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EVIDENCE THAT INHIBITION OF PHORBOL ESTER-INDUCED SUPEROXIDE ANION FORMATION BY CYCLOSPORIN A IN PHAGOCYTES IS NOT MEDIATED BY DIRECT INHIBITION OF PROTEIN KINASE C

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Abstract—Cyclosporin A (CsA) has been reported to inhibit phorbol myristate acetate (PMA)-induced superoxide anion (O_2^-) formation in human neutrophils and murine macrophages. We found that CsA inhibited O_2^- formation in HL-60 cells induced by PMA (30 nM) and phorbol dibutyrate (200 nM) with a half-maximal effect at 1 and 0.75 μM, respectively. One possible target of CsA action is protein kinase C (PKC) [EC 2.7.1.37] since phorbol esters activate this kinase. However, CsA did not inhibit PMA-mediated reduction of histamine-induced rises in cytosolic Ca^{2+} concentration in, and PMA-induced differentiation of, HL-60 cells and platelet aggregation. CsA did not reduce the activity of various recombinant *c*-PKC isoenzymes (α, β1 and γ), *n*-PKC isoenzymes (δ and ε), an *a*-PKC isoenzyme (ζ) nor of PKC purified from rat brain *in vitro*. These data show that CsA inhibits phorbol ester-induced O_2^- formation in HL-60 cells but not other phorbol ester-mediated events and that inhibition by CsA of O_2^- formation cannot readily be attributed to direct PKC inhibition. We also show that CsA does not change the activity of nucleoside diphosphate kinase [EC 2.7.4.6] in HL-60 membranes nor the latter's physical properties.

Key words: cyclosporin A; superoxide anion; phorbol esters; protein kinase C

Phagocytes possess a multi-component NADPH oxidase [EC 1.6.99.6] which catalyses the formation of O_2^-‖, NADPH being the electron donor [1–3]. Among the regulatory components of NADPH oxidase are small GTP-binding proteins and NDPK [EC 2.7.4.6] [1–3]. O_2^- formation can be stimulated by formyl peptides and PMA [1–3]. PKC [EC 2.7.1.37] plays a crucial role in NADPH oxidase activation [1–3]. PKC is a family comprising *c*-, *n*- and *a*-PKC isoenzymes [4–6]. *c*-PKC isoenzymes (α, β1, β2 and γ) are Ca^{2+}-dependent and are activated by PMA. *n*-PKC isoenzymes (δ, ε, η and θ) are activated by phorbol esters as well but are Ca^{2+}-independent. A third group of PKC isoenzymes, *a*-PKC (ζ and λ), is not substantially activated by phorbol esters and is also Ca^{2+}-independent. Recent data indicate that *n*-PKC isoenzymes are involved in PMA- and formyl peptide-induced activation of O_2^- formation [7].

The immunosuppressant, CsA [8], inhibits PMA-induced O_2^- formation in murine peritoneal macrophages and human neutrophils [9–11]. In addition, CsA inhibits formyl peptide-induced O_2^- formation in neutrophils and HL-60 cells [10–12]. The precise mechanism by which these effects are accomplished is poorly understood. With regard to *c*-PKC isoenzymes, both inhibitory effects and the lack of effect of CsA on enzyme activity have been reported [13, 14]. The aim of the present study was to learn more about the mechanism underlying the inhibitory effect of CsA on O_2^- formation, especially inasmuch as CsA is a direct inhibitor of *n*-PKC isoenzymes.

MATERIALS AND METHODS

Materials. CsA was kindly provided by Sandoz (Basel, Switzerland). A stock solution of CsA (1 mM) was prepared in 100% (v/v) dimethyl sulfoxide and stored in polypropylene tubes at −20° under light protection for up to 4 weeks. The final dimethyl sulfoxide concentration in assays was adjusted to 0.3% (v/v). PMA and PDB were obtained from Sigma Chemie (Deisenhofen, F.R.G.). [Serine^{25}]PKC(19–31) was obtained from Peninsula (Belmont, CA, U.S.A.). Gö 6850 and Gö 6976 were provided by Gödecke AG (Freiburg, F.R.G.). [γ-^{32}P]ATP (3 Ci/mmol) was obtained from Amersham (Braunschweig, F.R.G.). Sources of

