Brief Report

Caulerpenyne Affects Bradykinin-Induced Intracellular Calcium Kinetics in LoVo Cells

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Abstract: Sesquiterpene cauleronpenyne (CYN) is the major metabolite present in green macroalgae Caulerpa taxifolia. This metabolite has been shown to be cytotoxic in some cell lines and was found to be active in various assays of pharmacological interest. In addition, it exerts antibiotic, antiviral, phytotoxic, antidysslipidemic, and antiproliferative activities. In the present study, we report that pretreatment with CYN decreases the bradykinin-induced calcium peak in human colon LoVo cells. We hypothesize that CYN pretreatment may adversely affect bradykinin-induced intracellular calcium increases. The data suggest that CYN, by reducing the increase in intracellular calcium, exerts an inhibitory role on calcium homeostasis and, likely, intercellular transmission.

Keywords: cauleronpenyne; LoVo cells; intracellular calcium concentration; bradykinin; Fura-2

Highlights:
The human colon cell line LoVo was used to study the effects of cauleronpenyne.
The cauleronpenyne pretreatment affects bradykinin-induced intracellular calcium concentrations.
Cauleronpenyne’s effect on rising levels of intracellular calcium is dose-dependent.

1. Introduction

Caulerpa taxifolia (M. Vahl) C. Agardh (Chlorophyta, Caulerpales) is a macroalgae of tropical origin that was accidentally introduced into the Mediterranean in 1984. It has been reported that subjects who had food poisoning following the consumption of Sarpa salpa (known commonly as cow bream, dreamfish, salema, and salema porgy), a fish that feeds on C. taxifolia, also exhibited some neurological disorders, including amnesia, dizziness, and hallucinations [1]. These symptoms have been attributed to the major metabolite produced by C. taxifolia, sesquiterpene cauleronpenyne (CYN), which can permeate the plasma membrane. Caulerpa’s toxic metabolites are transferred through marine food chains, concentrating in herbivores [2]; CYN reduces their palatability and activates their antioxidant defenses [3]. CYN also presents antiproliferative, antibiotic, and antiviral activities [4–6]. This metabolite has exhibited cytotoxic properties in sea urchins and mammalian cells [7–10]. In particular, the effect of CYN on the human neuroblastoma SK-N-SH cell line was investigated [9]. Its antiproliferative activity in the cancer cells of SK-N-SH cell lines and the ability to modify the microtubule network and the long biopolymers that contribute to maintaining the cytoskeleton of the cells have been highlighted. In addition, CYN inhibits α-amylase, pancreatic lipase, and phospholipase A2 activity [11–13]. However, CYN has shown inhibitory effects on soybean lipoxygenase [14]. Therefore, CYN has been shown to have inhibitory effects on cell growth, with some variability between different cell lines. Brunelli et al. [15] and Mozzachiodi et al. [16] found that CYN exerts an inhibitory effect on Na⁺/K⁺-ATPase activity, analyzing the role of the metabolite on sensory T-cells in the leech Hirudo medicinalis (Annelida). In the leech swimming induction learning behavioral model, CYN application reproduces the effect of a nociceptive stimulation, causing an enhancement of the animal’s swimming behavior.
response to the test stimulus (sensitization) [15]. This effect is analogous to that induced by endogenous neurotransmitter serotonin. Barbier et al. [9] showed that CYN influences some ion channels, which exhibit a reduction in posthyperpolarization amplitude as well as a reduction in cell membrane resistance. Pesando et al. [17] found that CYN inhibited intracellular ATP-dependent Ca\(^{2+}\) storage in a dose-dependent manner without causing the release of Ca\(^{2+}\) stored in sea urchin eggs. The effect is similar to that of thapsigargin, a specific inhibitor of the Ca\(^{2+}\) pump of the endoplasmic reticulum, suggesting that CYN may prevent intracellular sequestration of Ca\(^{2+}\) by inhibiting the activity of the pump. Coordinated and efficient control of the processes that alter the concentration of free intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is fundamental to the maintenance of cellular Ca\(^{2+}\) homeostasis.

