Vol. 185, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1992 Pages 363-369

EFFECTS OF CHANGES IN MEMBRANE POTENTIAL ON THE CYCLOSPORIN-INDUCED INHIBITION OF T-CELL PROLIFERATION*

Attila Tordai, Reuven Or, and Erwin W. Gelfand**

Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, and The Raymond and Beverly Sackler Foundation, Denver, Colorado 80206

Received April 6, 1992

Cyclosporin A (CsA) exerts its major immunosuppressive effect by inhibition of T-lymphocyte proliferation. The precise mechanism and target of its action has not yet been completely identified. CsA is also known to induce a rapid membrane depolarization in T lymphocytes. We have tested the role of CsA-dependent depolarization in the inhibition of T-cell proliferation by the drug. In these studies, induced membrane depolarization (in the presence of gramicidin or by replacing the Na^+ content of the medium with K^+) or hyperpolarization (in the presence of valinomycin) had no influence on the induction of T-cell competence by phorbol dibutyrate/ionomycin or by submitogenic concentrations of PHA, a target for CsA immunosuppression. However, regardless of the state of membrane potential during the induction of T-cell competence, the inhibition by CsA was the same as seen in normally polarized cells. We conclude that the depolarization induced by CsA is not a critical element in its inhibitory effect on T-cell proliferation.

Cyclosporin A (CsA), an extensively used immunosuppressive agent, acts by inhibition of T-cell proliferation induced by antigen or mitogens. The inhibition of IL2 production by activated T cells is reported to be the major effect of CsA (1-3). However, other effects of this drug are being increasingly described. The influence of CsA on functional IL2 receptor expression remains controversial (4-7) and several effects of CsA have also been demonstrated in other cell types besides the T cell, including the inhibition of B-cell (8), macrophage (9) and basophil (10) activation. Evidence is accumulating indicating a direct effect of CsA on the nucleus of activated T cells (11), namely the inhibition of the binding capacity of specific nuclear factors (e.g. NFAT, AP-1) to the transcriptional regulatory sites of the IL2 gene (12-14), which may explain the inhibition of IL2 production. In previous studies the depolarization of lymphocytes by CsA and

*This work was supported by Grants AI-26490 and AI-29704 from the NIH (EWG). EWG is a Scholar of the Raymond and Beverly Sackler Foundation. EWG is a Scholar of the Raymond and Beverly Sackler Foundation.

**To whom correspondence should be addressed at Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206.

0006-291X/92 $4.00

the other immuno-suppressive cyclosporins (Dihydro-CsD and CsG) was among the earliest effects detected in lymphocytes (15-17). Since the non-immunosuppressive cyclosporin analogue CsH failed to trigger such a decrease in membrane potential we questioned whether the depolarizing effect was linked to the immunosuppressive activities.

Using a "two-phase activation system", we have shown that the effects of CsA are limited to the initiation (induction of competence) of T-cell activation (4), but have little or no effect on cell-cycle progression to DNA synthesis. We used this system to determine if the CsA-induced depolarization is involved in its immunosuppressive activity. Based on these data, we conclude that the decrease in transmembrane potential caused by CsA is not necessary for its inhibition of T-cell proliferation.

MATERIALS AND METHODS

Cells . T lymphocytes were prepared from the blood of healthy volunteers by Ficoll-Hypaque gradient centrifugation and by E-rosette enrichment as described (18). These cells were more than 90% CD3-positive.

Media and reagents. Complete medium was used for the 72 hr cultures and for control experiments. This was prepared by supplementing RPMI 1640 (Gibco, Grand Island, NY) with 4 mM L-glutamine, 1 mM Na pyruvate, 15 μM Hepes, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Gibco) and 10% (v/v) FCS (Hyclone, Logan, UT). The following media were used for the initial activation of T cells and for the membrane potential measurements. The Na^+-based medium contained 140 mM NaCl, 2 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM Hepes, 10 mM $NaHCO_3$, 10 mM Na_2HPO_4 and 10 mM glucose at pH 7.4. The K^+-based medium contained 140 mM KCl instead of NaCl and all the other constituents were the same. Phorbol dibutyrate (PDB) and ionomycin were purchased from Calbiochem (San Diego, CA), dissolved in DMSO, and stored at -20°C. PHA was from Difco (Detroit, MI). CsA was kindly provided by Dr. J. Borel (Sandoz, Basel, Switzerland), dissolved in ethanol at 1 mg/ml concentration and stored at 4°C under light protection. Gramicidin and valinomycin were from Sigma (St. Louis, MO), bis-oxonol and diS-C_3-(5) were from Molecular Probes. The latter four chemicals were dissolved in DMSO and stored at -20°C.

