Biochemical Pharmacology, Vol. 35, No. 6, pp. 947–951, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

EFFECT OF CYCLOSPORIN A ON THE MEMBRANE-ASSOCIATED EVENTS IN HUMAN LEUKOCYTES WITH SPECIAL REFERENCE TO THE SIMILARITY WITH DEXAMETHASONE*

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(*Received* 1 *July* 1985; *accepted* 1 *October* 1985)

Abstract—The effect of an immunosuppressive drug, cyclosporin A, and dexamethasone was assessed on the enzymatic reactions of membrane phospholipid in normal human lymphocytes and neutrophils. Incubation for 20 min with cyclosporin A markedly suppressed, in a dose dependent manner, phospholipase A_2 activity and the release of prostaglandin E_2 in lymphocytes, and slightly those in neutrophils, while no inhibition of phosphatidylethanolamine (PE)-N methyltransferase activity was observed. Choline phosphotransferase (CPT) activity was not inhibited by the drug, either. These inhibitory effects on enzyme activities of membrane phospholipid are similar to those of dexamethasone, although different incubation time of the drug was required to induce inhibitory effects. These findings suggest that cyclosporin A acts upon early membrane events in the activation of cells involved in inflammatory reactions; they further suggest that suppression of immune response by cyclosporin A is at least partly due to inhibition of phospholipase A_2 in the plasma membrane of inflammatory cells. This inhibition reduces the production of cell membrane lyso-phosphatidylcholine (PC) and arachidonic acid from PC, which is produced by transmethylation of PE and cytidine diphosphate (CDP) choline pathway of which the last reaction to PC is mediated by CPT.

Cyclosporin A (CS-A)** is a cyclic endecapeptide used clinically as an immunosuppressive agent to help organ transplantation [1, 2]. *In vitro* studies have shown a reversible inhibitory action of CS-A on early events in T cell activation [3]. Reported effects of CS-A include inhibition of T cells to help antibody production [4] and the generation of cytotoxic T lymphocytes [5], abrogation of Con A-induced suppressor T cell activity [6], unresponsiveness of T cells to interleukins 1 and 2 [7] and suppression of the synthesis of these lymphokines [8]. Although the mechanism of action of CS-A remains to be determined, recent evidence has suggested that the drug binds directly to lymphocyte plasma membranes [9, 10]. This was further supported by the observation of CS-A binding to phospholipid vesicles [10].

Recently, members of the Behçet's Disease Research Council of Japan, including two of us (YN and TS), have observed that CS-A is highly effective in the treatment of Behçet's disease [11–13]. In the pathogenesis of this disease, neutrophil activation, rather than lymphocyte immune dysfunction, appears to play a major role [14–18]. These facts prompted us to investigate the effect of CS-A on phospholipid metabolism associated with cell activation in both lymphocytes and neutrophils. The action of CS-A on cell membrane enzyme activities are compared to those of the glucocorticosteroid, dexamethasone, whose inhibitory action mechanism on membrane associated events has been already elucidated [19–22].

MATERIALS AND METHODS

Drug for each assay. Cyclosporin A preparation for injection was obtained from Sandoz pharmaceuticals (Basel, Switzerland) and olive oil, vehicle for the agent, from Nakarai Chemicals (Kyoto, Japan). For dose–response study, the following drug concentrations were added, before homogenization of the cells, and preincubated for 20 min or 12 hr in neutrophil, T-lymphocyte or lymphocyte suspensions which were obtained by the technique described below: Cyclosporin A 0.08, 0.8, 8 μg/ml; olive oil 1.6, 16, 160 nl/ml; dexamethasone 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M. Thereafter, the cells were washed with phosphate buffered saline (PBS) and used for each following assay system.

* Supported by a grant from Behçet Disease Research Committee of Japan Ministry of Welfare.