Ca\(^{2+}\) can be released from the intracellular storage compartments by specific signals, such as the activation of receptors for inositol 1,4,5-triphosphate (IP3), present in the endoplasmic reticulum. In particular, stimulation of the extracellular mediator at the G-protein-coupled receptor activates the enzyme phospholipase-C, which, in turn, catalyzes the production of second messengers IP3 and diacylglycerol. IP3 binds to IP3 receptors (IP3R) and mediates the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) deposits [18]. IP3R is regulated by a wide variety of interacting proteins, such as calmodulin, kinases, phosphatases, and components of the cytoskeleton, including bradykinin (BDK), a phosphoinositide agonist of membrane cell activity. BDK induces a fast translocation of protein kinase C isoforms from the cytosol to the plasma membrane and acute increases in [Ca\(^{2+}\)]\(_i\) [19].

In the present study, the human colon cell line LoVo is used as a model system to investigate the effects of CYN. The choice of the cell line was suggested because human colorectal cancer cells are particularly sensitive to CYN [4]. The effects of CYN on [Ca\(^{2+}\)]\(_i\) kinetics in cultured human colon adenocarcinoma LoVo cells are evaluated, and the modulatory effects of CYN on BDK-evoked intracellular calcium fluxes are examined.

2. Materials and Methods

2.1. Cell Cultures

The human colon cancer epithelial cell line LoVo was cultured in 10-cm\(^2\) culture dishes in F-12K medium (Kaighn’s modification of Ham’s F-12 medium) containing 2 mM l-glutamine and 1500 mg/L sodium bicarbonate, supplemented with 100 µg/mL penicillin, 100 µg/mL streptomycin, 1 mmol/L HEPES buffer, and 10% fetal bovine serum (FBS) in humidified air (5% CO\(_2\)) at 37 °C.

2.2. Solutions

CYN was purified from fresh algae collected in Imperia (Italy) using the extraction protocol used by Pesando et al. (1996). Briefly, algae were air-dried and extracted with n-hexane-dichloro-methane, filtrated, and concentrated under pressure. The extracted oily liquid was solubilized in n-hexane and subjected to chromatography. The last fraction containing CYN was applied to exclusion chromatography. The fraction that was retained was chromatographed on an open eluted silica column and subjected to two-step high-pressure liquid chromatography (HPLC). The yield of pure CYN was 0.16% on the dry weight of algae. Finally, CYN was dissolved in dimethylsulfoxide (DMSO), sonicated, and added to a saline solution (DMSO final concentration 0.1%).

Krebs–Ringer–Hepes (KRH) solution (in mM) was as follows: NaCl 136, KCl 4.7, CaCl\(_2\) 1.3, MgCl\(_2\) 1.25, glucose 10, and HEPES 10, with the pH adjusted to 7.4 by sodium hydroxide (NaOH).

2.3. Treatment with Caulerpenyne

LoVo cell monolayers were preincubated with CYN for three hours at different concentrations of CYN (1, 2, 5, 10, 20, 50, and 100 µM), washed three times in KRH, and analyzed 24 or 48 h after CYN removal.
2.4. Measurement of Intracellular Free Calcium Concentration ([Ca\textsuperscript{2+}])

Spectrofluorimetric measurement was performed according to Traina et al.’s [20] protocol. Briefly, LoVo cells (1 × 10\textsuperscript{6}/mL) were washed in KRH, dissociated, and loaded for 30 min at 37 °C, with Ca\textsuperscript{2+}-sensitive dye, fura-2 acetoxy-methyl (Calbiochem) ester, added to the final concentration of 2 µM with 0.2% bovine serum albumin. Loaded cells were resuspended in the incubation medium, supplemented with 2.5 × 10\textsuperscript{-4} M sulfinpyrazone to prevent dye leakage, transferred in a quartz thermostated cuvette (37 °C), and kept under continuous stirring as a homogeneous population. Fluorimetric measurements of [Ca\textsuperscript{2+}] were performed on a homogeneous population of LoVo cells with an LS-50B luminescence spectrometer (Perkin Elmer Life Sciences, Waltham, MA, USA) with a double excitation wavelength (340 and 380 nm) and an emission wavelength (510 nm). Data were collected every 40 ms and calibrated in terms of [Ca\textsuperscript{2+}]. The fura-2 fluorescence response was calibrated using calcium standards (Calcium Calibration Buffer Kit, Molecular Probes, Invitrogen, Karlsbad, CA, USA) in the absence of cells, as described in [20–22]. Baseline [Ca\textsuperscript{2+}] was measured for 100 s without any addition. Then, BDK was quickly added via pump injector at the final concentration of 100 nM. The resultant changes in [Ca\textsuperscript{2+}] were monitored for 8 min. Baseline [Ca\textsuperscript{2+}] levels were 80–100 nM. Cells with baseline > 100 nM were not considered for the study.

