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PHOTOACTIVATED INHIBITION OF SUPEROXIDE GENERATION AND PROTEIN KINASE C ACTIVITY IN NEUTROPHILS BY BLEPHARISMIN, A PROTOZOAN PHOTODYNAMICALLY ACTIVE PIGMENT

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Abstract—Blepharismin is an endogenous photosensitizing pigment found in the protozoan *Blepharisma*. This pigment inhibited the generation of superoxide anion ($O_2^{\cdot-}$) in neutrophils not only via a diacylglycerol-induced protein kinase C (PKC)-dependent reaction but also by an arachidonate-induced PKC-independent reaction. The inhibition was light and concentration dependent for both reactions. Light-activated inhibition was strong at wavelengths between 520 and 570 nm but not above 610 nm. PKC activity in neutrophils and from rat brain was inhibited by blepharismin in a light- and concentration-dependent manner. Moreover, arachidonate-activated NADPH oxidase activity in a cell-free system was also inhibited by the pigment in a light- and concentration-dependent manner. These results suggest that blepharismin inhibits NADPH oxidase activation through the non-specific inhibition of various membrane-bound enzymes and that this inhibition may also be correlated with that of PKC.

Key words: active oxygen generation; fluorescence of pigment; perylenequinones; enzyme inhibition; NADPH-oxidase; tyrosine kinase

Blepharisma, a protozoan, has a photosensitizing red pigment, blepharismin. The biological function of the pigment is problematic since the animal can live with or without it [1, 2]. The pigment is toxic to some other kinds of protozoa but only kills those cells when exposed to very high light intensity (in excess of 2000 ft. candles or 100×10^{-3} W/cm^2) [3–5]. Blepharismin is easily extracted from the organism by organic solvents such as ethanol, chloroform and acetone [6–10]. The natural pigment is red but is changed to various colors by exposure to visible light in the presence of oxygen [4, 6]. The color of the pigment also depends on the hydrogen ion concentration [6]. Its chemical structure is currently unknown but it is thought to have a basic structure similar to that of hypericin, a polycyclic anthrone which has been isolated from the St. Johnswort plant (herb *Hypericin triquetrifolium* Turra) [11]. From its unique properties it is thought to be a polymerized form of that pigment and a suggested structural formula is shown in Fig. 1 [12]. Hypericin was synthesized by Brockman *et al.* [13] and has been shown to have powerful anti-retroviral activity against FLV†† and radiation leukemia virus in mice [14]. Recently it has been reported that hypericin has virucidal activity against enveloped and non-enveloped DNA and RNA viruses [15] and that it inhibits protein kinase C [16]. It has also been found that various inhibitors of protein kinase C depress the production of phorbol ester-induced human immunodeficiency virus type 1 virion [17]. Furthermore, calphostin C, a recently isolated compound from *Cladosporium cladosporioides*, which has a structure that includes a perylene quinone, possesses extremely potent biological activity as shown by its antitumor action against murine lymphocytic leukemia P388 *in vivo* [18]. This compound also specifically inhibits protein kinase C in a light-dependent manner [18, 19]. Most PQPs act as photodynamic toxins and generate singlet oxygen($^1O_2$) and $O_2^{\cdot-}$ [20, 21], and induce lipid peroxidation of biological membranes. Thus, it is considered that generated active oxygen species are the cause of the photodynamic action of these pigments. On the basis of the above evidence we examined the effect of blepharismin on PKC-dependent and independent reactions related to

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†† Abbreviations: Cyt c, ferricytochrome c; DG, diacylglycerol; diC_8, L-α-1,2-dioctanoylglycerol; DPPC, dipalmitoyl-phosphatidylcholine; FLV, Friend leukemia virus; GPtPMN, guinea-pig peritoneal neutrophils; H-7, 1-(5-isoquinolinelsulfonyl)-2-methyl-piperazine; HPPMN, human peripheral neutrophils; KRP, Krebs–Ringer phosphate; $O_2^{\cdot-}$, superoxide anion; PC, phosphatidylcholine; PKC, protein kinase C; PQPs, perylenequinonoid pigments; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; SOD, Mn^{2+}-superoxide dismutase; TCA, trichloroacetic acid.