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** Abbreviations used: CS-A, cyclosporin A; EGTA, ethylene glycol β-aminoethylether-*N*,*N*,*N*′,*N*′-tetraacetic acid; [^{14}C]-CDP, ^{14}C-cytidine diphosphate; LPC, lysophosphatidyl-choline; PBS, phosphate buffered saline; PBSG, phosphate buffer with sodium azide and gelatin; PC, phosphatidyl-choline; PDME, phosphatidyl dimethyl ethanolamine; PE, phosphatidylethanolamine; PG, prostaglandin; PGE_2, prostaglandin E_2; PGI_2, prostaglandin I_2; PHA, phytohemagglutinin; PMA, phorbol myristate acetate; PMME, phosphatidyl monomethyl ethanolamine; RPMI, Roswell Park Memorial Institute; SIBA, 5′-*S*-Isobutyl-5′-deoxyadenosine; [^{3}H-methyl] SAM, *S*-adenosyl-L-[^{3}H-methyl]-methionine.

Preparations of neutrophils and lymphocytes. Heparinized venous blood was obtained from 16 healthy volunteers; either neutrophils or mononuclear cell fractions (including lymphocytes and monocytes) were separated from each sample by Ficoll-Hypaque centrifugation [14, 23]. Thereafter, non-adherent cells (lymphocytes) were separated from the mononuclear cell fractions by incubation on Petri dishes at 37° for 3 hr. Monocyte contamination in lymphocyte fractions was 6.3 ± 1.5%, which was identified by Giemsa staining and by reaction with OKM1 monoclonal antibody (Ortho Pharmaceutical Corp., Raritan, NJ) that binds to cells of the monocyte and myeloid series. Then, T lymphocyte fraction was further isolated from lymphocyte fraction by the sheep erythrocyte rosetting technique [24] and contained less than 1% monocytes.

Prostaglandin assay. Neutrophils or lymphocytes were suspended in RPMI 1640 containing 10% fetal calf serum at 33×10^4 cells/ml. The PG E_2 levels released from the following cells were assessed: (1) neutrophils stimulated with 1 mg/ml opsonized-zymosan, with or without pretreatment by PG synthetase inhibitors, indomethacin (0.1 μM; Sigma) or Ro 3-1314 (1 μM; Octadeca-9, 12-diyonic acid; Makor Chemicals, Jerusalem, Israel), (2) neutrophils stimulated with 1 μg/ml phorbol myristate acetate (PMA) (Sigma), with or without pretreatment by PG synthetase inhibitors, (3) lymphocytes stimulated with 10 μg/ml Con A, with or without pretreatment by PG synthetase inhibitors, (4) lymphocytes stimulated with 2.25 μg/ml PHA, with or without pretreatment by PG synthetase inhibitors, (5) homogenized neutrophils or lymphocytes.

PG E_2 was measured according to the method modified from Dray *et al.* [25]. Briefly, PG fractions in cell-free supernatant were extracted with ethyl acetate. After removal of the ethyl acetate by evaporation each fraction in a siliconized glass conical tube was dried under nitrogen gas. Thereafter, PG fractions were eluted with solvent (benzoate : ethyl acetate : methanol = 60 : 40 : 2) and again dried under nitrogen gas. Determination of the amount of PG E_2 was performed by radioimmunoassay, using anti-PG E_2 serum (Institute Pasteur Production, Paris). Standard PG E_2 (Sigma Chemical) dissolved in PBSG (0.1 M phosphate buffer with 0.9% NaCl, pH 7.4, containing 0.1% sodium azide and 0.1% gelatin), 1 pg [^{3}H] PG E_2 (New England Nuclear) and anti-PG E_2 were used to prepare a calibration curve. Extraction efficiency of PG E_2 in our assay condition was 82.8 ± 7.0%.

Preparation of leukocyte membrane fractions. We have recently described an assay in detail for methyltransferase activity in human neutrophils, lymphocytes and T-lymphocytes [26]. In the assay of membrane phospholipid enzyme activities of neutrophils and T-lymphocytes including methyltransferase, phospholipase A_2 and choline phosphotransferase, cells were suspended in 0.25 M sucrose and disrupted by sonication at 24 W for 10 sec on ice. The crude homogenates were centrifuged at 14,000 *g* for 10 min at 4° to remove mitochondria, nuclei and debris. The microsomal fraction was then recovered from the supernatant by centrifugation at 105,000 *g* for 1 hr at 4°. The so-obtained precipitates were suspended again in 0.25 M sucrose (*ca.* 4–6 mg/ml). Since 5′-nucleotidase activity was found to be high in our microsomal fraction, a considerably greater amount of plasma membrane was considered to be contained in the microsomes. Therefore, we refer to crude membrane fractions used in this study as the "microsomal" fraction [26].