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‖ Abbreviations: ATP[γS], adenosine 5′-[γ-thio]triphosphate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; CsA, cyclosporin A; Gö 6850, 2-(1*H*-indol-3-yl)-3-[1-(3-dimethylaminopropyl)-1*H*-indol-3-yl]-maleinimide; Gö 6976, 12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]-carbazole; GTP[γS], guanosine 5′-[γ-thio]triphosphate; NDPK, nucleoside diphosphate kinase; O_2^-, superoxide anion; PDB, phorbol dibutyrate; PKC, protein kinase C; PMA, phorbol myristate acetate.

other materials have been described elsewhere [7, 10, 12, 15–21].

Cell culture. HL-60 cells were cultured in suspension culture at 37° and differentiated towards neutrophil-like cells upon incubation with dibutyryl cAMP (0.2 mM) for 48 hr [15]. In other experiments, HL-60 cells were differentiated towards macrophage-like cells with PMA (10–100 nM) for 96 hr [22].

Assay for O_2^- formation in HL-60 cells. O_2^- formation was monitored at 550 nm by continuous measurement of ferricytochrome *C* reduction inhibitable by superoxide dismutase, using an Uvikon 810 dual beam spectrophotometer (Kontron, Eching, F.R.G.) [15]. Reaction mixtures (0.5 mL) contained 100 μM ferricytochrome *C* and a buffer consisting of 138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5.5 mM glucose and 20 mM Hepes/NaOH, pH 7.4. HL-60 cells (2.5×10^6 cells/cuvette) were suspended in the solution described above and incubated for 3 min in the presence of solvent (control) or various concentrations of CSA at 37°. O_2^- formation was initiated by the addition of stimuli. The maximum rates of O_2^- formation were calculated.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was determined using the fluorescent dye, Fura-2, as described previously [16]. Fluorescence of HL-60 cells (1.0×10^6 cells in 2 mL) was determined at 37° under constant stirring at 10^3 rpm using a Ratio II spectrofluorometer (Aminco, Silver Spring, MD, U.S.A.). Cells were incubated for 3 min in the absence or presence of CsA before the addition of solvent (control) or PMA. After an additional 3 min, stimulus was added to the cells.

Platelet aggregation. Isolation of platelets from healthy drug-free volunteers was performed as described previously [17]. Platelet aggregation was studied in an Aggrecorder II PA-3220 (Kyoto Daiichi Kagaku, Kyoto, Japan) according to Berg *et al.* [17]. Solvent (control) or CsA was added to platelets 3 min before the addition of stimuli.

Preparation of recombinant PKC isoenzymes and purification of PKC from rat brain. Full-length human cDNAs for PKC isoenzymes α, β1, and ζ, rat cDNA for PKC isoenzyme γ and mouse cDNAs for PKC isoenzymes δ and ε were inserted into the baculovirus expression vector, pVL1393 [18, 23]. Expression of PKC isoenzymes in Sf9 insect cells and purification of expressed enzymes were performed as described elsewhere [18]. PKC from rat brain was prepared according to the procedure described by Inagaki *et al.*[24]. This purification resulted in a mixed preparation of the four c-PKC isoenzymes as revealed by immunoblotting with isoenzyme-specific antibodies (data not shown).

Determination of PKC activity. For determination of PKC activity, reaction mixtures (200 μL) contained 5–10 units of PKC (1 unit transfers 1 pmol of phosphate/min), 10 μM [γ-^{32}P]ATP (0.2 μCi/tube), 40 μg of histone H_1, 5 mM $MgCl_2$, 1 mM EDTA, 1.25 mM EGTA, 1.32 mM $CaCl_2$, 1 mM dithiothreitol, 1 μg of phosphatidylserine and 0.2 μg of diolein in 50 mM Hepes/NaOH, pH 7.5. Reaction mixtures additionally contained solvent (control), CsA or Gö 6850. Reactions were initiated by the addition of [γ-^{32}P]ATP and conducted for 5 min at 30°. Reactions were terminated by the addition of 2 mL of 8.5% (w/v) H_3PO_4 and filtration through 0.45 μm Sartorius nitrocellulose filters. Radioactivity