2.5. Cytotoxicity of CYN

The cytotoxicity of CYN was assessed by colony formation assay. Cells were collected by trypsinization, counted, and plated at a density of 10,000 cells/well in 96-well flat-bottomed microtiter plates. CYN was tested at scalar concentrations, ranging from 1 to 15 µM, for 24 h, followed by 24 h culture in drug-free medium. The number of colonies was counted, and the surviving fraction was determined as the ratio of the number of colonies in the CYN-treated sample to the number of colonies in the control sample. Triplicate wells were set up for each concentration.

2.6. Statistical Analysis

The values of the [Ca\textsuperscript{2+}] variations (concentration reached by the spike-like component of the transient) were analyzed by ANOVA for variance and Fisher’s t-test for significance using Sigma Stat software. Significance is indicated with an asterisk (p < 0.001). Different concentrations were compared between them (same letter, no statistical significance; different letters, statistical significance, p < 0.01).

3. Results and Discussion

3.1. Cytotoxicity Assay

Since CYN is cytotoxic, a proliferation assay on LoVo cells was evaluated after treatment with CYN. Cytotoxicity was evaluated as a survival rate of cell clones at different concentrations of CYN (1–15 µM; Figure 1). The regression line was calculated using the least square method and is expressed by the following function: f (y) = −6.445 × + 94.447. CYN induced inhibition of LoVo cell proliferation, with an IC50 value of 6.9 µM after 24 h of incubation. This effect was in agreement with previous studies conducted with the toxin on other cell lines [4].

For the experiments of the present study, clones of CYN-resistant mutant LoVo cells were selected.

3.2. CYN on Intracellular Free Calcium Concentration, [Ca\textsuperscript{2+}]

It is well known that calcium ion (Ca\textsuperscript{2+}) concentration serves as an important intracellular signal for a very wide variety of cellular phenomena, including proliferation and differentiation, regulation of gene expression, stimulus–secretion coupling, and motility [23]. It is known that sustained and prolonged increases in [Ca\textsuperscript{2+}], over time, are toxic to cells and are involved in triggering events that lead to cell apoptosis. [24]. The role of
CYN on intracellular free calcium was investigated in human colon epithelial LoVo cells, utilizing fura-2 fluorescence.

Figure 1. Cytotoxicity assay. Regression line for the cytotoxic effect of sesquiterpene caulerpenyne (CYN) on LoVo cells. Control (100% survival) is provided by incubation in DMSO. Time of exposure: 24 h.

Bradykinin (BDK) is an agonist of cell membrane activity and was used as a positive control in this study. BDK induced transient increases in \([Ca^{2+}]_i\), likely by interacting with specific membrane receptors and activating intracellular mechanisms through the phosphoinositide-phospholipase pathway [25]. BDK was added to the cuvette at a final concentration of 100 nM. BDK elicits a rapid, transient elevation of \([Ca^{2+}]_i\) in LoVo cells (Figure 1). The effect has two components: a first phase is a spike-like increase, followed by a slow plateau. BDK mediates both the \(Ca^{2+}\) release from internal pools and a small entry of \(Ca^{2+}\) into the cytoplasm from the extracellular space, as suggested by the plateau phase (26–30 nM) [26] (Figure 2).

Figure 2. Typical trace of bradykinin (BDK) application in LoVo cells. BDK elicits a rapid, transient phase of elevation of \([Ca^{2+}]_i\), followed by a slow steady-state phase.
In LoVo cells that have not been pretreated with CYN, the spike-like component caused an increase in $[Ca^{2+}]_i$ of 91.2 ± 7.9 nM (Figure 2).

In a series of experiments, spectrofluorimetric analysis was performed 24 h after incubation time in CYN in a second series of experiments, 48 h after the incubation in CYN. Different CYN incubation times were tested, and CYN was applied without preincubation (data not shown). A choice was made for 3 h of preincubation.