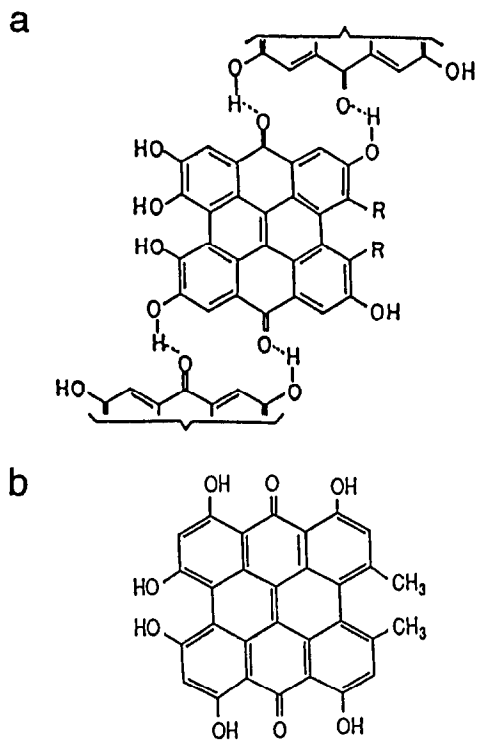

Fig. 1. The chemical structure of hypericin and a proposed structural formula of blepharismin. (a) Blepharismin [12]; (b) hypericin [13].

O_2^- generation in neutrophils, NADPH oxidase in a cell-free system and the activity of isolated rat brain PKC activity. This paper reports that blepharismin inhibited photodynamically not only PKC but also NADPH oxidase activity in a cell-free system of neutrophils.

MATERIALS AND METHODS

Chemicals. Cereal leaves, Cyt c, PMA, PMSF, leupeptin, H1 histone (type IIIS), SOD, sodium myristate and sodium arachidonate were purchased from Sigma Co. (St Louis, MO, U.S.A.). DEAE-Cellulose (DE-52), CM-Cellulose, and glass filter (GF/C) were supplied by Whatman Ltd (Maidstone, WI, U.S.A.). TSK G-3000 SW was obtained from Tosoh Co. (Tokyo, Japan). [γ-^{32}P]ATP and [^{32}P]-orthophosphate were obtained from ICN Radiochemicals (Irvine, CA, U.S.A.), and diC_8 was purchased from Avanti Polar Lipid (U.S.A.). Ficoll-Hypaque and RPMI-1640 medium were purchased from ICN Biochemicals Inc. (Aurora, OH, U.S.A.) and Nikken Inc. (Kyoto, Japan), respectively. All other chemicals were from Nacalai Tesque (Kyoto, Japan). diC_8 and PMA were dissolved in ethanol, and the final concentrations of ethanol in the reaction mixture were less than 0.5%.

Blepharismin. Blepharismin was cultured in infusion medium of 1% cereal leaves containing Enterobacter aerogenes at 23° as described in a previous paper [22] and extracted from frozen 5 mL packs of Blepharisma with 50 mL of acetone. After 1 min, the acetone extract was centrifuged at 800 *g* for 10 min. and the acetone evaporated to complete dryness in a rotary evaporator. Blepharismin was separated from other residual materials by thin-layer chromatography using ethyl acetate/acetone (4/1, v/v)[22].

Preparation of neutrophils and subcellular fractions. HPPMN were isolated from fresh blood of healthy volunteers by a procedure which utilized a Ficoll/Hypaque gradient [23]. GPtPMN were isolated from guinea-pigs 16 hr after intraperitoneal injection of 2% Nutrose and washed twice with calcium-free KRP, pH 7.4, as described by Takahashi *et al.* [24]. Isolated neutrophils were washed twice with KRP and resuspended at a concentration of 1×10^8 cells/mL. The viability of cells was determined by trypan blue exclusion. The subcellular fractions of GPtPMN were prepared using the method of Borregaard *et al.* [25], except that relaxation buffer contained 100 mM KCl, 3 mM NaCl, 10 mM HEPES buffer (pH 7.3), 3.5 mM $MgCl_2$, 1.25 mM EGTA, 100 μM leupeptin, and 0.5 mM PMSF. Purified neutrophils were resuspended in relaxation buffer and disrupted by 10-sec burst of sonication, using a microprobe sonicator at low power. Nuclei and unbroken cells were pelleted by centrifugation at 750 *g* for 10 min at 4°. The supernatant was decanted and loaded on to Percoll gradient precooled to 4°. For discontinuous Percoll gradient, the Percoll was adjusted to a density of 1.08 g/mL and 1.10 g/mL and centrifuged for 15 min at 48,000 *g* at 4°. The γ-fraction was washed with relaxation buffer by centrifugation at 100,000 *g* for 60 min at 4°. The cytosolic fraction was further centrifuged at 100,000 *g* for 60 min at 4°. diC_8 (5×10^{-6} M), 1×10^{-9} M PMA and 3×10^{-5} M sodium arachidonate were used to stimulate neutrophils.