Phospholipid transmethylation assay. The standard assay mixture contained 100 mM Tris–HCl (pH 8.0), 0.1 mM ethylene glycol β-aminoethyl-ether-*N,N,N′,N*-tetraacetic acid (EGTA), 50 μM *S*-adenosyl-L [^{3}H-methyl] methionine ([^{3}H-methyl] SAM) (2 μCi) and a microsomal fraction containing approximately 200 μg protein in a final volume of 200 μl. After incubation at 37° for 30 min, the reaction was stopped by adding 0.6 ml of 0.25 N HCl. In order to extract phospholipids from the incubation mixture, 3 ml of chloroform/methanol (1 : 2) was added and vortexed, and then 1 ml of 1% KCl and 1 ml of chloroform were further added and vortexed according to the method described by Bligh and Dyer [27]. After the two water-soluble layers were discarded, 2 ml of 0.5% KCl in 50% methanol was added, vortexed and centrifuged. Finally, 1 ml of the chloroform phase was removed and transferred into a counting vial; the radioactivity was measured after drying at 70–80° in an oven and the addition of universal gel (Nakarai Chemicals, Kyoto, Japan) or aqua gel liquid scintillation cocktail by a liquid scintillation spectrometer (Packard TRI-CARB, IL) and expressed as pmol of [^{3}H]-methyl incorporated into phospholipids per min per mg protein. Control tubes, lacking the microsomal fraction, were treated identically throughout the assay.

The reaction products (radiolabeled phospholipids) were separated and analysed [28, 29] by two-dimensional thin-layer chromatography (Kiesel gel 60 plate, 20 × 20 cm, 0.25 mm thickness, E, Merck) with chloroform/acetone/methanol/acetic acid/water (5/2/1/1/0.5 v/v) as the solvent in the first dimension and *n*-propyl alcohol/propionic acid/chloroform/water (3/2/2/1) in the second. The spots of methylated products corresponding to individual phospholipids were scraped off and transferred into counting vials for measuring the radioactivity. The individually identified radioactive methylated phospholipid is expressed as a percentage of the total radioactivity (CPM) ± S.E.M. recovered from the plate.

The preincubation of the cells with the methyltransferase inhibitor, 10 μM SIBA (5′-*S*-isobutyl-5′-deoxyadenosine) for 60 min inhibited the incorporation of the [^{3}H]-methyl group into cell lipids by 47–55% (not shown). As previously confirmed by us [26], two-dimensional thin layer chromatography demonstrated that most of the [^{3}H]-methyl incorporated into phospholipid was recovered as PC. Modest amounts of PDME, LPC, and PMME were labeled, while only negligible amounts of incorporation were observed to other phospholipids. As PMME, PDME, PC were surely labeled as determined by TLC, the validity of our assay method for phospholipid methylation was well supported [26].

Phospholipase A_2 assay. According to our original method [30], the substrate, 10 μl (0.5 μCi) β-[^{14}C] arachidonyl PC (β-[1-^{14}C] arachidonyl-α′-stearoyl-L-

β-PC) (Amersham, 59.3 mCi/nmol) was isolated by evaporation, then vortexed at 4° for 30 min and incubated at 30° for 30 min. Thereafter, this isolated agent was added to a 200 μg microsomal fraction suspended in 0.25 M sucrose, obtained as described above. These mixtures were vortexed at 30° and incubated at 30° for 90 min. The reaction was stopped by adding 1.5 ml of ice-cold chloroform/methanol (2/1 v/v) and then vortexed for 1 min and centrifuged at 2000 *g* for 5 min to separate the two layers. The chloroform layer was evaporated to dryness in vacuo at 30° and the residue was dissolved in a few drops of chloroform/methanol (2/1 v/v) and then applied to a thin-layer chromatography and developed with petroleum ether (b.p. 30–70°)/diethyl ether/acetic acid (80/30/1). The spots of reaction products corresponding to individual lipids were scraped off and counted.

