Cyclosporine A Inhibits Ca^{2+}-dependent Stimulation of the Na^+/H^+ Antiport in Human T Cells

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Abstract. The cyclic undecapeptide cyclosporine A (CsA) is a potent immunosuppressive agent that inhibits the initial activation of T lymphocytes. This agent appears to be most effective in blocking the action of mitogens such as concanavalin A and the calcium ionophore A23187, which cause an influx of Ca^{2+}, but not those that may act by alternate mechanisms. These observations suggest that CsA may block a Ca^{2+}-dependent step in T cell activation. We have shown that stimulation of the T3-T cell receptor complex-associated Ca^{2+} transporter activates the Na^+/H^+ antiport (Rosoff, P. M., and L. C. Cantley, 1985, *J. Biol. Chem.*, 260: 14053–14059). The tumor-promoting phorbol esters, which are co-mitogenic for T cells, activate the exchanger by a separate pathway which is mediated by protein kinase C. Both the rise in intracellular Ca^{2+} and intracellular pH may be necessary for the successful triggering of cellular activation. In this report we show that CsA blocks the T3-T cell receptor-stimulated, Ca^{2+} influx-dependent activation of Na^+/H^+ exchange, but not the phorbol ester-mediated pathway in a transformed human T cell line. CsA inhibited mitogen-stimulation of interleukin-2 production in a separate cell line. CsA also inhibited vasopressin stimulation of the antiporter in normal rat kidney fibroblasts, but had no effect on serum or 12-*O*-tetradecanoyl phorbol 13-acetate stimulation. CsA did not affect serum or vasopressin or serum stimulation of normal rat kidney cell proliferation. CsA also had no effect on lipopolysaccharide or phorbol ester stimulation of Na^+/H^+ exchange activity or induction of differentiation in 70Z/3 pre-B lymphocytes in which these events are initiated by the protein kinase C pathway. These data suggest that mechanisms of activation of Na^+/H^+ exchange that involve an elevation in cytosolic Ca^{2+} are blocked by CsA but that C kinase-mediated regulation is unaffected. The importance of the Na^+/H^+ antiport in the regulation of growth and differentiation of T cells is discussed.

STIMULATION of quiescent cells by growth factors and, in the case of T and B lymphocytes, specific antigen, is followed by the activation of a number of membrane signal transduction systems resulting in rapid alterations in the cytoplasmic levels of several critical cations. In many cell systems, proliferation or cellular activation will not occur when certain specific inhibitors of the activated transport systems are added (8, 9, 18, 19, 33, 42, 46, 47). Some mitogens act by initially stimulating phosphatidylinositol turnover, generating the second messengers, diacylglycerol and inositol trisphosphate (2, 26). The former activates protein kinase C while the latter effects the release of Ca^{2+} from intracellular stores (2, 26). In many cells, mitogens also stimulate a Ca^{2+} influx from outside the cell (23, 26, 43). Both an elevation in cytosolic calcium and activation of protein kinase C have been shown to stimulate the Na^+/H^+ antiport (3, 24, 35, 36). In human T cells, the initial event in this process appears to be the stimulation of the T cell antigen receptor and the closely associated T3 complex by either lectin, monoclonal antibodies, or antigen (14, 18, 21, 25, 28–30, 35, 36, 39–41, 45–47). Both the rise in intracellular pH (pH_i)[1] and cytosolic free calcium are thought to be important for cell activation and differentiation (8, 9, 13, 33, 34), although two recent reports have questioned the physiological importance of Na^+/H^+ antiport activation in the subsequent induction of T cell proliferation (22) or pheochromocytoma neuronal cell differentiation (4).

The human T3-T cell receptor complex (T3-TCR), found on all functional human T lymphocytes, is composed of an invariant heterotrimer (the T3 complex) and the 90-kD antigen receptor heterodimer (21, 27). The monoclonal antibodies OKT3 and WT-31 bind to the T3 complex and the constant region domain of the receptor, respectively, and are mitogenic for resting T cells (18, 38–40). This laboratory and others have previously shown that these antibodies induce a

1. *Abbreviations used in this paper*: $[Ca^{2+}]_i$, intracellular Ca^{2+}; CsA, cyclosporine A; DMCF, dimethylcarboxyfluorescein; IL-2, interleukin 2; LPS, lipopolysaccharide; pH_i, intracellular pH; T3-TCR, T3-T cell receptor complex; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; VSP, arginine vasopressin.

rapid, membrane potential-sensitive Ca^{2+} influx in the leukemic human T lymphocyte cell lines, HPB-ALL (28) and Jurkat (14, 15, 46, 47), respectively. Jurkat cells may also be stimulated to produce interleukin 2 (IL-2) (47). The potent tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), has no effect on intracellular Ca^{2+} ($[Ca^{2+}]_i$) or cellular activation/proliferation (28, 43), although it is known to be a co-mitogen for T cells by substituting for the requirement that monocytes/macrophages be present in the culture system (5, 47).