Measurement of O_2^- generation. O_2^- production was assayed by Cyt c reduction as described previously using a dual beam spectrophotometer (Shimadzu UV 3000) equipped with a water-jacketed cell holder and magnetic stirrer [24]. Briefly, the reaction was started by adding neutrophils ($0.5–2 \times 10^6$ cells/mL) at 37° in KRP medium containing 10 mM glucose, 100 μM Cyt c, 1 mM NaN_3 and 1 mM $CaCl_2$ in the presence or absence of various ligands. The change in absorbance at 550–540 nm ($A_{550-540}$) was monitored continuously [24, 26].

Measurement of NADPH oxidase. NADPH-oxidase in a cell-free system from GPtPMN, plasma membranes (7–10 μg protein) and a cytosolic fraction (150–200 μg protein) was used in a medium containing 140 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 5 mM glucose, 1.5 mM NaN_3, 80 μM Cyt C and 5 mM Tris–HCl (pH 7.4), supplemented with 250 μM EGTA, 10 mM NaF, 20 μM GTP, 250 μN NADPH and 5 mM HEPES buffer (pH 7.8) [27]. The reaction was carried out by preincubating cytosol with plasma membranes for 2 min prior to the addition of 250 μM NADPH. 3×10^{-5} M μM arachidonate was added 2 min after the addition of NADPH, and the rate was calculated from the linear phase of the Cyt c reduction. The activity was monitored by the Cyt c reduction using a

double beam spectrophotometer in the presence of 100 U/mL SOD in the reference cell.

Preparation of PKC. PKC was partially purified from the soluble fraction of rat brain according to the method of Kikkawa *et al.* [28]. Briefly, rat brains were homogenized in the medium comprising 20 mM Tris–HCl (pH 7.5), 0.25 M sucrose, 5 mM EDTA, 2 mM PMSF, 10 mM 2-mercaptoethanol and 0.1% Triton X 100 at 4°, then centrifuged at 100,000 *g* for 60 min. The supernatant was loaded onto a DE-52 column (0.7 × 7 cm) equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 30 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, 6 mM 2-mercaptoethanol, 1 mM PMSF and 100 μM leupeptin (buffer A). The enzyme was eluted from the column by a one-step gradient of NaCl (30 mM to 100 mM) using buffer B which was identical to buffer A except that the NaCl concentration was 100 mM. The eluate (0.2 mL) was then loaded on to a TSK-G 300 SW column (1 × 30 cm) and the enzyme eluted with buffer C (20 mM Tris–HCl buffer, pH 7.5, containing 100 mM NaCl, 0.5 mM EGTA, 0.5 mM EDTA, and 6 mM 2-mercaptoethanol).

Assay of PKC activity. The activity of PKC was routinely assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone (type IIIS) [28–31]. Preincubation was carried out for 3 min in a mixture (250 μL) containing 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 0.3 mM $CaCl_2$ and 100 μM phospholipid (DPPC/PS, 4/1), 0.1 μM PMA or 5 μM diC_8 and various concentrations of blepharismin under dark or constant light (29×10^{-3} W/cm^2). The reaction was started by addition of 0.1 μM [γ-^{32}P]ATP and 0.2 mg/mL H1 histone (type IIIS) as substrate and incubated for 3 min at 30°. The reaction was stopped by the addition of ice-cold 25% TCA. The TCA precipitable material was collected on a glass filter (GF/C, Whatman Ltd). Its radioactivity was counted with a liquid scintillation counter.

In vivo *phosphorylation.* HPPMN were washed four times with phosphate-free RPMI-1640 medium and suspended in the same medium at a final concentration of 1×10^6 cells/mL. The cells were incubated with 0.5 μCi/mL of [^{32}P]orthophosphate at 37° for 3 hr and then washed with 20 mM HEPES buffer containing 0.9% NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 10 mM glucose. HPPMN were incubated with blepharismin (200 ng/mL) at 37° in the light (29×10^{-3} W/cm^2) or dark and the cells stimulated by 5 μM diC_8. After 5 min ice-cold 20% TCA was added to the suspension to yield a final concentration of 10%. The precipitated proteins were then subjected to SDS–PAGE analysis, after which they were transferred to an Immobilon-P membrane (millipore, Bedford, MA, U.S.A.). After staining with Coomassie brilliant blue, the membrane filters were developed on Kodak X-Omat films overnight or for 3 days at −60° [32].

Statistical analysis. At least three independent experiments were performed except where indicated. Results are presented as the mean value ± SD.