Activation procedure (competence induction phase). T cells (1.5×10^6) were placed in the appropriate medium and were stimulated either by the combination of 10 nM PDB and 500 nM ionomycin for 30 min or by 500 ng/ml PHA for 60 min. Activation was carried out in the presence or absence of CsA (1 μg/ml). Cells were then washed 3 times and plated in 96-well flat bottom plates (1.5×10^5 T cells/well) in complete medium. There were no significant differences between cells activated in complete medium or in Na^+-based medium as measured in the proliferation assay.

Cell proliferation (progression phase). Activated cells were cultured in complete medium, in the presence or absence of 10 nM PDB, and in the presence or absence of 1 μg/ml CsA for 72 hrs. [^{3}H]TdR incorporation was assessed by harvesting (PhD Cell Harvester, Cambridge, MA) and scintillation counting (Beckmann Scintillation Counter, Fullerton, CA) at 72 hr after a 6-hr pulse with 0.5 μCi/well [^{3}H]TdR (ICN, Irvine, CA). Competent T cells (treated either with PDB/ionomycin for 30 min or with submitogenic PHA for 60 min and washed) did not show significant [^{3}H]TdR incorporation in the 72 hr culture if the second stimulus (PDB) was not present.

Measurement of membrane potential. The membrane potential of T lymphocytes was determined with two different fluorescent probes, bis-oxonol and diS-C_3-(5). Cells (2×10^6) were placed in 2 ml of the appropriate medium and equilibrated with one of the probes in a cuvette at 37°C with continuous stirring in a Hitachi F-4010 fluorescent spectrophotometer. The excitation and emission wavelengths were 540 and 580 nm for bis-oxonol and 620 and 670 nm for diS-C_3-(5). The final concentration of bis-oxonol in the cuvette was 0.3 μM, while that of diS-C_3-(5) was 1 μM. Calibration of the bis-oxonol fluorescence was done as described by replacing the Na^+ content of the media by varying concentrations of N-methyl glucamine (19). The diS-C_3-(5)

fluorescence was calibrated using the valinomycin null-point titration method at different extracellular K^+ concentrations (20,21).

RESULTS

Monitoring resting membrane potential in T cells and the effect of CsA. In resting T cells, membrane potential averaged 59 ± 3 mV when measured with either bis-oxonol or diS-C_3-(5). Addition of PHA resulted in a transient hyperpolarization (averaging between -9 and -12 mV) with a return to baseline in bis-oxonol-loaded cells (Fig. 1A). Addition of gramicidin caused a rapid depolarization (Fig. 1A). In K^+-based medium, the cells were also depolarized, and the further addition of gramicidin had little additional depolarizing effect (Fig. 1B).

Since valinomycin is known to form a complex with bis-oxonol unrelated to the changes in membrane potential, we used diS-C_3-(5) to monitor the effects of valinomycin. As shown in Fig. 1C, addition of valinomycin caused a -14 ± 2 mV increase in membrane potential, i.e. hyperpolarized the cells.