Stimulation of the T3-TCR on HPB-ALL cells with OKT3 and WT-31 results in a rapid rise in pH_i via stimulation of a dimethylamiloride-sensitive Na^+/H^+ antiport system (36). The T3-TCR-activated mechanism is dependent on the presence of extracellular Ca^{2+} and can be blocked by La^{3+}. This mechanism may be analogous to that activated after epidermal growth factor stimulation of A431 cells (23). Addition of TPA to HPB-ALL cells also activates the exchanger by a mechanism similar to that described in TPA induction of differentiation in the pre-B cell line 70Z/3 (33, 34) and the promyelocytic leukemia line, HL-60 (3). Curiously, the TPA stimulation of Na^+/H^+ exchange in HPB-ALL cells is transient while the anti-T3-TCR induction persists for >20 min. In addition, pretreatment of these cells with TPA results in an inhibition of the anti-T3-TCR antibody stimulation of the antiport without affecting the receptor-associated Ca^{2+} transporter. These results suggest that the antibody-mediated Ca^{2+} influx is activating a Ca^{2+}-dependent intermediate which is in turn responsible for directly stimulating the antiport (36).

A recent report has shown that the potent immunosuppressive drug cyclosporine A (CsA) binds to calmodulin and inhibits in vitro calmodulin-dependent phosphodiesterase activity (7). These findings suggested to us that the T lymphotrophic immunosuppressive properties of this agent may be a consequence of inhibition of responses to the T cell receptor-stimulated Ca^{2+} influx. In this report we show that CsA does not block anti-TCR antibody stimulation of Ca^{2+} influx but does block the subsequent activation of Na^+/H^+ exchange. This is associated with an inhibition of mitogen-stimulated IL-2 production by Jurkat cells. CsA also inhibits a homologous Ca^{2+}-dependent pathway in mitogen-stimulated fibroblasts, although it has no effect on arginine vasopressin- (VSP) or serum-stimulated proliferation. These results do not support the theory that the biological effects of CsA are not due to a primary inhibition of calmodulin. Our data suggest that stimulation of the Na^+/H^+ exchanger by the Ca^{2+}-dependent pathway is integral to T cell activation, although it may play a reduced role in transmembrane signal transduction in serum-stimulated fibroblasts. At least some of the immunosuppressive effects of CsA may therefore be related to a lineage-specific effect on mitogen stimulation of the T cell Na^+/H^+ antiport.

Materials and Methods

Cells

The human T lymphocytic leukemia cell line HPB-ALL was maintained in liquid suspension culture in RPMI 1640 medium supplemented to 5% (vol/vol) with fetal calf serum (FCS), 10 mM Hepes buffer (pH 7.35), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate at 37°C in 95% air/5% CO_2 (28). Jurkat cells were obtained from Cox Terhorst and Hans Oettgen (Dana-Farber Cancer Institute, Boston, MA). They were maintained in the above supplemented RPMI medium (made 10% in FCS). Normal rat kidney (NRK) cells were the generous gift of Dr. R. J. Goldberg of Merck Sharpe and Dohme Research Laboratories (Rahway, NJ) and maintained in Dulbecco's modified Eagle's medium (DME) with 10% FCS supplemented as above and grown in monolayers in tissue culture flasks or 100-mm tissue culture dishes (Nunc, Roskilde, Denmark) at 37°C in 95% air/5% CO_2. For measurement of pH_i, NRK cells were transferred to Leighton tubes (Bellco Glass Inc., Vineland, NJ) and grown in monolayers on 9 × 50 mm glass coverslips as described (41). 70Z/3.B3 cells were maintained as described (33–35).