Phospholipase A_2 activity was expressed as a percentage of arachidonic acid:

$$\frac{\text{isolated } \beta\text{-}[^{14}C] \text{ arachidonic acid}}{\beta\text{-}[^{14}C] \text{ arachidonyl PC} + \text{isolated } \beta\text{-}[^{14}C] \text{ arachidonic acid}} \times 100$$

Choline phosphotransferase activity. The standard assay mixture contained 100 mM Tris–HCl (pH 8.0), 0.1 mM EGTA, 5 mM $MgCl_2$, 50 μM cytidine-5′-diphospho [^{14}C-methyl] choline (0.1 μCi) and microsomal fraction containing 20–50 μg protein in a final volume of 200 μl. Subsequent reaction procedures were performed in the same way as in above-described transmethylation assay. [^{14}C]-methyl incorporation was assessed and expressed as pmol of [^{14}C]-methyl incorporated into phospholipids per min per mg protein. Control tubes, lacking the microsomal fraction, were treated identically throughout the assay.

Triplicate assays were performed simultaneously in each experiment; the results are expressed as the mean ± S.E.M. of replicate assays. Statistical significance was determined by Student's *t*-test.

RESULTS

20 min incubation with CS-A and dexamethasone

The release of prostaglandin E_2 from T-lymphocytes was markedly inhibited by the addition of cyclosporin A in a dose dependent fashion (0.08 μg/ml; $0.01 < P < 0.05$; 0.8 μg/ml; $P < 0.01$, 8 μg; $P < 0.001$). PG E_2 release from neutrophils was also slightly suppressed by CS-A (0.8 μg and 8 μg/ml; $0.01 < P < 0.05$) (Fig. 1). The pretreatment of the cells with the two prostaglandin synthesis inhibitors (0.1 μM indomethacine and 0.1 μM Ro-B14) inhibited the release of prostaglandin E_2, attesting the specificity of the prostaglandin assay by our method (data not shown).

Cyclosporin A markedly inhibited phospholipase A_2 activity in T-lymphocytes (8 μg/ml; $P < 0.0001$) and slightly that in neutrophils (8 μg/ml; $0.01 < P < 0.025$). In contrast, no significant effect on the activity of methyltransferase and CPT was found ($P > 0.05$) though the drug showed a tendency to decrease methyltransferase activity slightly ($P > 0.05$).