As previously reported CsA caused a rapid depolarization of resting cells. Interestingly, CsA had the same effect on competent T cells, i.e., on cells treated with PDB/ionomycin for 30

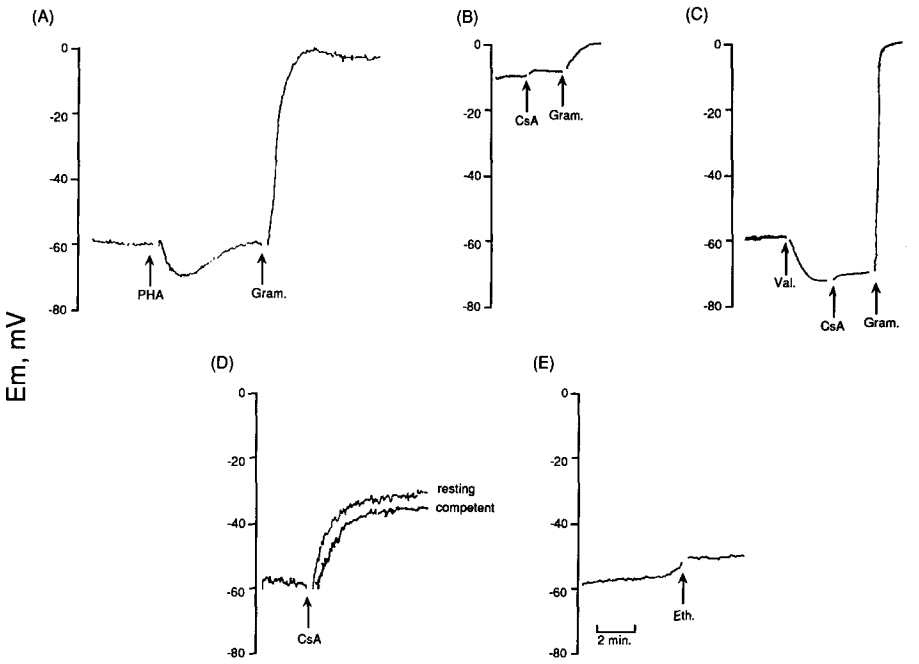

Figure 1. Effect of PHA, CsA and ionophores on the membrane potential of T lymphocytes. 2 x 10^6 T cells were equilibrated with 0.3 μM bis-oxonol (panels A, D and E) or with 1 μM diS-C_3-(5) (panels B and C). Reagents were added where indicated by the arrows in the following final concentrations: PHA: 10 μg/ml; gram (gramicidin) 250 nM; CsA: 1 μg/ml; val (valinomycin): 100 nM; eth (ethanol) 0.1% (v/v). Panels A, C, D and E show experiments carried out in Na^+-based media, while panel B shows an experiment done in K^+-based medium. Panel D shows the results of experiments in resting and competent (after 30 min PDB/ionomycin treatment) T cells. Traces are representative of at least 3 separate experiments.

min (Fig. 1D). Ethanol alone, the vehicle for suspending CsA, had only a small effect on membrane potential (Fig. 1E).

In cells depolarized by gramicidin (not shown), or suspended in high K^+ medium (Fig. 1B), or in cells hyperpolarized by valinomycin (Fig. 1C), addition of CsA did not further depolarize the cells or reverse the state of hyperpolarization. Thus the effects of CsA on membrane potential were neutralized and it was under these conditions that we tested the imunosuppressive activity of the drug.

Effect of CsA on T-cell proliferation in depolarizing media. As shown previously (4), CsA showed an exclusive effect on the induction of competence with little effect in the progression phase. Using PDB in the progression phase, the presence of CsA in the competence phase resulted in approximately 85% inhibition of the response initiated by PDB/ionomycin (data not shown). Those cells which were initially stimulated in the absence of CsA for 30 min proliferated to a similar extent regardless of the presence or absence of CsA in the progression phase. Fig. 2 illustrates the results of experiments carried out with cells suspended in media which result in a decrease in membrane potential. In K^+-based medium T cells are known to decrease their transmembrane potential to almost zero while in the presence of gramicidin in Na^+-based medium a maximum depolarization occurs. These conditions were maintained only for the time of the initial activation (induction of competence) of T cells, after which time the cells were restored to complete medium. Cell proliferation in response to PDB in the progression phase was observed to a similar extent regardless of the state of membrane potential during the induction of competence. The data were similar if we induced competence by incubating T cells with PDB/ionomycin for 30 min (Fig. 2A) or by a submitogenic concentration of PHA for 60 min (Fig. 2B) . Thus despite

