Cochlioquinone A, an Inhibitor of Diacylglycerol Kinase

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The effects of cochlioquinone A, isolated from *Drechslera sacchari*, were studied *in vitro* and *in vivo*. This compound specifically inhibited diacylglycerol kinase activity with $Ki=3.1\,\mu M$. The kinetics revealed that cochlioquinone A inhibited diacylglycerol kinase in competition with ATP, and non-competitively with diacylglycerol. The compound inhibited neither protein kinase C, epidermal growth factor receptor-associated protein tyrosine kinase, nor phospholipase C. Cochlioquinone A reduced the concentration of phosphatidic acid in T cell lymphoma with a half maximal concentration of $3\,\mu M$, and simultaneously augmented the phosphorylation of 80 kDa protein, a known substrate of protein kinase C. The degree of the phosphorylation of 80 kDa protein in the presence of cochlioquinone A was similar to that in the presence of phorbol myristate acetate ($0.1\,\mu g/ml$).

These results demonstrate that cochlioquinone A is a specific inhibitor of diacylglycerol kinase, which regulates the activity of protein kinase C.

It is known that cell proliferation is regulated by several protein kinases. The regulation of protein kinase C is particularly well understood. Its activator has been identified as diacylglycerol which is a product of phospholipase C, a priming-enzyme of signal transduction[1,2]. Phorbol myristate acetate, a potent tumor-promoter, directly activates protein kinase C. This suggests a significant role of protein kinase C in the proliferative signaling of cells.

Diacylglycerol is produced from phospholipids by the action of phospholipase C, and is converted to phosphatidate by the action of diacylglycerol kinase, while phosphatidate is reversed to diacylglycerol by phosphatidic acid phosphatase. Thus, diacylglycerol kinase plays a crucial role in the regulation of the concentration of diacylglycerol and in the activation of protein kinase C. Some evidence suggests that diacylglycerol kinase activity is augmented in epidermal growth factor (EGF)-treated A431 cells, *erb-B*-transformed fibroblasts, and vasopressin-stimulated hepatocytes[3~5]. Recent studies suggest that diacylglycerol kinase is classified into at least four distinct subtypes. Localization of these subtypes in specific tissues is also reported by SAKANE *et al.*[6]

We have screened inhibitors of diacylglycerol kinase, and isolated cochlioquinone A and stemphone from *Drechslera sacchari*[7]. In this report, we describe the inhibitory effects of cochlioquinone A *in vitro* and *in vivo*.

Materials and Methods

Materials

[γ-^{32}P]-ATP (6000 Ci/mmol) and [^{32}P]-orthophosphoric acid (8500~9120 Ci/mmol) were purchased from New England Nuclear Inc. All subtypes of diacylglycerol and other lipids were purchased from Sigma Chemical Co. Diacylglycerol kinase from *Escherichia coli* was purchased from Calbiochem. Co. BW5147 and Swiss 3T3 cells were obtained from Japan Cancer Research Bank. All other reagents used in this study were of the highest grade available.

Diacylglycerol Kinase Assay

Activity of diacylglycerol kinase was determined by the measurement of [^{32}P]-phosphatidic acid formed from [γ-^{32}P]-ATP and diacylglycerol[6]. A reaction mixture ($50\,\mu l$) containing Tris-HCl 50 mM (pH 7.5), NaCl 50 mM, bovine serum albumin 0.1%, ethyleneglycol bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid 0.5 mM, $MgCl_2$ 5 mM, sodium deoxycholate 0.04%, dilauroyl glycerol 0.22 mM, [γ-^{32}P]-ATP $100\,\mu M$ ($1\,\mu Ci$), and enzyme protein (40 ng), was incubated at 37°C for 20 minutes with or without $2\,\mu l$ of inhibitor solution in methanol. The reaction was terminated with 0.125 ml of 1 N HCl and 0.5 ml of $CHCl_3$ - MeOH - HCl (200 : 100 : 1) was added to the mixture for separation of [^{32}P]-phosphatidic acid from [γ-^{32}P]-ATP. The mixture was vigorously aggitated and spun down for removal of the aqueous phase from the organic phase. The organic phase was washed once with 0.5 ml of distiled water, evaporated to dryness, and extracted three times with $10\,\mu l$ of $CHCl_3$. These extracts were spotted at the same origin on a silica gel thin layer plastic plate (Merk Kieselgel 60 F_{254}). The TLC plate was developed with $CHCl_3$ - MeOH - AcOH - H_2O (170 : 25 : 25 : 6). [^{32}P]-phosphatidic acid was detected by autoradiography and the radioactivity was measured by Cerenkov counting.