RESULTS

Effect of blepharismin on the diC_8 and arachidonate-induced $O_2^{\cdot-}$ generation of neutrophils

Neutrophils generate $O_2^{\cdot-}$ on stimulation by various stimuli such as diC_8, PMA, opsonized zymosan and arachidonate, and there are more than two pathways. One is a PMA- or DG-induced PKC-dependent pathway [33] and the other a fatty acid- or SDS-dependent pathway [27, 34]. The former is sensitive to PKC inhibitors, such as staurosporine and H-7, but the latter is insensitive to these inhibitors. The $O_2^{\cdot-}$ generation of HPPMN induced by diC_8 was 3.2 nmol $O_2^{\cdot-}$/min/10^6 cells and was stimulated by blepharismin (50–400 ng/mL) in the dark in a concentration-dependent manner (Fig. 2). However, when HPPMN were preincubated with blepharismin under constant illumination with visible light (29×10^{-3} W/cm^2 for 3 min), diC_8-induced $O_2^{\cdot-}$ generation was inhibited strongly in a concentration-dependent manner (Fig. 2). Under these conditions,

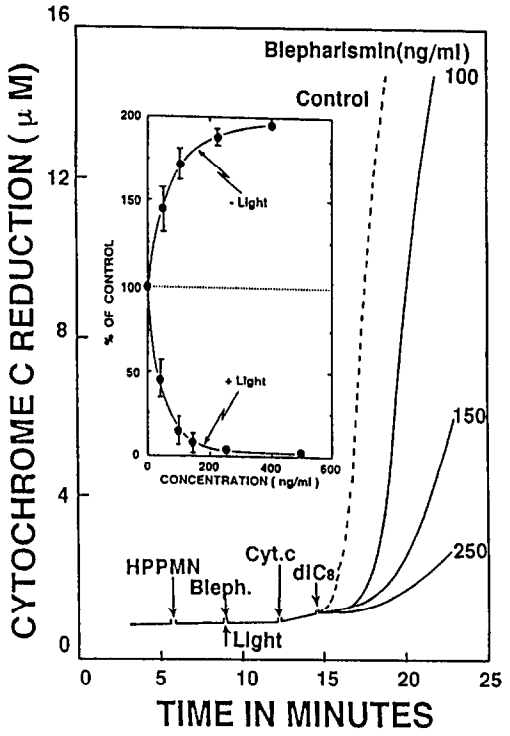

Fig. 2. Effect of blepharismin on $O_2^{\cdot-}$ generation by human neutrophils induced by diC_8. Human peripheral neutrophils (HPPMN, 1×10^6 cells/mL) were incubated at 37° in KRP medium containing 10 mM glucose, 1 mM NaN_3, 1 mM $CaCl_2$ and various concentrations of blepharismin. The reaction was started by illumination with light of constant intensity (29×10^{-3}W/cm^2) for 3 min followed by the addition of 100 μN Cyt c. $O_2^{\cdot-}$ generation was triggered by adding 5 μM diC_8 and the process was monitored continuously by the change in absorbance at 550–540 nm ($A_{550-540}$). The broken line shows the control without blepharismin. Data were obtained 3 min after addition of diC_8. The insert shows the effects of varying the concentration of blepharismin in light or dark on Cyt c reduction (data show means ± SD from three independent experiments). HPPMN, 1×10^6cells/mL, human peripheral neutrophils; blepha., various concentrations of blepharismin; light, constant tungsten illumination (29×10^{-3} W/cm^2, 3 min); Cyt c, 100 μM Cyt c; diC_8, 5 μM diC_8. Numbers are concentrations of blepharismin (ng/mL).

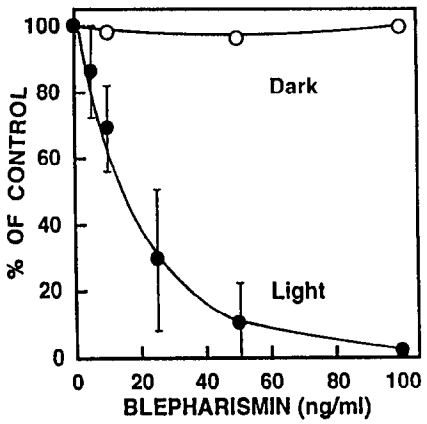

Fig. 3. Effect of blepharismin on arachidonate-induced $O_2^{\cdot}$ generation of GPtPMN. Experimental conditions were the same as those described in Fig. 2 except that 1×10^6 cell/mL of GPtPMN and 3×10^{-5} M arachidonate were used (data show means ± SD from three separate experiments). Dark, in dark; Light, in constant tungsten illumination (29×10^{-3} W/cm^2, 3 min).