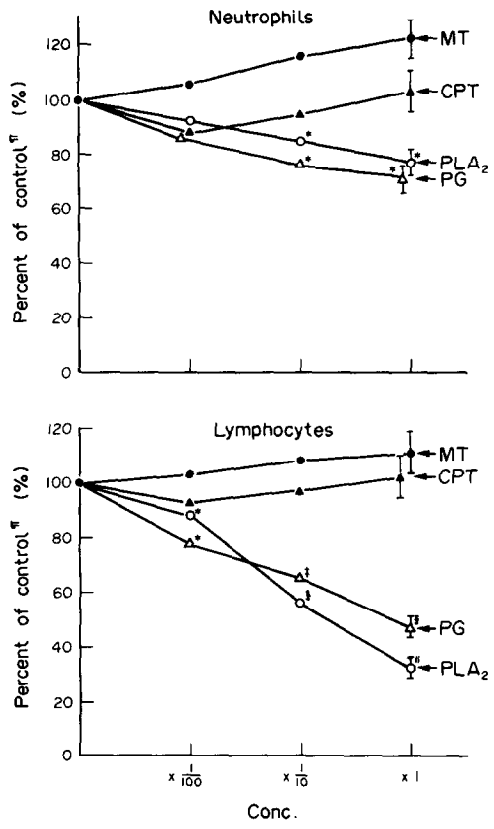

Fig. 1. Effect of 20 min incubation with cyclosporin A on the activities of methyltransferase, phospholipase A_2, choline phosphotransferase and PG E_2 release in normal human neutrophils and lymphocytes: ●—● MT, methyltransferase activity of unstimulated cells; ○—○ PLA_2, phospholipase A_2 activity of unstimulated cells; ▲—▲ CPT, choline phosphotransferase activity of unstimulated cells; △—△ PG, PG E_2 released from 33×10^4 neutrophils or lymphocytes, stimulated with 1 mg/ml opsonized zymosan for neutrophils or 10 μg/ml Con A for lymphocytes. Cyclosporin A was preincubated with the cells for 20 min before sonication. The effect of the agent on the activity of MT, PLA_2 and CPT was assessed for unstimulated neutrophils and T-lymphocytes and that on PG E_2 release was determined with stimulated neutrophils and lymphocytes; no effect of CS-A on PG release from unstimulated cells was detectable because of very low values without stimulation [33]. On the other hand, regarding MT, PLA_2 and CPT, enough amounts of their activities were readily obtained even in the case of no stimulation of membrane [26]. Therefore, the activation was required for assessing the PG levels and not for other enzyme activities. Each symbol in the figures represents the mean of triplicate determinations, the vertical bars ± one standard error of the mean (S.E.M.). Similar S.E.M.s were found in all of the plots. However, only S.E.M.s in the highest concentrations were described for avoiding complicated figures. ×1 = 8 μg/ml cyclosporin A; *$0.01 < P < 0.05$ vs. control (in the absence of cyclosporin A); ‡$P < 0.01$, §$P < 0.001$, ‖$P < 0.0001$, vs. control; ¶Each value was expressed as percent of each normal level (control level without CS-A) (normal values for neutrophils: MT 0.62 ± 0.04 pmol/min/mg protein, PLA_2 3.8 ± 0.23%, CPT 18.1 + 1.5 pmol/min/mg protein, and PG E_2 43.0 ± 2.4 pgr/33 × 10^4 cells, and normal values for lymphocytes: MT 0.85 ± 0.06 pmol/min/mg protein, PLA_2 5.9 ± 0.15%, CPT 54.2 ± 5.1 pmol/min/mg protein, and PG E_2 32.1 ± 1.3 pgr/33 × 10^4 cells. See Materials and Methods for further details.

The olive oil vehicle in which cyclosporin A was dissolved did not significantly affect the three membrane phospholipid enzyme activities or prostaglandin release ($P > 0.05$) (not shown).

As for the effect of dexamethasone, any dose of dexamethasone by 20 min incubation with the cells did not affect prostaglandin release or enzyme activities of methyltransferase, phospholipase A_2 or CPT.

12 hr incubation with CS-A and glucocorticosteroids

CS-A had no significant effects on any of the measured activities under these conditions (not shown). Olive oil vehicle alone did not affect the activities of these three membrane enzyme activities either (not shown). This may be explained as follows; the inhibitory effect of the drug on membrane enzyme activity is reduced or lost in 12 hr presumably because the agent is metabolized or inactivated.

On the other hand, dexamethasone significantly suppressed PG E_2 release from both T-lymphocytes (10^{-4} M; $0.01 < P < 0.025$) and neutrophils (10^{-4} M; $P < 0.01$) (Fig. 2). Phospholipase A_2 activity was also markedly suppressed by dexamethasone in both lymphocytes (10^{-4} M; $P < 0.01$) and neutrophils (10^{-4} M; $P < 0.001$) (Fig. 2). In contrast, methyltransferase and CPT activities were not significantly affected by dexamethasone although trend to increase methyltransferase activity was observed ($P > 0.05$) (Fig. 2).