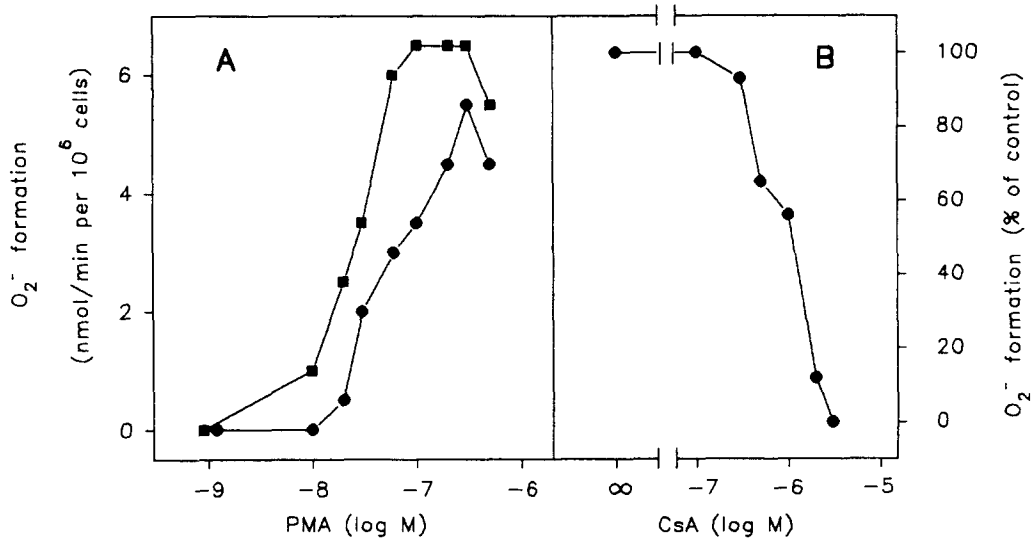

Fig. 1. Effect of CsA on PMA-induced O_2^- formation in HL-60 cells. O_2^- formation was determined as described in Materials and Methods. (A) Concentration–response curve to PMA. CsA (1 μM) (●) or solvent (control) (■) was added to reaction mixtures 3 min before the addition of PMA at the indicated concentrations. (B) Concentration–response curve to CsA. CsA was added to reaction mixtures 3 min before the addition of PMA (30 nM). O_2^- formation in the presence of solvent (control) amounted to 3.64 ± 0.21 nmol/min per 10^6 cells. Data shown are the means of four experiments carried out with different preparations of HL-60 cells. The SD values of the data were generally <10% of the means.

bound to filters was determined in a liquid scintillation spectrometer. Reaction mixtures for determination of PKC isoenzyme ζ activity contained 2 μg of [serine^{25}]PKC(19–31) rather than histone H_1 as phosphate acceptor, and P81 Whatman phosphocellulose filters were used instead of nitrocellulose filters [25]. As phospholipid-dependent activity of PKC isoenzyme ζ is very low [5, 18], the total incorporation of phosphate into [serine^{25}]PKC(19–31) was regarded as enzyme activity.

Measurement of NDPK activity. GTP[γS] formation was determined as described previously [23]. Reaction mixtures (50 μL) contained HL-60 membranes (40 μg of protein/tube), 0.5 μM [^{3}H]-GDP (1 μCi/tube), 50 μM ATP[γS], 2 mM $MgCl_2$, 0.1 mM EGTA, 1 mM dithiothreitol and 0.2% (w/v) BSA in 50 mM triethanolamine/HCl, pH 7.4. Reaction mixtures additionally contained solvent (control) or CsA. Reactions were conducted for 15 min at 25°. Nucleotides were separated by TLC and eluted from TLC plates as described [19].