Measurement of Phosphatidic Acid

BW5147 T cell lymphoma (1×10^6 cells) was labeled

with 20 μCi of [^{32}P]-orthophosphoric acid in 0.5 ml of Hanks solution for 1 hour in the presence or absence of the inhibitor. The cells were spun down at 800 × g for 5 minutes and the cell pellet was suspended with 1 ml of Dulbecco's phosphate-buffered saline, and spun down again. The cells were suspended in 0.1 ml of Dulbecco's phosphate-buffered saline, and mixed with 0.5 ml of $CHCl_3$-MeOH-HCl (200:100:1) and 0.16 ml of 1 N HCl. [^{32}P]-phosphatidic acid in the organic phase was concentrated to dryness and separated by TLC and the radioactivity was measured as described in the section of "Diacylglycerol kinase assay".

Preparation of Diacylglycerol Kinase

Diacylglycerol kinase was partially purified by the method of KANOH *et al.*[8]. Twelve grams of bovine thymus was minced with 20 ml of buffer A (sucrose 0.25 M, Tris-HCl 25 mM (pH 7.5), dithiothreitol 0.5 mM, ATP 10 μM, ethylenediamine N,N,N′,N′-tetraacetic acid 1 mM and leupeptin 1 μg/ml), and centrifuged at 12,000 × g for 30 minutes. The supernatant solution was applied on a DE-52 ion exchange chromatographic column (1.5 × 5 cm) which had been equilibrated with buffer A, and step-wisely eluted with 270 ml of 0.05 M NaCl, 180 ml of 0.1 M NaCl, and 150 ml of 0.2 M NaCl in buffer A. Fractions were collected in a volume of 10 ml. Diacylglycerol kinase activity was eluted in fractions of 0.2 M NaCl in buffer A. The active fractions (No. 48~52, 50 ml) were combined and directly applied on an AF-heparin-Toyopearl column (0.5 cm × 5 cm) which had been equilibrated with 0.2 M NaCl in buffer A. After washing with the equilibration buffer, the enzyme was eluted from the column with 20 ml of a linearly increasing concentration gradient of NaCl from 0.2 M to 1 M in buffer A, and collected in 1-ml fractions. Diacylglycerol kinase activity was detected at around 0.7 M NaCl. The active fractions (No. 9~16, 8 ml) were combined and stored at −80°C.