Reagents

OKT3 and WT-31 monoclonal antibodies were the gift of Dr. Cox Terhorst (Dana-Farber Cancer Institute). They were purified from mouse ascites and stored at 1 mg/ml at −20°C in phosphate-buffered saline (PBS) as described (28). Arginine vasopressin (VSP) and *Escherichia coli* lipopolysaccharide (LPS; serotype 055:B5) were from Sigma Chemical Co., St. Louis, MO. Dimethylcarboxyfluorescein diacetate (DMCF) was from Molecular Probes, Inc. (Junction City, OR). Phorbol esters were from LC Services Corp. (Woburn, MA). CsA was from Sandoz Pharmaceuticals Div., Sandoz Inc., Hanover, NJ, and was stored in a stock solution of 2.5 mM in absolute ethanol at −70°C. Dimethylamiloride was the generous gift of Dr. Edward Cragoe, Jr. (Merck Sharp and Dohme Research Laboratories, West Point, PA). [^{3}H]Thymidine was from New England Nuclear, Boston, MA. Recombinant human IL-2 was the generous gift of Dr. Vicki Sato (Biogen, Inc., Cambridge, MA).

Measurement of pH_i

pH_i was determined in HPB-ALL, 70Z/3.B3 and NRK cells as previously described using the pH-sensitive fluorescent dye, DMCF (34, 36). For experiments with HPB-ALL and 70Z/3.B3 cells, 2–3 × 10^7 cells were loaded with the membrane permeant acetomethoxy ester of DMCF in a buffer containing 145 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4, 0.8 $MgSO_4$, 1.8 $CaCl_2$, 25 mM Hepes (pH 7.4), and 10 mM glucose, as described (28). This was also the buffer used in all other experiments. NRK cells were loaded with DMCF diacetate in Leighton tubes. pH_i was measured by comparing the ratio of absorbance at λ_{505} nm/λ_{470} nm to a standardized calibration curve that was individually determined for each cell line used (33, 36). All measurements were performed in a Uvikon dual beam spectrophotometer (Kontron Analytical, Kontron AG, Everett, MA) with the cuvette chamber constantly maintained at 37°C. OKT3 and WT-31 antibodies were added to a concentration of 2.5 μg/ml (28, 36) and TPA at 50 nM from a stock in dimethylsulfoxide at 1.62 × 10^{-5} M (28, 33–36). CsA was added 20 s before the addition of any other agent and was diluted to 5 μM from a stock of 2.5 × 10^{-3} M in ethanol; this concentration was used as it was reported to be the minimal amount required to inhibit mitogen stimulation of IL-2 receptor expression (17) and was nontoxic to the cell lines used (data not shown). VSP was added to a final concentration of 1 μM (30, 44). LPS was used at 10 μg/ml (33, 36). For experiments performed in the absence of extracellular Ca^{2+} with added EGTA, the buffer used consisted of 145 mM NaCl, 4 mM KCl, 0.8 mM $MgSO_4$, 1.0 mM KH_2PO_4, 0.5 mM EGTA, 10 mM glucose, and 25 mM Hepes (pH 7.4).

IL-2 Measurements

Production of IL-2 was determined by measuring the proliferative response of the IL-2-dependent T cell line to supernatants of treated Jurkat. Supernatants of Jurkat cell cultures subjected to various treatments were assayed for IL-2 content by Smith's method (37) using the cloned murine helper T cell line 1.31 or the cytotoxic line, CTTLL-2 (10). Jurkat cells were grown in 25-cm^2 tissue culture flasks, pulsed with the desired treatment for 24 h, then washed and resuspended in fresh medium for an additional 24 h before testing for the production of IL-2. This procedure eliminated the possibility of contamination of the supernatants with the original additives. Equal volumes (0.1 ml) of diluted supernatants and test cells at an original concentration of 10^5 cells/ml were mixed in duplicate wells of flat-bottomed microtiter plates. All supernatant samples were set up in duplicate wells and maintained in a humidified 95% air/5% CO_2 incubator at 37°C. The cultures were maintained in RPMI 1640/10% FCS, 10 mM Hepes (pH 7.4), and antibiotics as described above. After 15 h of culture, cell proliferation was measured by the incorporation of [^{3}H]thymidine as described (37). Cell cultures were harvested 24 h after the [^{3}H]thymidine pulse onto glass filter paper disks, dried, and suspended in Scintiverse II liquid scintillation cocktail (Fisher Scientific Co., Pittsburgh, PA) and counted in a beta-

scintillation counter (model LS 7500; Beckman Instruments, Inc., Fullerton, CA). Positive controls were performed with purified, recombinant human IL-2.