Steady-state and differential polarized phase fluorometry. Determination of steady-state and differential phase fluorescence of 1,6-diphenylhexa-1,3,5-triene-loaded HL-60 membranes was performed as described [20]. Membranes were incubated for 10 min at 25° in the presence of solvent (control) or CsA. Fluorescence measurements were performed at 25° in a SLM 4800 spectrofluorometer (SLM Instruments, Urbana, IL, U.S.A.) equipped with a thermostated cuvette holder.

ATPase [EC 3.6.1.3] *assays.* Determination of the activities of Na^+/K^+-ATPase and Mg^{2+}-ATPase in HL-60 membranes was performed as described by Ebel *et al.* [21]. Reaction mixtures (100 μL) contained 6.0 μg of protein and solvent (control) or CsA. The amount of inorganic phosphate in supernatant fluids of reaction mixtures was determined according to Ames [26].

Miscellaneous. Protein was determined according to Lowry *et al.* [27]. HL-60 membranes were prepared as described [19].

RESULTS

We first studied the effects of CsA, Gö 6850 and Gö 6976 on phorbol ester-induced O_2^- formation in HL-60 cells. PMA activated O_2^- formation with a half-maximal effect at 25 nM and a maximum effect at 100 nM (Fig. 1). When compared to PMA, PDB was similarly effective but about 10-fold less potent at activating O_2^- formation (Fig. 2). CsA (1 μM) inhibited O_2^- formation induced by PMA and PDB at submaximally and maximally effective concentrations of the stimuli. The inhibitory effect of CsA on O_2^- formation induced by PMA (30 nM) was half-maximal at 1 μM and was complete at 3 μM (see Fig. 1). With respect to O_2^- formation induced by PDB (200 nM), the inhibitory effect of CsA was half-maximal at 0.75 μM and maximal at 3 μM (see Fig. 2). Gö 6850 (1 μM), an inhibitor of *c-* and *n-*PKC isoenzymes [18], abolished the stimulatory effects of PMA on O_2^- formation, whereas Gö 6976 (1 μM), a selective inhibitor of c-PKC isoenzymes [18], was ineffective (data not shown). These data are in agreement with those obtained with human neutrophils and substantiate the view that *n*-PKC