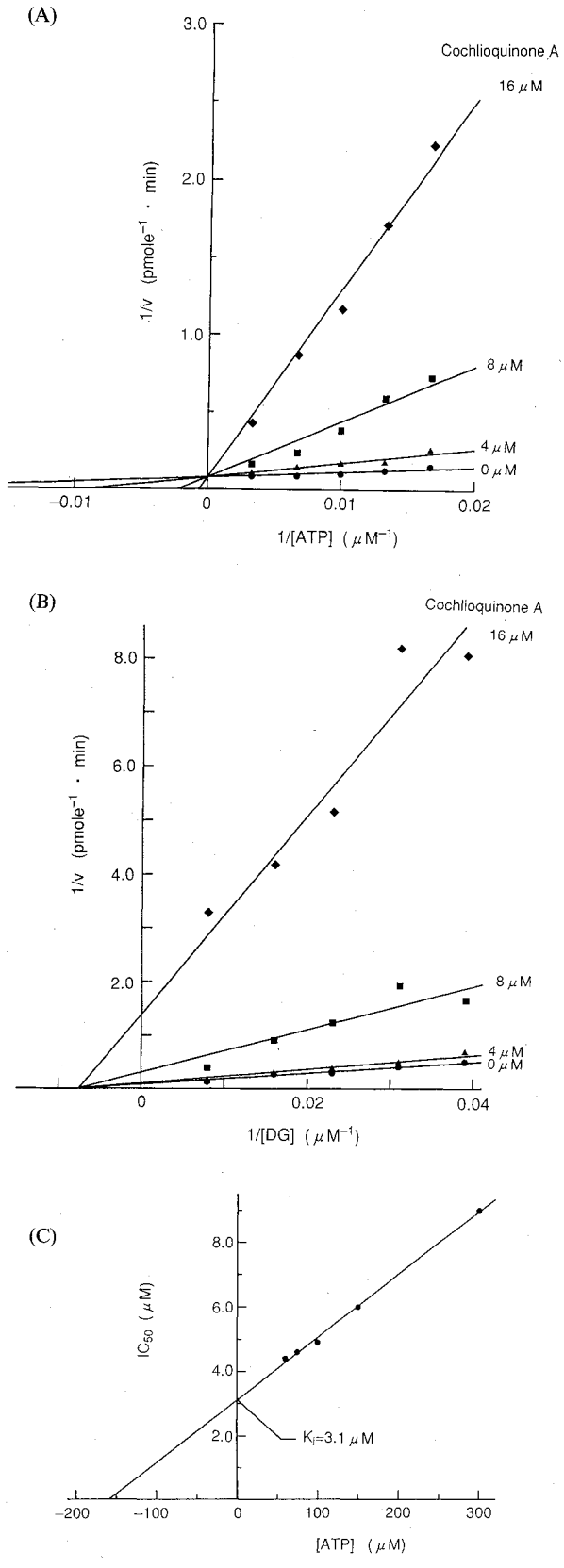
Measurement of Other Enzymes

Protein kinase C, phosphatidylinositol kinase, EGF receptor-associated protein tyrosine kinase, choline phosphotransferase, and hexokinase were measured by the method of ERUSALIMSKY *et al.*[9], YAMAKAWA *et al.*[10], AKIYAMA *et al.*[11], ISHIDATE *et al.*[12], and BÜCHER *et al.*[13], respectively. Phospholipase C activity was measured as described previously[14,15].

Results and Discussion

Cochlioquinone A and stemphone were isolated from *Drechslera sacchari* as inhibitors of diacylglycerol kinase[7]. Because of the very low yield of fermentation, the available amount of stemphone did not allow the kinetic study on the mode of inhibition of stemphone. Moreover, as stemphone structurally differs in the side chain only, the inhibitory properties of cochlioquinone

Fig. 1. Inhibition kinetics of cochlioquinone A on the activity of diacylglycerol kinase.



Diacylglycerol kinase activity was assayed with varying concentrations of each substrate, *i.e.*, ATP (A) or 1,2-dilauroylglycerol (B) in the combination with cochlioquinone A (0, 4, 8, 16 μM). *Ki* for cochlioquinone A was determined from the secondary plot of IC_{50} of cochlioquinone A versus ATP concentrations (C).

A may correspondingly be similar to those of stemphone. Thus, we further investigated the properties of cochlioquinone A.

The results of kinetic studies are shown in Fig. 1. Lineweaver-Burk plots show that the inhibition mode of cochlioquinone A was competitive with ATP (Fig. 1A) and non-competitive with diacylglycerol (Fig. 1B). When the apparent *Km* values of Fig. 1A or the reciprocals of the apparent V_{max} values of Fig. 1B were replotted against the concentration of cochlioquinone A, the lines of the plots curved upwards (data not shown), indicating the positive cooperative binding or micellar formation of cochlioquinone A. We determined therefore the *Ki* value as IC_{50} by extrapolating the ATP concentration to zero, because the inhibition mode of cochlioquinone A was apparently competitive with ATP (Fig. 1A). This analysis is based on the following equations.