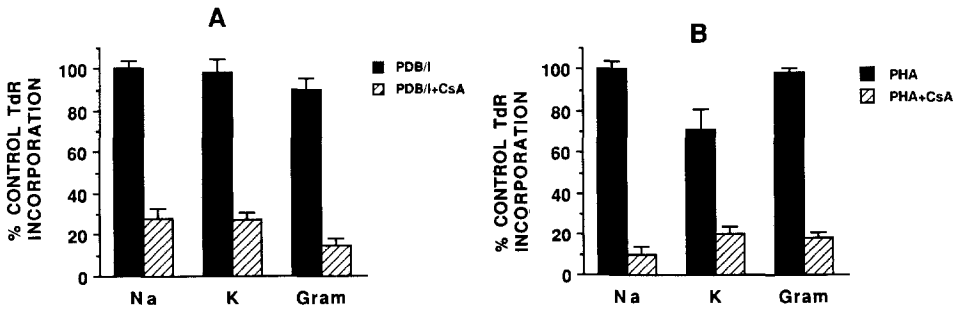

Figure 2. Effect of CsA on the induction of competence by PDB/ionomycin or submitogenic PHA in T lymphocytes suspended in different media or in the presence of gramicidin. T cells were suspended in 3 different media: Na^+-based (Na), K^+-based (K) and Na^+-based + 250 nM gramicidin (Gram). Under these conditions 10 nM PDB and 500 nM ionomycin (PDB/I) were added for 30 min (panel A) or 500 ng/ml PHA was added for 60 min (panel B) with or without 1 μg/ml CsA. Cells were then washed and cultured for 72 hr in the presence of 10 nM PDB and the cell proliferation was measured by [^{3}H]TdR incorporation. In cultures without PDB, cpm values ranged between 1-2 X 10^3 (not shown) while in cultures with PDB they were similar if the initial stimulation was in complete medium or in Na^+-based medium: $2.6 \pm 0.4 \times 10^4$ cpm for PDB/ionomycin and $4.2 \pm 0.6 \times 10^4$ cpm for PHA. Results are means (± SEM) of 3 separate experiments.

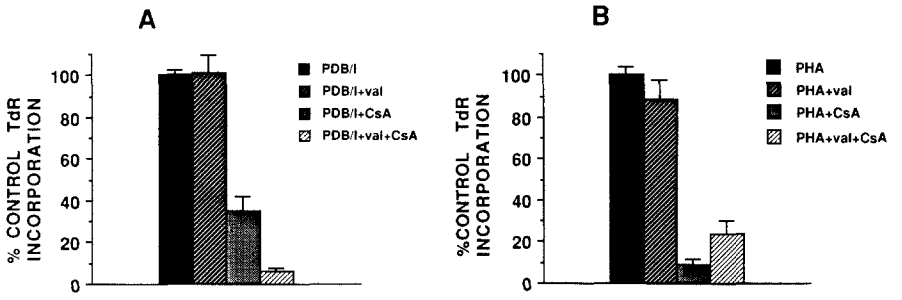

Figure 3. Effect of valinomycin on CsA-mediated inhibition of competence induction by PDB/ionomycin or submitogenic concentrations of PHA. T cells, suspended in Na^+-based medium (Na) in the presence or absence of 100 nM valinomycin, were treated with 10 nM PDB and 500 nM ionomycin (PDB/I) for 30 min (panel A) or 500 ng/ml PHA (panel B) for 60 min with or without 1 μg/ml CsA. Cells were then washed and cultured for 72 hr in the presence of 10 nM PDB and cell proliferation was measured by [^{3}H]TdR incorporation. In cultures without PDB, cpm values ranged between 0.8-1.8 x 10^3 (not shown). In cultures containing PDB, the control cpm values were 1.3 ± 0.2 x 10^4 for PDB/ionomycin and 4.6 ± 0.6 x 10^4 for PHA respectively. Results are means (± SEM) of 3 separate experiments.

substantial depolarization of the cells, induction of competence proceeded normally with either mitogen. In addition, the inhibitory effects of CsA in the competence induction phase were similarly maintained despite the alterations in transmembrane potential induced by the K^+-based medium or gramicidin (Fig. 2).