Proliferation Assays

NRK cells were grown in 30-mm tissue culture dishes. When the cultures were observed to be in the exponential phase of growth, they were washed and placed in serum-free DME. After an overnight incubation, the cells were washed and fresh medium ± FCS or other treatments was added back. The cells were pulsed with 10 μCi [^{3}H]thymidine/30-mm culture dish for 24 h. The cells were then harvested, washed, and lysed in 1% SDS, suspended in Scintiverse II, and radioactivity determined by liquid scintillation counting.

Differentiation Assay

The differentiation of 70Z/3 cells to surface IgM(+) cells in response to LPS or TPA was determined by immunofluorescence microscopy as described (33, 34).

$[Ca^{2+}]_i$ Measurements

HPB-ALL or Jurkat cells were loaded with the fluorescent $[Ca^{2+}]_i$ chelator dye, Quin2/AM, and fluorescence intensity measured with excitation at λ_{339} nm and emission at λ_{492} nm as described (28, 35).

Results

CsA Inhibits T Cell Na^+/H^+ Exchange

HPB-ALL cells were loaded with the pH-sensitive fluorescent dye dimethylcarboxyfluorescein diacetate and pH_i was determined. Fig. 1 *A* shows the results. The monoclonal anti-T3-TCR antibodies, OKT3 and WT-31, and the phorbol ester, TPA, induced a rapid increase in pH_i consistent with previous findings (36). Stimulation of the T3-TCR by the antibodies produced a sustained rise in pH_i while TPA caused a transient pulse (36). 4-α-phorbol 12,13-didecanoate, a biologically inactive phorbol ester, had no effect on pH_i (34, 36, and data not shown). As Fig. 1 *B* shows, 5 μM CsA was able to completely block the increase in pH_i caused by OKT3 and WT-31 treatment but not that caused by TPA. The dose used in these experiments is inhibitory for a wide variety of T cell functions, including inhibition of mitogen stimulation of IL-2 receptor expression (17). CsA by itself had no effect on pH_i. The ability of the antibodies to cause a rapid influx of Ca^{2+} in either HPB-ALL or Jurkat cells was not impaired by CsA, as Fig. 2 shows. These data indicate that the T3-TCR associated Ca^{2+} transporter remains functionally intact when cells are exposed to inhibitory concentrations of CsA. These data are consistent with the drug inhibiting a step after ligand-induced Ca^{2+} influx.

CsA Blocks Mitogen Stimulation of IL-2 Production

The data presented above suggest that CsA inhibits Ca^{2+}-dependent, but not protein kinase C activation, of the Na^+/H^+ antiport in human T cells. It has been proposed that activation of the exchanger with a consequent increase in intracellular Na^+ and pH_i, is a required mechanism in membrane signal transduction by certain mitogens (32). In many experimental cell systems, activation of the antiport accompanies treatment of the cells with mitogens (3, 11, 17, 24, 26, 33, 34, 44). Indeed, in at least one system, that of LPS-treated 70Z/3 pre-B lymphocytes, activation of the antiport