In general, the competitive inhibition kinetics can be written as follows[17]:

$$v = \frac{V_{max}}{1 + \frac{Km}{[S]}\left(1 + \frac{[I]}{Ki}\right)}$$

Let v_i = the initial velocity at a given [S] in the presence of inhibitor,

v = the initial velocity at a given [S] in the absence of inhibitor,

and $\frac{v_i}{v}$ = the fractional activity

$$\frac{v_i}{v} = \frac{Km + [I]}{Km\left(1 + \frac{[I]}{Ki}\right) + [S]}$$

Because IC_{50} is an inhibitor concentration giving a fractional activity = 0.5, an expression for IC_{50} at a given substrate concentration can be written as follows:

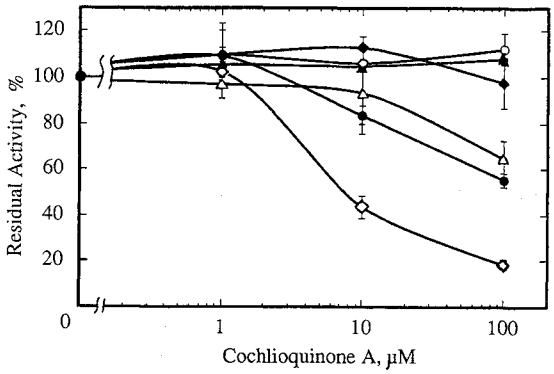
$$IC_{50} = \frac{([S] + Km)Ki}{Km}$$

This equation reasonably expects a linear relationship between the IC_{50} of cochlioquinone A and the competitor substrate ATP. Consequently, when IC_{50} values are plotted against substrate concentrations, the intercept on the IC_{50} axis gives the *Ki* value, while the intercept on the substrate concentration axis yields the *Km* value.

Fig. 1C shows the linear plot of IC_{50} of cochlioquinone A versus ATP concentration. The *Ki* value was determined as 3.1 μM. The *Km* value for ATP obtained from the horizontal axis of Fig. 1C was also similar with that obtained from Fig. 1A. The result suggests that cochlioquinone A shares a binding site with ATP.

Fig. 2. Specificity of cochlioquinone A.

Diacylglycerol kinase (◇), protein kinase C (○), choline phosphotransferase (●), EGF-receptor-associated protein tyrosine kinase (◆), inositol phospholipid-specific phospholipase C (▲), and phosphatidylinositol 4-kinase (△) activities were assayed with varying concentrations of cochlioquinone A.

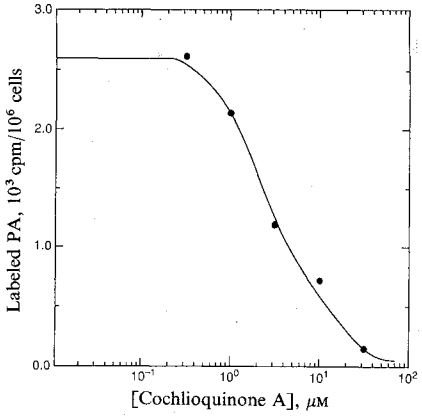


Enzyme assays were carried out as described in the section of materials and methods. The bar represents the standard error of triplicate experiments.

Several kinases and inositol phospholipid specific enzymes were assayed in the presence or absence of cochlioquinone A (Fig. 2). One hundred μM of cochlioquinone A inhibited neither phospholipase C, protein kinase C, nor EGF-receptor-associated protein tyrosine kinase. Cochlioquinone A (100 μM) decreased the activity of hexokinase from baker's yeast as much as 86% of the control activity (data not shown). Phosphatidylinositol 4-kinase and choline phosphotransferase were inhibited less significantly ($IC_{50} \geq$ 100 μM) than diacylglycerol kinase. Diacylglycerol kinase from *E. coli* was not inhibited, but rather activated as much as 6-fold at 100 μM of cochlioquinone A (data not shown). In conclusion from the kinetic analyses, cochlioquinone A specifically inhibited bovine diacylglycerol kinase.