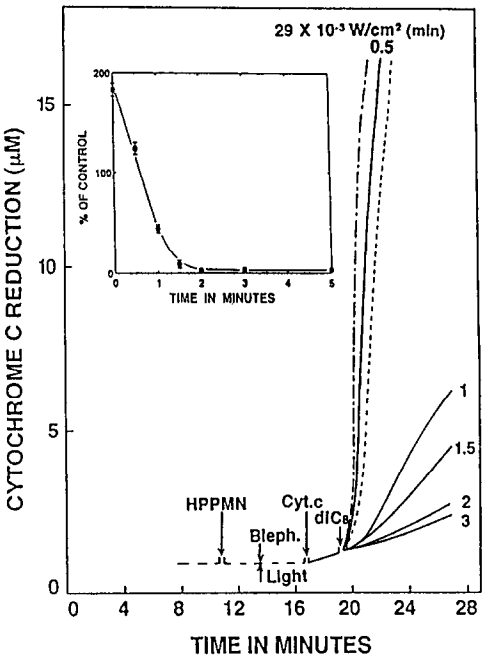

Fig. 4. Photodynamic inhibition of neutrophil $O_2^{\cdot}$ generation by blepharismin. Experimental conditions were the same as those in Fig. 2 except that the time of exposure to light of constant intensity (29×10^{-3} W/cm^2) was variable. The concentration of added blepharismin was 200 ng/mL. The insert shows the time-dependent inhibition curve for blepharismin (data show means ± SD from three separate experiments). The broken line (----) shows the control without blepharismin; (—·—·—·—) shows the curve with blepharismin under dark conditions. Numbers are times of illumination (min).

the concentration of blepharismin for half-maximum inhibition was 55 ng/mL. A similar inhibitory action of blepharismin was observed with PMA-stimulated $O_2^{\cdot}$ generation of HPPMN and GPtPMN and the ID_{50} of blepharismin was approx. 25 ng/mL. Furthermore, arachidonate-induced $O_2^{\cdot}$ generation (5.5 nmol $O_2^{\cdot}$/min/10^6 cells) in GPtPMN, which are very sensitive to arachidonate, was also inhibited by blepharismin in a light-dependent manner (Fig. 3). The ID_{50} was also 17 ng/mL. Similar inhibition was observed on the neutrophils stimulated with myristate or SDS. However, no stimulation of $O_2^{\cdot}$ generation was observed in the dark (Fig. 3). In contrast, preincubation of blepharismin alone in the light, followed by incubation with stimulated neutrophils in the dark did not inhibit $O_2^{\cdot}$ generation (data not shown). The results of the above experiments imply that photoactivation of blepharismin generates a short-lived active species that reacts with a target protein or proteins in neutrophils, resulting in the suppression of NADPH oxidase activation. The data also suggest that blepharismin has a general and non-specific effect on some membrane components.

Photodynamic inhibition of neutrophil $O_2^{\cdot}$ generation by blepharismin

To characterize the photodynamic activity of blepharismin on diC_8-induced $O_2^{\cdot}$ generation in HPPMN, the effects of light intensity and time of exposure to constant illumination were investigated. Preincubation of neutrophils with blepharismin (200 ng/mL) in either total darkness or constant illumination (tungsten, 29×10^{-3} W/cm^2) followed by incubation with 5 μM diC_8 resulted in stimulation or inhibition of $O_2^{\cdot}$ generation (Fig. 4). Complete inhibition was observed at 3 min by exposure to light of 29×10^{-3} W/cm^2. Decreasing light intensity over the same period of illumination resulted in a loss of inhibition (Fig. 5). The time for half-maximum inhibition under constant illumination (29×10^{-3} W/cm^2) was 0.5 min. The light intensity for half-maximum inhibition was 5×10^{-3} W/cm^2 for 3 min. Similar results were observed on PMA- or arachidonate-induced $O_2^{\cdot}$ generation (data not shown). These results indicate that the inhibitory action of blepharismin on $O_2^{\cdot}$ production depends on the total energy of the incident light.

Effect of different wavelengths of light on the inhibitory action of blepharismin

To investigate the mechanism of the light-dependent inhibitory action of blepharismin, the effect of varying the wave length of the incident light was examined. Blepharismin fluoresces when irradiated with visible light. The wavelength for maximum emission was 585 nm (Fig. 6b). The excitation spectrum for 585 nm fluorescence of blepharismin was similar to that of the absorption spectrum of the pigment (Fig. 6a). Illumination in the range 455–570 nm (6×10^{-3} W/cm^2) inhibited $O_2^{\cdot}$ generation by HPPMN, whereas no inhibition was observed with light of 619 nm or above (Fig. 7). These experimental results suggest that the inhibitory effect of blepharismin is dependent on the absorption of light.