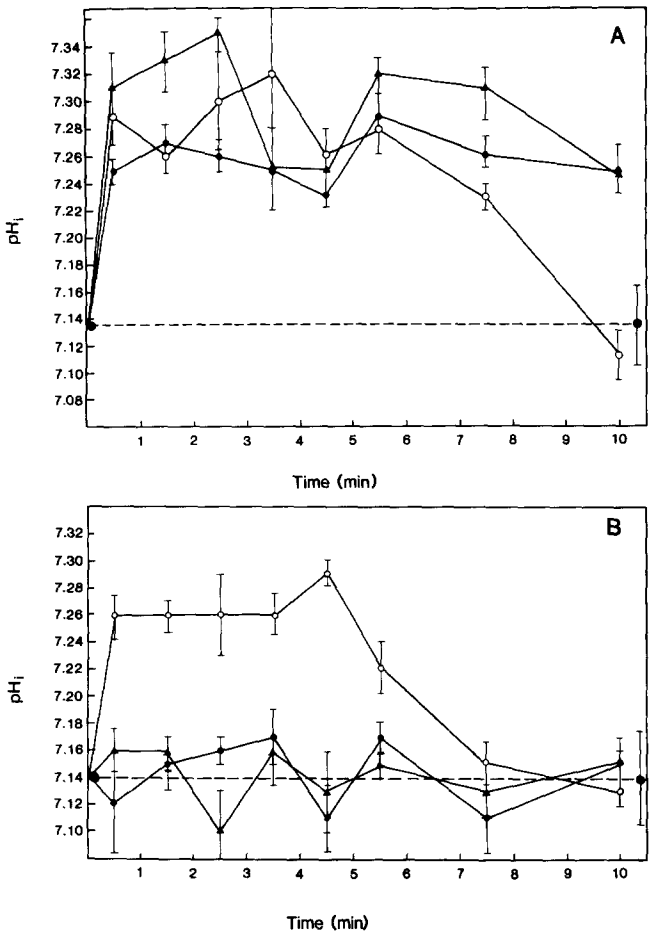

Figure 1. Stimulation of protein kinase C and the T3-T cell antigen receptor complex increases pH_i inhibition of the receptor stimulated pathway by CsA. HPB-ALL cells were loaded with the pH-sensitive dye DMCF, as described in Materials and Methods and pH_i followed with time. OKT3 and WT-31 antibodies were added to a final concentration of 2.5 μg/ml. TPA was added to 50 nM. CsA was added to 5 μM. (*A*) OKT3 and WT-31 monoclonal antibodies and the phorbol ester TPA increase pH_i. The activity of the former is dependent on the presence of extracellular Ca^{2+} while the latter is not (24). ▲, OKT3; —●—, WT-31; ○, TPA; ---●---, untreated control. (*B*) CsA inhibits the rise in pH_i stimulated by the anti-receptor complex antibodies, but not by phorbol esters. ▲, OKT3 + CsA; —●—, WT-31 + CsA; ○, TPA + CsA; ---●---, CsA alone. Data points represent means ± SD of at least four independent experiments.

appears to be rate-limiting for differentiation to proceed (33, 34). Recent evidence from Mills et al. (22), however, suggest that, under physiological conditions, i.e., in bicarbonate-containing medium, activation of the antiport is not necessary for growth factor-induced proliferation in T lymphocytes. Since CsA appears to selectively inhibit a major mechanism for growth factor stimulation of the exchanger in T lymphocytes, we therefore tested the effects of CsA on mitogen-induced IL-2 production in T cells. The results are shown in Table I. It is clear that CsA inhibits IL-2 production by mitogenic anti-T3-TCR antibodies in the Jurkat T lymphocyte cell line. Furthermore, dimethylamiloride, which selectively inhibits the antiport, also blocked the OKT3 and TPA stimulation of IL-2 release. These experiments were conducted in bicarbonate-containing medium, thereby mimick-

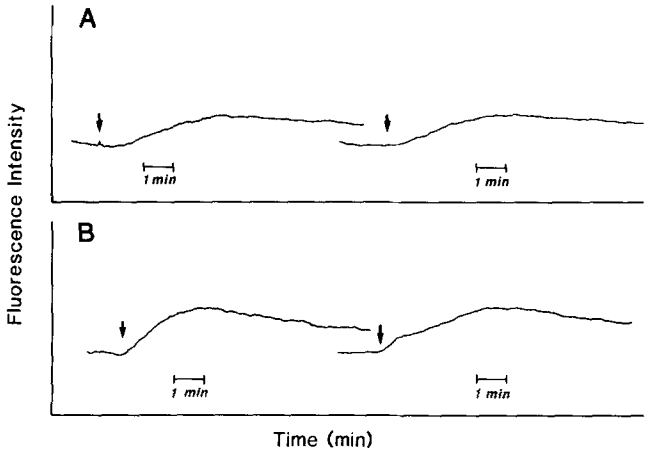

Figure 2. Anti-T3/T cell receptor antibody stimulation of Ca^{2+} influx is unaffected by CsA. HPB-ALL or Jurkat cells were loaded with Quin2 as described in Materials and Methods and fluorescence intensity measured (ex λ_{339} nm/em λ_{492} nm). CsA was added to 5 μM 20 s before the addition of 2.5 μg/ml OKT3 (arrow). (*A*) HPB-ALL cells. (*Left*) OKT3 alone; (*right*) OKT3 and CsA. (*B*) Jurkat cells. (*Left*) OKT3 alone; (*right*) OKT3 and CsA. The mean $[Ca^{2+}]_i$ in unstimulated cells was 170 nM with peak $[Ca^{2+}]_i$ after OKT3 treatment, rising to 350–400 nM in HPB-ALL and Jurkat cells, respectively. $[Ca^{2+}]_i$ was calculated as described (31).