Effect of CsA on T-cell proliferation in hyperpolarizing medium

To hyperpolarize the cells, valinomycin was used. As shown in Fig. 3, the increase in membrane potential by this ionophore did not influence the induction of competence or the ability of CsA to inhibit competence induction in hyperpolarized cells. We observed this with T cells stimulated by PDB/ionomycin for 30 min (Fig. 3A) or by a submitogenic concentration of PHA for 60 min (Fig. 3B). The presence of valinomycin did result in some toxicity to the cells, accounting for some reduction (~20%) in the degree of T-cell proliferation.

DISCUSSION

The goal of the present study was to determine whether the immunosuppressive activity of CsA was linked or dependent on its ability to rapidly depolarize human T cells. This possibility was underscored by the previous findings that only the immunosuppressive cyclosporins had this effect in contrast to a non-immunosuppressive analogue (15). To address this question we manipulated the membrane potential of the cells by altering the extracellular ionic milieu or by addition of the conductive ionophores gramicidin or valinomycin. To decrease the toxicity to the cells as a consequence of these manipulations, we utilized a previously developed two-phase culture protocol that isolates a competence induction phase and a progression phase (22). In this system, CsA only exerts its immunosuppressive effects when present in the competence induction phase (4).

We chose two approaches to depolarize the T cells. Since K^+ is the major contributor to the membrane potential of resting T cells, increasing the extracellular K^+ concentration results in a substantial depolarization of these cells. Gramicidin, a K^+/Na^+ ionophore, strongly increases the permeability of the plasma membrane to these ions and similarly results in membrane depolarization. Under these conditions, cell depolarization did not affect the ability of the combination of PDB/ionomycin or submitogenic concentrations of PHA to induce a state of competence in these cells; i.e., subsequent incubation with PDB resulted in a full proliferative response. Since depolarization of the cells is known to inhibit ligand-activated Ca^{2+} entry in T cells (23-25) and submitogenic concentrations of PHA do not result in detectable changes in intracellular Ca^{2+} concentrations, Ca^{2+} entry does not appear to be necessary for induction of competence with PHA. Induction of competence with PDB/ionomycin is, however, dependent on the availability of extracellular Ca^{2+} (4).

CsA may alter Ca^{2+} entry in cells stimulated with mitogenic concentrations of PHA but does not affect the increases in cytosolic Ca^{2+} triggered by the ionophore ionomycin (15). Regardless of its effects on Ca^{2+}, CsA induced a rapid depolarization in both resting and competent T cells. In cells already depolarized by gramicidin or K^+-based medium, CsA did not further depolarize the cells nor interfere with the depolarizing effects of gramicidin or high K^+. Despite this, CsA was fully effective in suppressing the induction of competence.

Similar results were obtained when the depolarizing effects of CsA were eliminated by prior exposure of the cells to valinomycin. This drug is a conductive K^+-selective ionophore which results in hyperpolarization of the cell membrane. Addition of CsA to these hyperpolarized cells had no effect on membrane potential yet still remained inhibitory for induction of competence. Competence induction itself was unaffected by hyperpolarization of the cells.

A number of different cell types have now been demonstrated to be susceptible to the additon of CsA. In many, the activation of the cells is blocked, in others more global effects on gene transcription are being delineated. It appears likely that there are several targets for CsA action on T cells as well (15). In both human and murine T and B cells, the binding of CsA results in a rapid alteration of transmembrane potential by an as yet undefined mechanism (15,17,26). However, based on the results of the present study, these effects on membrane potential appear unrelated to the potent immunosuppressive activity of this group of drugs.

ACKNOWLEDGMENTS

We thank Dr. J. Borel for providing CsA and Jane Watkins for her help in preparation of the manuscript.