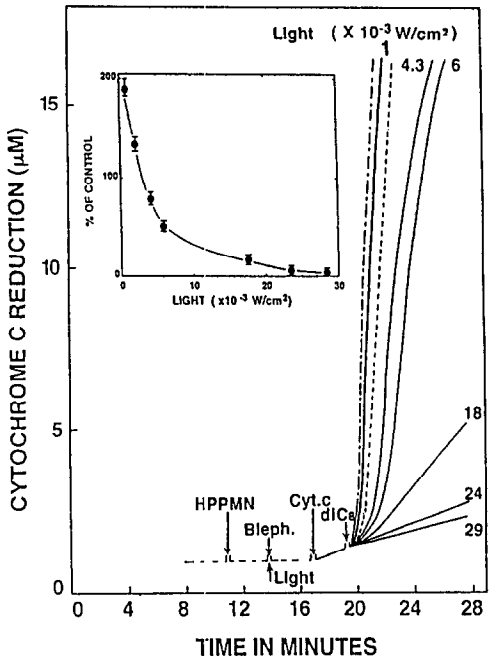

Fig. 5. Effect of light intensity on the inhibition of neutrophil O_2^- generation by blepharismin. Experimental conditions were as for Fig. 3 except that the light intensity was variable. The duration of illumination was 3 min. Blepharismin concentration was 200 ng/mL. The insert shows the effect of light intensity on inhibition by blepharismin. (Data show means ± SD from three independent experiments). The broken line (----) shows the control without blepharismin. (-·-·-·-) shows the curve with blepharismin under dark conditions. Numbers are light intensity ($\times 10^{-3}$ W/cm^2).

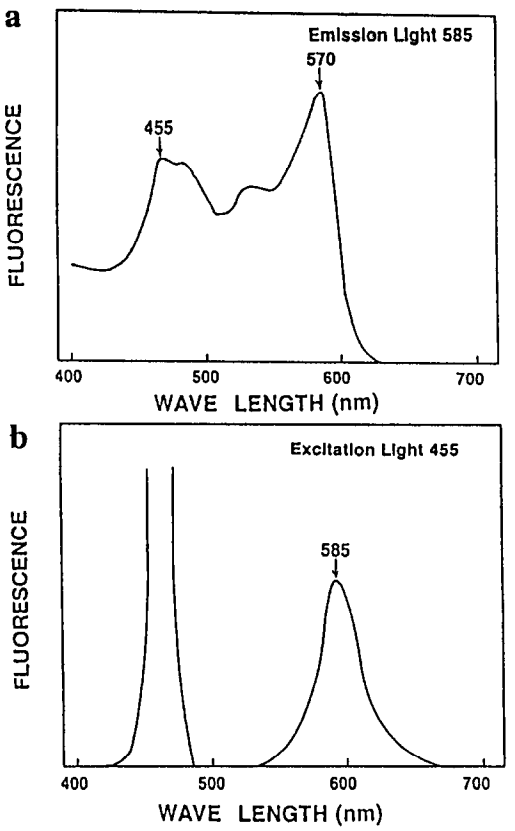

Fig. 6. Excitation (a) and emission (b) spectra of blepharismin.

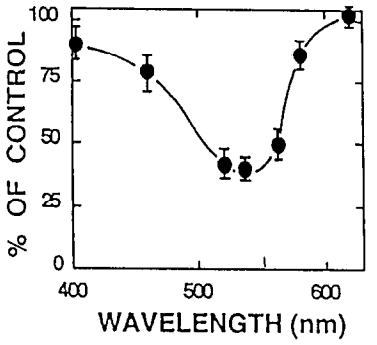

Fig. 7. Effect of different wavelengths of light on the inhibitory action of blepharismin. Experimental conditions were as described in Fig. 2 except for the wavelength of light which was variable. Light intensity at each wavelength was constant (29×10^{-3} W/cm^2, 3 min). The concentration of blepharismin was 200 ng/mL. (data show means ± SD from three independent experiments).

Effect of blepharismin on NADPH oxidase in a cell-free system

To obtain further insight into the mechanism of inhibition of O_2^- generation, the effect of the agent on NADPH oxidase activity in a cell-free system was investigated. NADPH oxidase activity was activated by 3×10^{-5} M arachidonate and assayed by SOD-inhibitable Cyt c reduction. The generated O_2^- was 112 nmol/min/mg protein of cytoplasmic membrane. Arachidonate-stimulated NADPH oxidase activity was inhibited by blepharismin in a light- and concentration-dependent manner (Fig. 8). The ID_{50} of blepharismin for the inhibition of NADPH oxidase was 40 ng/mL. This value was slightly higher than that for O_2^- generation induced by the same concentration of arachidonate in intact GPtPMN (Fig. 3). A similar inhibitory action of blepharismin was observed with SDS-stimulated NADPH oxidase activity in a cell-free system (data not shown).