ing in vivo conditions. These data suggest that enhanced Na^+/H^+ exchange plays a critical role in the T3-TCR-mediated activation of IL-2 production. However, since CsA only blocks the receptor-mediated increase in antiport activity, without affecting the TPA-stimulated, protein kinase C pathway, these data would suggest that the additive effect on pH_i observed with dual stimulation is required for IL-2 production. CsA is known to preferentially affect early T cell activation events that may not require cell division to occur (48). Therefore, it is possible that stimulation of the antiport may play a reduced role in other events, such as the initiation of proliferation as shown by Mills et al. (22).

CsA Inhibits Ca^{2+}-activated Na^+/H^+ Exchange in Fibroblasts

CsA has a remarkably restricted range of inhibitory activity, affecting only a very few types of cells both in vivo and in vitro (48). Fibroblasts have not been reported to be sensitive to growth inhibition by this drug. It was therefore important

Table I. Effect of CsA on IL-2 Production in Jurkat Cells

Treatment	IL-2 Activity secreted
	units
Untreated	1.0
OKT3 + TPA	14.75
CsA	0.88
OKT3 + TPA + CsA	0.95
DMA	0.70
OKT3 + TPA + DMA	0.30

Jurkat cells were maintained in RPMI 1640 medium/10% FCS. Cells were treated with various additives for 24 h, washed, and placed in fresh medium and the supernatants collected 24 h later and tested for IL-2 activity in the IL-2-dependent proliferation assay as described in Materials and Methods. A unit of activity is defined as the amount of IL-2 activity present in the untreated supernatants relative to a known positive control. The results are the means tabulated from three independent experiments.

Table II. Effect on CsA on Mitogen Stimulation of Na+/H+ Antiport Activity in NRK Fibroblasts and 70Z/3.B3 Cells

Cells/treatment:	Change in pH_i	Percent inhibition
NRK fibroblasts		
VSP	0.21	–
VSP + EGTA	0.01	–
VSP + CsA	0.00	100
TPA	0.22	–
TPA + EGTA	0.21	0
TPA + CsA	0.21	0
FCS	0.22	–
FCS + EGTA	0.20	0
FCS + CsA	0.21	0
70Z/3.B3 Pre-B cells		
LPS	0.15	–
LPS + CsA	0.15	0
TPA	0.13	–
TPA + CsA	0.14	0

The maximum change in pH_i was recorded 30 s after addition of the drug. The values shown represent the mean of at least three independent experiments. NRK cells were grown in DME with 10% FCS on 9 × 50 mm glass coverslips in Leighton tubes as described in Materials and Methods. When subconfluent, the cells were washed twice and placed in serum-free DME for 18 h. They were then loaded with DMCF diacetate and pH_i measured as described. CsA was added to 5 μM, LPS was added at 10 μg/ml, VSP was added to 1 μM, and TPA was used at 50 nM. FCS was added to 10% (vol/vol). For experiments performed in the absence of extracellular Ca^{2+} with added EGTA, the buffer used consisted of 145 mM NaCl, 4 mM KCl, 1.0 mM KH_2PO_4, 0.8 mM $MgSO_4$, 0.5 mM EGTA, 10 mM glucose, and 25 mM Hepes (pH 7.4).

to test whether the effect of CsA on HPB-ALL and Jurkat cells was one restricted to T lymphocytes or could also be observed in other types of cells in which Na^+/H^+ exchange can be enhanced by an influx of Ca^{2+} and in which the activation of this transport system appears to be important for the regulation of cellular proliferation and pH_i homeostasis. Villereal and his associates have reported that VSP and serum addition to serum-starved human foreskin fibroblasts stimulates a Ca^{2+} influx (44). This increase in cellular calcium activates the Na^+/H^+ antiport (28, 36). We have performed similar experiments using serum-starved NRK fibroblasts. Table II shows the results. In agreement with previous reports, both VSP and serum stimulated an increase in pH_i that was dependent on the presence of extracellular Ca^{2+}. TPA also caused a rise in pH_i that was unaffected by EGTA. As in HPB-ALL cells, CsA inhibited the Ca^{2+}-dependent pathway of Na^+/H^+ antiport activation in NRK cells, but not that activated by phorbol esters. The rise in pH_i observed after treatment of NRK cells with 10% FCS, which stimulates the antiport via both pathways, was unaffected by CsA. These data suggest that both human T lymphocytes and NRK fibroblasts have in common a CsA-inhibitible mechanism for enhancing the activity of the Na^+/H^+ antiport.

