivasan, A.; Vig, P. J. S.; Desaiah, D. Modulation of calmodulin and proteins kinase C activities by *Penicillum* mycotoxins. *Int. J. Toxicol.* **1999**, *91*, 91−96.

(5) Vertessy, B. G.; Ramat, V.; Böcskei, Z.; Náray-Szabó, G.; Orosz, F.; Ovádi, J. Simultaneous binding of drug with different chemical structures to Ca^{2+}-calmodulin: Crystallographic and spectroscopic studies. *Biochemistry* **1998**, *37*, 15300−15310.

(6) Nishino, H.; Naitoh, A.; Umezawa, K. Quercetin interacts with calmodulin, a calcium regulatory protein. *Experientia* **1984**, *40*, 184−185.

(7) Macías, M.; Ulloa, M.; Gamboa, A.; Mata, R. Phytotoxic compounds from the new coprophilous fungus *Guanomyces polythrix. J. Nat. Prod.* **2000**, *63*, 757−761.

(8) Macías, M.; Ulloa, M.; Gamboa, A.; Toscano, R. A.; Mata, R. Phytotoxic naphthopyranone derivates from the coprophilous fungus *Guanomyces polythrix. Phytochemistry* **2001**, *58*, 751−758.

(9) Sharma, R. K.; Wang, J. H. Preparation and assay of the Ca^{2+}-dependent modulator protein. In *Advances in Cyclic Nucleotide Research*; Greengard, P., Robinson, G. A., Eds.; Raven Press: New York, 1979; Vol. 10, pp 187−198.

(10) Leung, P. C.; Taylor, W. A.; Wang, J. H.; Tripton, C. L. Ophiobolin A a natural product inhibitor of calmodulin. *J. Biol. Chem.* **1984**, *259*, 2742−2747.

(11) Sumner, J. B. Scientific apparatus and laboratory methods. A method for the colorimetric determination of phosphorous. *Science* **1944**, *100*, 413−415.

(12) Harmon, A. C.; Jarret, H. W.; Cormier, M. J. An enzymatic assay for calmodulins based on plant NAD kinase activity. *Anal. Biochem.* **1984**, *141*, 168−178.

(13) Zhang, Y.; Mills, G. L.; Nair, M. G. J. Cyclooxygenase inhibitory and antioxidant compounds from the mycelia of the edible mushroom *Grifolia frondosa. J. Agric. Food Chem.* **2000**, *50*, 7581−7585.

(14) Ovadi, J. Effect of drugs on calmodulin-mediated enzymatic actions. In *Progress in Drug Research*; Jucker, E., Ed.; Birkhauser Verlag: Basel, Switzerland, 1989; Vol. 33, pp 353−395.