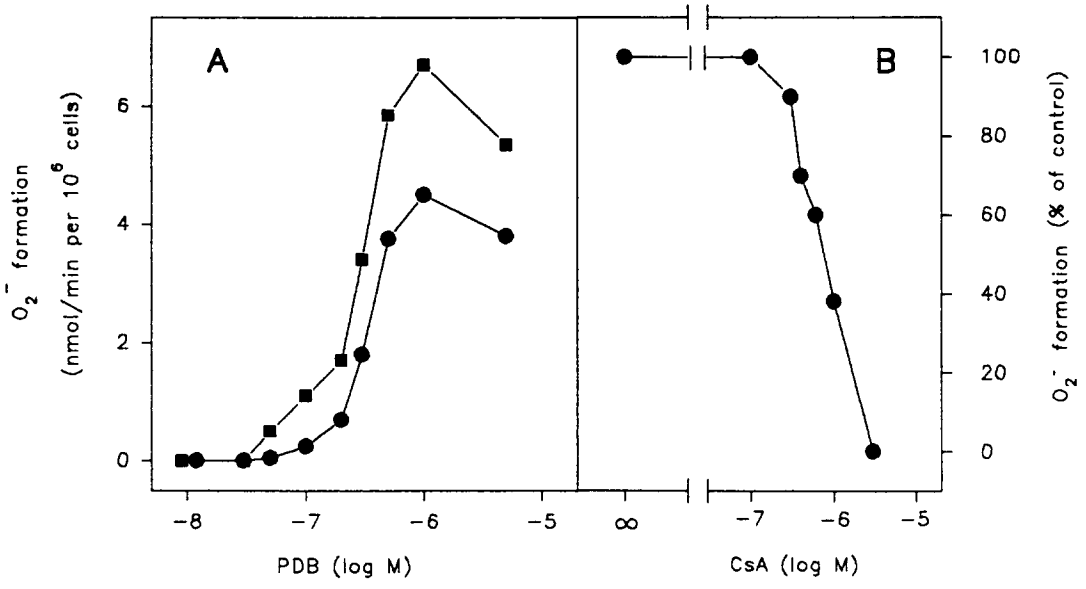

Fig. 2. Effect of CsA on PDB-induced O_2^- formation in HL-60 cells. O_2^- formation was determined as described in Materials and Methods. (A) Concentration–response curve to PDB. CsA (1 μM) (●) or solvent (control) (■) was added to reaction mixtures 3 min before the addition of PDB at the indicated concentrations. (B): Concentration–response curve to CsA. CsA was added to reaction mixtures 3 min before the addition of PDB (200 nM). O_2^- formation in the presence of solvent (control) amounted to 1.85 ± 0.10 nmol/min per 10^6 cells. Data shown are the means of four experiments carried out with different preparations of HL-60 cells. The SD values of the data were generally <10% of the means.

Table 1. Effects of CsA and Gö 6850 on the activity of recombinant PKC isoenzymes α, $\beta1$, γ, δ, ε and ζ and PKC purified from rat brain

Enzyme	Enzyme activity (% of control) CsA	Gö 6850
PKC isoenzyme α	98	6
PKC isoenzyme $\beta1$	100	18
PKC isoenzyme γ	113	12
PKC isoenzyme δ	104	2
PKC isoenzyme ε	108	0
PKC isoenzyme ζ	104	ND
PKC from rat brain (α, $\beta1$, $\beta2$, γ)	100	3

The activity of PKC was determined as described in Materials and Methods. Enzyme activity refers to that observed in the presence of solvent (control). CsA was employed at a concentration of 10 μM throughout. In experiments with PKC isoenzymes α, $\beta1$ and γ and PKC purified from rat brain, Gö 6850 was employed at a concentration of 300 nM, and in experiments with PKC isoenzymes ε and δ, Gö 6850 concentration was 10 μM. Data shown are the means of two independent experiments performed in triplicate. The SD values of the data were generally <5% of the means.

ND, not done.

isoenzymes are involved in PMA-mediated activation of NADPH oxidase [7].

In addition to activation of O_2^- formation, PMA shows numerous other biological effects: e.g., inhibition of receptor agonist-induced rises in $[Ca^{2+}]_i$ [16], induction of macrophage-like differentiation of HL-60 cells [22] and activation of platelet aggregation [28]. PMA (2 nM) abolished the stimulatory effect of histamine (100 μM) on $[Ca^{2+}]_i$ in HL-60 cells, but CsA (1 μM) could not revert this inhibition (data not shown). In addition, PMA (10–100 nM) induced macrophage-like differentiation of HL-60 cells as assessed by adherence and changes in morphology. Again, CsA (1 μM) did not prevent the phorbol ester effect (data not shown). Moreover, PMA induced aggregation of human platelets with a half-maximal effect at 10 nM and a maximum at 100 nM, but CsA (1 μM) did not affect platelet aggregation induced by PMA (data not shown).

The effects of CsA on the activity of recombinant PKC isoenzymes α, $\beta1$, γ, δ, ε and ζ and on *c*-PKC isoenzymes purified from rat brain were studied *in vitro* (Table 1). CsA (1 and 10 μM, data for CsA at 1 μM not shown) did not substantially reduce the activity of any of the PKC isoenzymes studied. In addition, CsA (1 and 10 μM) did not affect the activity of PKC purified from rat brain. By contrast to CsA, Gö 6850 strongly reduced the activity of *c*- and *n*-PKC isoenzymes and of PKC purified from rat brain (see Table 1).

The effect of CsA on NDPK-catalysed GTP[γS] formation in HL-60 membranes was studied. NDPK catalysed the formation of 9.4 ± 0.8 pmoles of GTP[γS] from GDP and ATP[γS]/mg (mean ± SD, N = 6). CsA (1 and 10 μM) had no effect on this GTP[γS] formation (data not shown).

We also studied the effect of CsA on physical properties of HL-60 membranes using steady-state polarization and differential polarized phase fluorometry with 1,6-diphenylhexa-1,3,5-triene probe [20]. We assessed limiting anisotropy and rotational correlation time. Limiting anisotropy in HL-60 membranes treated with solvent (control) was 0.175 ± 0.002, and rotational correlation time was 1.445 ± 0.106 nsec (means ± SD, N = 3). CsA (1 μM) did not change these values (data not shown).