Based on the above findings, it was important to examine a cell line that does not use the Ca^{2+}-dependent pathway of Na^+/H^+ antiport stimulation. 70Z/3.B3 is a murine pre-B cell lymphoma that may be induced to differentiate to a surface IgM(+) phenotype by both TPA and the B cell mitogen, LPS (33, 34). In these cells, stimulation of enhanced Na^+/H^+ exchange is critical and rate-limiting for differentiation to proceed (33, 34). Both LPS and TPA induce increased Na^+/H^+ exchange by a Ca^{2+}-independent mechanism that is

mediated by protein kinase C (34, 35). Therefore, 70Z/3.B3 cells were treated with LPS or TPA in the presence and absence of CsA and pH_i was monitored. The results are shown in Table II. It is clear that CsA had no effect on the ability of either TPA or LPS to induce a rise in pH_i. These data suggest that CsA inhibits a pathway of mitogen/growth factor-mediated activation of the Na^+/H^+ antiport, i.e., that stimulated by a major increase in $[Ca^{2+}]_i$. 70Z/3.B3 cells, which lack this pathway, are unaffected by CsA.

CsA Does Not Inhibit Mitogen-stimulated Fibroblast Proliferation

The data presented above show that CsA blocks a common pathway of Na^+/H^+ antiport activation in both fibroblasts and T cells. Since this drug also inhibits mitogen stimulation of IL-2 production, it was important to determine whether CsA had any effect on growth factor stimulation of fibroblast proliferation. Exponentially growing NRK cells were serum-starved overnight and incorporation of [^{3}H]thymidine was determined during a 24-h pulse with either VPS or FCS. Table III shows the results. It is clear that CsA had no effect on the ability of either VSP or serum to stimulate DNA synthesis and proliferation as measured by incorporation of radiolabeled thymidine. In addition, as would be predicted from the lack of effect of CsA on LPS-stimulated enhanced Na^+/H^+ exchange in 70Z/3 pre-B lymphocytes, CsA did not inhibit LPS induction of surface IgM expression in these cells (Table IV). Thus, while CsA may be a general inhibitor of the Ca^{2+}-dependent pathway of mitogen-stimulated Na^+/H^+ antiport activity, this effect by itself is not sufficient to inhibit mitogen initiation of proliferation in rat fibroblasts.

Discussion

The data presented in this paper suggest that CsA is a general inhibitor of the Ca^{2+}-dependent pathway of Na^+/H^+ antiport activation in mitogen-stimulated cells as diverse as human T lymphocytes and rat fibroblasts. This effect is not due to inhibition of the T3-TCR-associated Ca^{2+} transporter as an influx of Ca^{2+} is still stimulated by mitogenic anti-T3-TCR antibodies in the presence of CsA (Fig. 2). These data suggest that CsA is specifically inhibiting a Ca^{2+}-dependent intermediate in this pathway. We have previously presented evidence supporting the existence of such an intermediate (32). It is possible that the intracellular target for CsA is the same step inhibited by pretreatment of HPB-ALL cells with phorbol esters (36).

Table III. Effect of CsA on Mitogen-induced Proliferation

Cells/treatment	cpm
NRK fibroblasts	*mean ± SD*
Untreated	8,100 ± 2,300
FCS	19,483 ± 1,540
VSP	18,400 ± 1,230
CsA	8,030 ± 1,840
FCS + CsA	23,000 ± 2,250
VSP + CsA	17,075 ± 1,620
DMA	7,950 ± 1,920
FCS + DMA	21,500 ± 1,600
VSP + DMA	19,050 ± 1,100

NRK cells were maintained in DME supplemented to 10% with FCS in 30-mm plastic tissue culture dishes. They were placed in serum-free DME at the beginning of each experiment and 24 h later serum (to 10%), or VSP (1 μM) ± DMA (50 μM) or CsA (5 μM) were added with 10 μCi [^{3}H]thymidine/plate of cells. They were harvested 24 h later and incorporation of radioactivity was determined as described in Materials and Methods. The results are tabulated from three independent experiments.