In particular, preincubation with CYN and spectrofluorimetric analysis 24 h after CYN removal caused a decrease in the $[Ca^{2+}]_i$ transient peak (mean ± SEM, n = 4; Figure 3).

![Figure 3](image-url)

**Figure 3.** Histograms summarizing the effects of pretreatment of CYN at different concentrations (3 h of incubation) on LoVo cells. Pretreatment with CYN and spectrofluorimetric analysis 24 h after CYN removal. Data are means ± SEM (n = 4); significance * $p < 0.001$ compared to control group. Different concentrations were compared between them (same letter, no statistical significance; different letters, statistical significance, $p < 0.01$). BDK, bradykinin; CYN, caulerpenyne; $[Ca^{2+}]_i$, intracellular calcium concentration.

Thus, it appears that the preincubation of cells with CYN induces a concentration-dependent decrease in the first component (spike-like) of the BDK increase in $[Ca^{2+}]_i$.

After the transient peak, $[Ca^{2+}]_i$ dropped to a plateau level of 10–20 nM for all different CYN incubation concentrations. This plateau persisted for up to 7 min of observation.

In the second series of experiments, the application of BDK and the analysis of $[Ca^{2+}]_i$ were evaluated 48 h after preincubation in CYN.

BDK alone in LoVo cells that were not pretreated with CYN (control) caused a rise in $[Ca^{2+}]_i$ of 88.25 ± 8.8 nM (spike-like component). Preincubation with CYN and spectrofluorimetric analysis 48 h after CYN removal caused a decrease in the $[Ca^{2+}]_i$ transient peak (mean ± SEM, n = 4). Again, the second component of BDK-induced calcium kinetics was a plateau level of 10–20 nM. Even in this condition, preincubation of cells with CYN induced a concentration-dependent decrease in the first component (spike-like) of the BDK-induced $[Ca^{2+}]_i$ increase (Figure 4).

A major effect is observed 24 h after preincubation in CYN (Figure 3). The effect could be related to the inhibitory action of CYN on phospholipase activity. CYN could affect the kinetics of IP3R and the calcium channel, possibly by reducing the time of the channel’s open state.
Figure 4. Histograms summarize the effects of pretreatment of CYN at different concentrations (3 h of incubation) on LoVo cells. Pretreatment with CYN and spectrofluorimetric analysis 48 h after CYN removal. Data are means ± SEM (n = 4); significance * p < 0.001 in comparison to control group. Different concentrations were compared between them (same letter, no statistical significance; different letters, statistical significance, p < 0.01). BDK, bradykinin; CYN, caulerpenyne; [Ca^{2+}]_i, intracellular calcium concentration.

Another aspect that emerges from the results is that pretreatment with CYN leads to a typical biphasic response on [Ca^{2+}]_i levels, suggesting that CYN could have a hormetic effect, depending on its concentration. As is well known, hormesis is considered an adaptive function characterized by a dose-dependent biphasic response [27]. Pesando et al. [17] reported that CYN reduced intracellular ATP-dependent Ca^{2+} storage in a dose-dependent manner without causing sequestered Ca^{2+} release. The study has suggested that the ionic signals involved in cell dynamics would be altered.

4. Conclusions

Ca^{2+} is the paradigm of intracellular messengers, and it presides over numerous cellular functions. However, if its intracellular concentration remains high for a long time, it can become highly toxic and lead to cell death. The selective control of intracellular calcium is, therefore, of particular importance. Pretreatment with CYN is able to influence the BDK-induced [Ca^{2+}]_i increase in LoVo cells. The functional significance of CYN in the control of intracellular calcium can be expressed in the ability to inhibit calcium and, therefore, also intercellular transmission.

Although this study has limitations, as it lacks mechanistic analysis, it is the first spectrofluorimetric work on cytosolic calcium that analyses the effect of CYN in LoVo cells. Future studies will delve into the signaling pathway and its functional role.

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Abbreviations

BDK  bradykinin
CYN  caulerpenyne
[Ca\(^{2+}\)]  intracellular calcium concentration
IP3  inositol 1,4,5-trisphosphate
IP3R  inositol 1,4,5-trisphosphate receptor
KRH  Krebs–Ringer–Hepes
PLC  phospholipase C

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