Effect of blepharismin on the activity of isolated protein kinase C

Figure 9 shows the effect of blepharismin on the activity of partially purified rat brain PKC prepared by the method of Kikkawa *et al.* [28]. This PKC fraction contained a number of isoforms of PKC such as α, β and γ. PKC activity stimulated by 5 μM diC_8 was inhibited by blepharismin provided that the incubation was illuminated with visible light (Fig. 9). Similar inhibition was also observed on PMA-

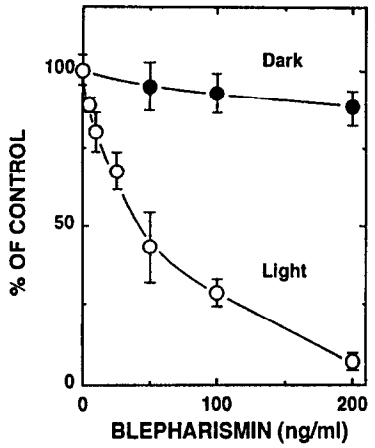

Fig. 8. Effect of blepharismin on NADPH oxidase activity in a cell-free system. The subcellular fractions obtained from GPtPMN were prepared using a modified method of Borregaard *et al.* [25, 27], and NADPH-oxidase was measured as described in Methods in a medium containing plasma membranes (7–10 μg protein) and cytosolic fraction (150–200 μg protein). Various concentrations of blepharismin were added to the complete system and exposed to constant light (29×10^{-3} W/cm^2) for 3 min at 37°, and the reaction was started by addition of 250 μM NADPH after addition of 3×10^{-5} M arachidonate, (data show mean ± SD from three independent experiments). Dark, in dark; light, 29×10^{-3} W/cm^2, 3 min.

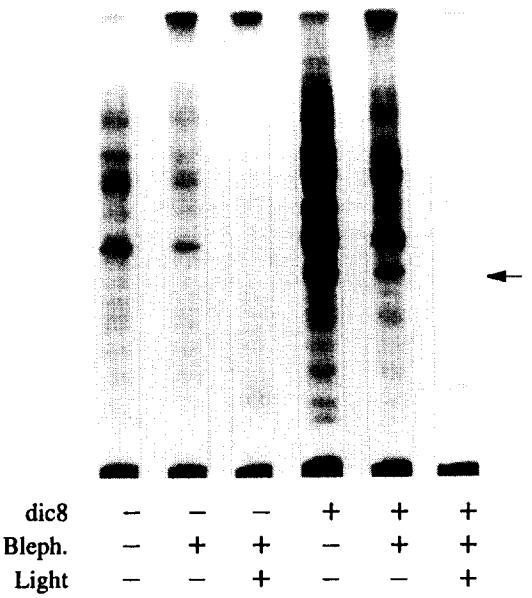

Fig. 10. Effect of blepharismin on the phosphorylation of neutrophil protein stimulated by diC$_8$. Phosphorylation was assayed by the incorporation of [^{32}P]orthophosphate into the neutrophil proteins as described in Materials and Methods. An arrow shows the p47^{phox}; protein. Bleph., 200 ng/mL blepharismin; diC$_8$, 5 μM diC$_8$; light, 29×10^{-3} W/cm^2 visible for 3 min.

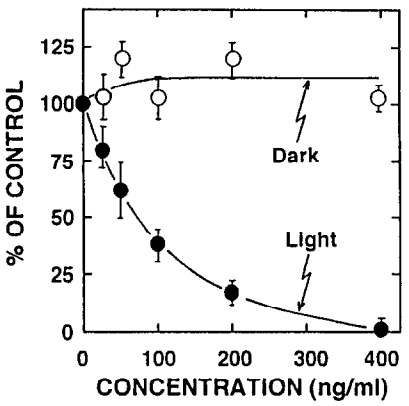

Fig. 9. Effect of blepharismin on the activity of protein kinase C isolated from rat brains. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into H1 histone (type IIIS) as described in Materials and Methods. Partially purified rat brain protein kinase C was incubated in a mixture containing 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 1 mM $CaCl_2$, 100 μM phospholipid (DPPC/PS, 4/1), 5 μM diC$_8$ and various concentrations of blepharismin. The reaction was started by incubation for 3 min at 30° either in darkness or constant light (29×10^{-3} W/cm^2) and then incubated for a further 3 min at 30° after addition of 0.2 mg/mL H1 (type IIIs) and 0.1 μM [γ-^{32}P]ATP (data show mean ± SD from three independent experiments). Dark, without exposing to light; light, with 29×10^{-3} W/cm^2 visible light for 3 min.

stimulated PKC activity (data not shown). The inhibitory effect of blepharismin was both concentration and light dependent. The ID_{50} of blepharismin for the inhibition of PKC was 70 ng/mL. The value was slightly higher than that for O_2^- generation induced by diC$_8$ in intact HPPMN. In contrast, blepharismin when preilluminated alone and incubated further under light had no inhibitory activity (data not shown).