Finally, we studied the effects of CsA on the activity of Mg^{2+}-ATPase and Na^+/K^+-ATPase in HL-60 membranes. The activity of these enzymes was 0.25 ± 0.03 μmol/mg/min and 0.14 ± 0.01 μmol/mg/min, respectively (means ± SD, N = 4). CsA (1, 3 and 10 μM) had no effect on Mg^{2+}-ATPase and Na^+/K^+-ATPase (data not shown).

DISCUSSION

In agreement with the data obtained with human neutrophils and murine peritoneal macrophages [9–11], we found that CsA inhibits phorbol ester-induced O_2^- formation in HL-60 cells (see Figs 1 and 2). We tested various hypotheses in an attempt to explain these effects of CsA.

PMA- and formyl peptide-mediated O_2^- formation involves activation of *n*-PKC isoenzymes [7], and CsA inhibits O_2^- formation induced by both stimuli (see Figs 1 and 2) [9–12]. From these findings, the question arises as to whether CsA inhibits these PKC isoenzymes. Due to the fact that purification to homogeneity of *n*-PKC isoenzymes from tissues or cells is difficult to perform, we used recombinant *n*-PKC isoenzymes. Unlike Gö 6850, CsA had no effect on two *n*-PKC isoenzymes (see Table 1). Additionally, CsA did not inhibit a recombinant *a*-PKC isoenzyme (see Table 1). Furthermore, and in close agreement with the data obtained by Szamel *et al.* [14] who studied human lymphocytes, we could not detect an inhibitory effect of CsA on *c*-PKC isoenzymes, regardless of whether recombinant enzymes or enzymes purified from rat brain were used (see Table 1). Moreover, CsA failed to revert PMA-mediated inhibition of agonist-induced rises in $[Ca^{2+}]_i$ in HL-60 cells, PMA-mediated differentiation of these cells, PMA-mediated platelet aggregation or various effects of PMA in human lymphocytes [14]. All these data render it unlikely that PMA inhibits O_2^- formation through direct inhibition of a known PKC isoenzyme.

Thus, does CsA interfere with a component specifically involved in NADPH oxidase activation? A candidate in this regard is NDPK which catalyses thiophosphorylation of GDP to GTP[γS], ATP[γS] being the thiophosphoryl group donor [19]. The newly formed GTP[γS] then potentiates O_2^- formation through activation of GTP-binding proteins [19]. However, CsA does not interfere with NDPK. Previous studies have already shown that CsA is not a formyl peptide receptor antagonist and does not interfere with GTP-binding proteins or the mechanisms leading to rises in $[Ca^{2+}]_i$ [9, 10, 12]. Moreover, CsA does not inhibit the catalytic domain of NADPH oxidase and does not scavenge O_2^- or inhibit glucose transport or energy metabolism [9, 10].

CsA interacts with phospholipid bilayers and has been shown to alter the physical properties of lymphocyte membranes [29, 30]. In addition, it has been reported to inhibit NA^+/K^+-ATPase, a membrane-bound enzyme which is sensitive to changes in physical membrane state [31], in lymphocytes [32]. These findings raise the question of whether CsA inhibits O_2^- formation through alterations in physical membrane properties. However, CsA did not affect limiting anisotropy (a static measure) [20] and rotational correlation time (a dynamic measure) [20] of a fluorescent probe in HL-60 membranes, nor did it affect the activity of NA^+/K^+-ATPase and Mg^{2+}-ATPase in HL-60 membranes. These findings render it unlikely that CsA inhibits O_2^- formation through changes in physical membrane properties.

In conclusion, we have shown that CsA inhibits phorbol ester-induced O_2^- formation in HL-60 cells but not other phorbol ester-mediated events. Inhibition by CsA of O_2^- formation cannot readily be attributed to direct PKC inhibition, interference with known components of NADPH oxidase or changes in physical membrane properties. Thus, our results clearly emphasize the need for additional work to elucidate the mechanism by which CsA inhibits O_2^- formation.

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