Table IV. Effect of CsA on LPS-induced Differentiation in 70Z/3 Pre-B Cells

Cells/treatment	Percent surface IgM(+)
70Z/3.B3 Pre-B lymphocytes	*mean ± SD*
Untreated	1.5 ± 0.5
LPS	83 ± 1.2
CsA	1.2 ± 0.3
DMA	1.4 ± 0.6
LPS + CsA	85 ± 1.8
LPS + DMA	1.8 ± 1.0

70Z/3.B3 cells were maintained in RPMI 1640 medium supplemented to 15% with FCS. LPS was added at 10 μg/ml, DMA at 50 μM, and CsA at 5 μM. 24 h later, expression of surface IgM was assayed by immunofluorescence microscopy as described (33, 34). A minimum of 500 cells/experiment was counted. The results are tabulated from three independent experiments.

The importance of the activation of the Na^+/H^+ exchanger to the mitogenic stimulation of IL-2 production in human T lymphocytes is shown by the data in Table I. Even in the presence of bicarbonate-containing medium, CsA was able to inhibit IL-2 secretion. DMA, a potent and specific inhibitor of the Na^+/H^+ antiport (49), had the same effect as CsA. By washing the cells after the 24-h treatment period, we have removed any of the stimulating agents present in the medium. Therefore, the stimulation of proliferation in the IL-2-dependent cell line could only come from IL-2 present in the added supernatants. These data would apparently contradict those of Mills et al. (22), who demonstrated that while IL-2-induced proliferation of T lymphocytes was accompanied by a stimulation of the antiport, DMA was unable to inhibit this induction. It is possible that the role of the exchanger in effecting alterations in intracellular pH and Na^+ levels may be radically different during different stages of cellular development. Alternatively, the activation of cells may require the generation of different types and levels of messengers than does the stimulation of proliferation. Of interest are the findings of Gschwendt et al. (11), who found that CsA inhibited the effects of TPA on some in vitro T cell functions.

This differential utilization of the various processes in growth factor/mitogen-dependent signal transduction may explain in part our data on the effects of CsA on NRK fibroblasts and 70Z/3 pre-B lymphocytes. CsA clearly inhibits the Ca^{2+}-dependent pathway of Na^+/H^+ antiport activation in NRK cells (Table II) but has no effect on either VSP or serum stimulation of proliferation (Table III). The relative lack of importance of the antiport in bicarbonate-containing medium to mitogen-stimulated DNA synthesis in NRK cells is confirmed by the lack of effect of DMA in this cell system. The immature pre-B cell line, 70Z/3, however, is unaffected by CsA, but is inhibited by DMA (Tables II and IV; 33–35). In this cell line, which lacks a Ca^{2+}-dependent pathway of antiport activation (28), the activation of the antiport is rate-limiting for LPS-induced surface IgM expression (33, 34).

Of interest are the recent findings of Aiello et al. (1), who found that CsA was able to inhibit the OKT3-induced proliferation of human peripheral blood T lymphocytes by

blocking the production of Interleukin 1. In their system, the stimulation of proliferation was an IL-2-dependent process, the production of which was itself dependent upon the synthesis and secretion of Interleukin 1. Therefore, according to this model, one would predict that CsA would not inhibit IL-2-stimulated proliferation of a (presumably) previously activated cell. This argument, however, is not enough to explain all the effects of CsA on mitogen-induced cell activation. Specifically, it does not explain the restricted number of cell types susceptible to CsA inhibition. One would have to predict the existence of a regulatory intermediate step in the Ca^{2+}-dependent pathway of antiport activation that is directly inhibited by CsA and that displays cell lineage specificity. This restricted range of activity strongly argues against CsA inhibiting in vivo calmodulin activity as previously suggested (7). CsA has been reported to bind to a 16-kD cytosolic protein ("cyclophilin") that is biochemically distinct from calmodulin (12). It is possible that this protein is a necessary component of the Ca^{2+}-dependent Na^+/H^+ exchange activation mechanism and is inhibited by the binding of CsA. Further investigation is required to answer these questions.

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