Effect of blepharismin on the diC$_8$-induced phosphorylation of neutrophil protein

To confirm the inhibition of diC$_8$-induced phosphorylation of cytoplasmic protein by blepharismin, the effect of the pigment on the incorporation of orthophosphate ^{32}P into cytoplasmic proteins of HPPMN such as p47^{phox} was examined. Many cytoplasmic proteins were phosphorylated without stimulation with diC$_8$ but phosphorylation was greatly enhanced by treatment with either diC$_8$ or PMA. Blepharismin (200 ng/mL) in the presence of light (29×10^{-3} W/cm^2, 3 min) strongly inhibited phosphorylation of cytoplasmic proteins including p47^{phox} [35, 36] in either the presence or absence of diC$_8$ (Fig. 10). This inhibitory activity was diminished in the absence of light. These results suggest that inhibition of the phosphorylation of cytoplasmic proteins such as p47^{phox} might be correlated with the inhibition of diC$_8$-dependent O_2^- generation in neutrophils by blepharismin.

DISCUSSION

The observed potent inhibitory action of bleph-

arismin on O_2^- generation stimulated by various stimuli, such as diC_8, PMA, arachidonate, myristate and SDS, and phosphorylation of cytoplasmic proteins in neutrophils, is light (visible range of spectrum) and concentration dependent. However, blepharismin when preincubated alone in light has no such inhibitory activity. These observations suggest that photoactivation of blepharismin generates a short-lived active species that reacts with a target protein or proteins in neutrophils, resulting in the suppression of NADPH oxidase activity.

Blepharismin appears to be structurally related to a series of PQPs and related compounds, such as calphostin C [19] and hypericin [37]. Several lines of evidence indicate that various inhibitors of PKC depress the production of phorbol ester-induced human immunodeficiency virus type 1 virion [17]. Hypericin photoinactivates viruses (especially HIV) [14]. To understand the mechanism of inhibition, many studies have been carried out, and it was found that PKC plays an important role in the inhibitory mechanism of PQPs. Calphostin C has been demonstrated to be a potent and specific inhibitor of PKC [18]. A similar inhibition of PKC was observed with hypericin by Takahashi *et al.* [16] and with blepharismin in this experiment. Thus, as reported by Diwu *et al.* [37], the structurally related PQPs have a strong inhibitory effect on PKC. $^1O_2$ plays a key role in the photosensitization of these PQPs [38], and all the potent PKC inhibitors of the PQP class were good $^1O_2$ generators [37]. However, no critical suppressive effect of azide, an efficient $^1O_2$ scavenger, on PKC was observed [39]. Thus no conclusion has yet been reached as to whether $^1O_2$ is involved in PKC inhibition or not. In this context, it is possible that additional active oxygen species, including the superoxide anion, hydroxyl radical and hydrogen peroxide, may play supplemental roles. These active oxygens induce lipid peroxidation of biological membrane [40]. Thus, we cannot exclude the possibility of the involvement of membrane modification by generated active oxygen species in inhibited membrane bound enzymes. In fact, blepharismin inhibited not only PKC in neutrophils and from rat brain but also NADPH oxidase, both of which are membrane-bound enzymes. However, O_2^- generation by neutrophils was inhibited by blepharismin not only via a PKC-dependent pathway but also via a pathway which is independent of PKC. These results appear that NADPH oxidase plays an essential role in the inhibitory action of blepharismin in O_2^- generation and that PKC may not be correlated with the inhibitory action of blepharismin in the PKC-independent reaction but may have an important role in the diC_8- or PMA-dependent O_2^- generation reaction.

Related to the specificity of blepharismin action against enzyme activity, the PQP semiquinone radical has been postulated to be a possible active species for photoinactivation of the PKC enzyme [37] based on its ability to inhibit other enzymes, such as epidermal growth factor-receptor kinase (membrane-bound tyrosine kinase) [41]. Preliminary experiments in this laboratory have shown that both blepharismin and hypericin have no photoinhibitory activity against non-membrane-bound tyrosine kinases, such as $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ [42]. Furthermore, DeWitte *et al.* [38] and Takahashi *et al.* [16] have reported that hypericin does not inhibit Ser/Thr protein kinases such as protein kinase A, casein kinase 1 and 2, and myosin light chain kinase and the enzyme of 5′-nucleotidase. These results indicate that the photoactivation of blepharismin exerts a general and non-specific effect on membrane-bound enzymes due to the hydrophobicity of the pigment, although it still binds to a specific site of the enzyme, such as an -SH of PKC [37]. Therefore, further studies are needed to elucidate the molecular mechanism of inhibition and specificity of blepharismin on neutrophil O_2^- generation.

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