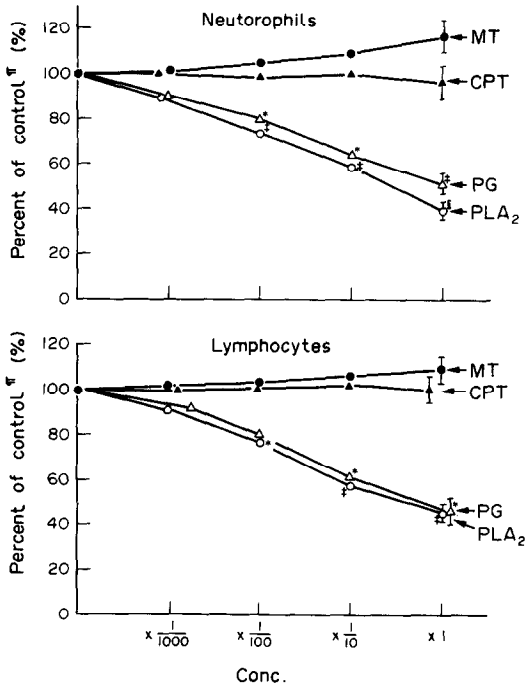

Fig. 2. Effect of 12 hr incubation with dexamethasone on the activities of methyltransferase, phospholipase A_2, choline phosphotransferase and PG E_2 release in normal human neutrophils and lymphocytes. The agent was preincubated with the cells for 12 hr before sonication. $\times 1 = 10^{-4}$ M dexamethasone. Regarding other symbols: see the legend to Fig. 1.

DISCUSSION

In the present study, the 20 min incubation of cyclosporin A was found to inhibit markedly the activity of phospholipase A_2; since this enzyme plays a critical role in particular membrane associated events, it is not surprising that other investigations have found it to inhibit various lymphocyte functions [4–8]. The inhibition of phospholipase A_2 by CS-A is also consistent with our observations that prostaglandin E_2 release, but not methyltransferase activity, was inhibited by the drug, since phospholipase A_2 acts at a distant reaction site to methyltransferase in the production of membrane arachidonic acid, and PG E_2 release occurs following arachidonic acid production mediated by phospholipase A_2. The inhibition of phospholipase A_2 appears to underlie the decreased prostaglandin synthesis, and this fact may result in the suppression of lymphocyte responses, and the generation of oxygen radicals by this agent.

The above-described effects of 20 m incubation of CS-A on membrane associated events are quite similar to those of 12 hr incubation of steroids which were already reported by Hirata [21] and others [22]. However, as shown in Figs 1 and 2, one obvious difference of drug action between CS-A and glucocorticosteroids was found: it requires less time for CS-A to exert on phospholipid metabolism, although the sites of action of two agents were found to be the same. This dissociation of the time required for the drug action may be due to the difference of the activity on the receptor between the two agents, or to the possibility that CS-A may exert inhibitory effect more shortly after it binds to the receptor presumably because it is rapidly metabolized to be activated, while it may take longer time for corticoids after binding to the receptor to be activated. This difference in time requirement may also speculate that CS-A elicits the inhibition of phospholipase A_2 without synthesizing phospholipase inhibitory protein, lipomodulin which is reported to require 12 hr more to be activated (by corticoids) [21, 22] if lipomodulin really exists. In view of these assumptions, it is likely that CS-A has a direct action on the enzyme activity, while corticoids act indirectly on the cells through the induction of inhibitory protein. Anyway, from the above-stated considerations, it is reasonable that CS-A is used not only for organ transplantation and the disorders with immune abnormalities but also for the treatment of the patients with potentiated neutrophil functions. In addition, it supports the present study that active Behçet's patients showed a marked increase in phospholipase A_2 activity of their leukocyte membrane [31], and this activity was reduced after the treatment of CS-A (data not shown).

As the effect of 20 min incubation with CS-A was found to be quite similar to that of 12 hr incubation with steroids (Figs 1 and 2), the trend to increase transmethylation was observed in the presence of both drugs although the statistical significance was not obtained (Figs 1 and 2). This finding by dexamethasone is in agreement with the previous report (in lymphoid cells) by Ramachandran and Melnykovych [32]. This tendency seems to be due to the result of decreased PC degradation.

Recently, Ryffel *et al.* [9] demonstrated cyclosporin receptors on human lymphocytes and competition of binding between ^{3}H-cyclosporin C and T cell mitogens. On the other hand, LeGrue *et al.* [10] presented conflicting data against the existence of a specific CS-A receptor on the surface of human T lymphocytes; from their results, they speculated that the immunosuppressive effects of the drug might be achieved by the partitioning of the hydrophobic CS-A molecule in the membrane lipid bilayer, with resultant perturbation of homeostatic control of membrane function. Although they did not show any concrete evidence for membrane phospholipid metabolism, their prediction is in part supported by our results.

How CS-A might exert such an effect remains to be determined; however, its strong lipophilic nature suggests that it may interact directly with membrane phospholipids or glucolipids.

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