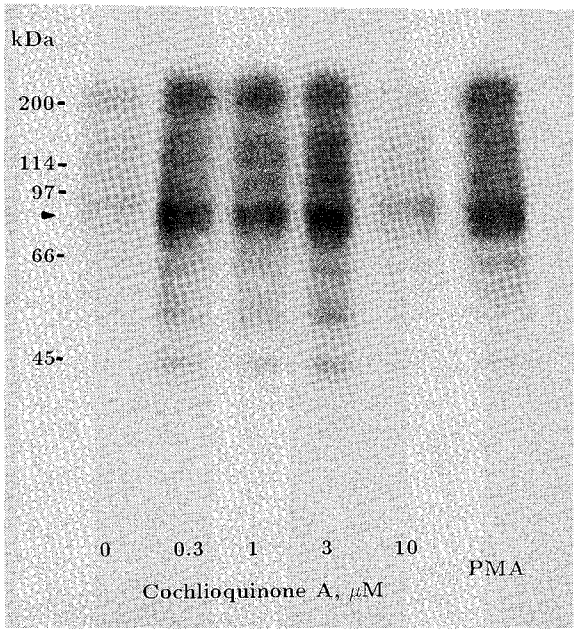
Theoretically speaking, inhibition of diacylglycerol kinase in cultured cells might lead to the decreased concentration of phosphatidate as well as the increased concentration of diacylglycerol, consequently resulting in the activation of protein kinase C. For experimental validation, the *in vivo* effects of cochlioquinone A on BW5147 T cell lymphoma (JCRB9002), which had a high level of phosphatidate, were examined by measureing the phosphatidate concentration. Fig. 3 shows that cochlioquinone A significantly reduced the concentration of phosphatidate of BW5147 T cell lymphoma with

Fig. 3. Effect of cochlioquinone A on phosphatidic acid.



T cell lymphoma BW5147 cells were loaded with 50 μCi of orthophosphoric acid for 1 hour, then the cells were treated with or without indicated concentrations of cochlioquinone A for 20 minutes. Extraction and measurement of phosphatidic acid were described in the section of materials and methods. Values are expressed as means ±S.D. of triplicated experiments.

Fig. 4. Activation of protein kinase C by cochlioquinone A.



Swiss 3T3 cells, which had been loaded with 50 μCi of [^{32}P]-orthophosphoric acid for 1 hour, were treated with or without indicated concentrations of cochlioquinone A for 20 minutes or with 0.1 μg/ml of phorbol myristate acetate for 5 minutes. Cell lysate was subjected to sodium dodecylsulfate polyacrylamide gel electrophoresis and autoradiography. Lanes from left to right: 0, no addition; 0.3, 1, 3, 10, 0.3, 1, 3, and 10 μM of cochlioquinone A; PMA, phorbolmyristate acetate (0.1 μg/ml), respectively. Molecular weights of each marker were shown on the leftmost side. Arrow indicats the position of 80 kDa protein kinase C substrate (MARCKS).

$IC_{50}=3\,\mu M$. This IC_{50} value conformed with the *Ki* value, 3.1 μM, against diacylglycerol kinase (Fig. 1C). This suggests that cochlioquinone A also inhibits diacylglycerol kinase *in vivo*. It is of interest whether the inhibition of diacylglycerol kinase induces the activation of protein kinase C *in vivo*. Thus, protein kinase C activity was measured by the phosphorylation of 80 kDa protein kinase C substrate known as MARCKS[9,18], a typical protein kinase C substrate established in Swiss 3T3 cells. Fig. 4 shows the stimulation of the phosphorylation of the 80 kDa protein by cochlioquinone A. In comparison with the result without cochlioquinone A, the addition of cochlioquinone A enhanced the phosphorylation of 80 kDa protein as much as that of phorbol myristate acetate (0.1 μg/ml) did.

These results suggest that cochlioquinone A inhibited cellular diacylglycerol kinase and consequently stimulated protein kinase C. It is of interest that cochlioquinone A was reported earlier as a nematocidal drug[16], although relationship between nematocide and the inhibition of diacylglycerol kinase is not obvious. It seems likely that cochlioquinone A may be employed as a specific tool for the study of the regulatory mechanisms of diacylglycerol kinase and protein kinase C.

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