REFERENCES

1. Elliot, J. F., Lin, Y., Mizel, S. B., Bleakley, R. C., Harnish, D. G., and Paetkau, V. (1984) *Science* **226**, 1439-1441.
2. Kronke, M., Leonard, W. J., Depper, J. M., Arya, S. K., Wong-Staal, F., Gallo, R. C., Waldmann, T. A., and Greene, W. C. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5214-5218.
3. Granelli-Piperno, A., Inaba, K., and Steinman, R. M. (1984) *J. Exp. Med.* **160**, 1792-1802.

4. Kumagai, N., Benedict, S. H., Mills, G. B., and Gelfand, E. W. (1988) *J. Immunol.* **141**, 3747-3752.
5. Gauchat, J. F., Khandjian, E. W., and Weil, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6430-6434.
6. Reed, J. C., Abidi, A. H., Alpers, J. D., Hoover, R. G., Robb, R. J., and Nowell, P. C. (1986) *J. Immunol.* **137**, 150-154.
7. Miyawaki, T., Yachie, A., Ohzeki, S., Nagaoki, T., and Taniguchi, N. (1983) *J. Immunol.* **130**, 2737-2742.
8. Wicker, L. S., Boltz, R. C., Jr., Matt, V., Nichols, E. A., Peterson, L. B., and Sigal, N. H. (1990) *Eur. J. Immunol.* **20**, 2277-2283.
9. Chiara, M. D., Bedoya, F., and Sobrino, F. (1989) *Biochem. J.* **264**, 21-26.
10. Hultsch, T., Rodriguez, J. L., Kaliner, M. A., and Hohman, R. J. (1990) *J. Immunol.* **144**, 2659-2664.
11. Granelli-Piperno, A. (1990) *J. Exp. Med.* **171**, 533-544.
12. Brini, A. T., Harel-Bellan, A., and Farrar, W. L. (1990) *Eur. Cytokine Net.* **13**, 131-139.
13. Emmel, E. A., Verweij, C. L., Durand, D. B., Higgins, K. M., Lacy, E., Crabtree, G. R. (1989) *Science* **246**, 1617-1620.
14. Randak, C., Brabletz, T., Hergenröther, M., Sobotta, I., and Serfling, E. (1990) *EMBO J* **9**, 2529-2536.
15. Gelfand, E. W., Cheung, R. K., and Mills, G. B. (1987) *J. Immunol.* **138**, 1115-1120.
16. Mátyus, L., Balázs, M., Aszalós, A., Mulhern, S., and Damjanovich, S. (1986) *Biochim. et Biophys. Acta* **886**, 353-360.
17. Damjanovich, S., Aszalós, A., Mulhern, S. A., Szöllösi, J., Balázs, M., Trón, L., and Fulwyler, M. J. (1987) *Eur. J. Immunol.* **17**, 763-768.
18. Cheung, R. K., Grinstein, S., and Gelfand, E. W. (1982) *J. Clin. Invest.* **70**, 632-638.
19. Grinstein, S., Goetz, J. D., and Rothstein, A. (1984) *J. Gen. Physiol.* **84**, 565-584.
20. Waggoner, A. S. (1979) *Meth. Enzymol.* **55**, 689-695.
21. Grinstein, S., Clarke, C. A., DuPre, A., and Rothstein, A. (1982) *J. Gen. Physiol* **80**, 801-823.
22. Kumagai, N., Benedict, S. H., Mills, G. B., and Gelfand, E. W. (1988) *J. Cell Physiol.* **137**, 329-336.
23. Sarkadi, B., Tordai, A., and Gárdos, G. (1990) *Biochim. et Biophys. Acta* **1027**, 130-140.
24. Gelfand, E. W., Cheung, R. K., Mills, G. B., and Grinstein, S. (1987) *J. Immunol.* **138**, 527-531.
25. Di Virgilio, F., Lew, D. P., Andersson, T., and Pozzan, T. (1987) *J. Biol. Chem.* **262**, 4574-4579.
26. Damjanovich, S., Aszalós, A., Mulhern, S., Balázs, M., and Mátyus, L. (1986) *Molecular Immunol.* **23**, 175-180.