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Cyclosporin A Inhibits Inositol 1,4,5-Trisphosphate Binding to Its Receptors and Release of Calcium from Intracellular Stores in Peritoneal Macrophages

Uma K. Misra, Govind Gawdi, and Salvatore V. Pizzo

We have studied the effects of the immunosuppressive drug cyclosporin A (CsA) on the generation of inositol 1,4,5-trisphosphate (IP₃) and intracellular Ca²⁺ levels elicited upon ligation of murine macrophage receptors for α₂-macroglobulin, bradykinin, epidermal growth factor, and platelet-derived growth factor. Preincubation of cells with CsA (500 ng/ml), either alone or with the various ligands, did not inhibit the synthesis of IP₃. However, we observed 70–80% inhibition of the binding of [³H]IP₃ to IP₃ receptors on macrophage membranes isolated from CsA-treated macrophages. Preincubation of macrophages with CsA abolished IP₃-mediated release of Ca²⁺ from intracellular stores and Ca²⁺ entry from the extracellular medium observed when macrophage receptors were stimulated with ligands in the absence of CsA. Preincubation of macrophages with CsA also significantly inhibited DNA synthesis induced by ligands for all four receptors studied. Thus in macrophages, as in T cells, CsA blocks receptor-activated signal transmission pathways characterized by an initial increase in intracellular Ca²⁺ concentration. This inhibition appears to result from a drug effect on IP₃ receptors. The Journal of Immunology, 1998, 161: 6122–6127.

The structurally different immunosuppressant agents cyclosporin A (CsA) and FK506 are front line drugs used to prevent graft rejection following organ transplantation (see Refs. 1–3 for review). These drugs block T cell function by preventing transcriptional activation of genes that encode the T cell growth factor IL-2 and other immunologically important T cell-derived lymphokines following antigenic stimulation (1–3). Triggering of the T cell Ag receptor as well as ligation of many other receptors, including the α₂-macroglobulin signaling receptor (α₂MSR) (4, 5), bradykinin receptor (BKR) (6, 7), epidermal growth factor receptor (EGFR) (8, 9), and platelet-derived growth factor receptor (PDGFR) (10, 11), results in activation of tyrosine kinase as well as rapid hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate. This activation generates inositol 1,4,5-trisphosphate (IP₃) and diaacylglycerol. The latter is important because of its role in activating protein kinase C (12). IP₃ binds to intracellular IP₃ receptors (IP₃R), changes their conformation, and opens the Ca²⁺ channel causing an increase in intracellular Ca²⁺ ([Ca²⁺]ᵢ) (13). Receptor-mediated Ca²⁺ release from internal stores is often followed by Ca²⁺ influx across the plasma membrane (14, 15). The mechanism of this capacitative Ca²⁺ entry is not fully understood but is thought to involve a novel diffusible activator, a G protein, tyrosine kinase, cGMP, and direct coupling between proteins of the endoplasmic reticulum and the plasma membrane (14, 15). Intracellular Ca²⁺ release is predominantly mediated by the ryanodine receptor (RyR) and IP₃R (13, 16, 17). These Ca²⁺ release channels display extensive amino acid homology and functional similarities. Prolonged elevation of [Ca²⁺]ᵢ is linked to IL-2 gene expression and to DNA synthesis in activated T cells (16, 17).

In T cells, CsA and FK506 inhibit signal transmission pathways from the cell surface to the nucleus that are characterized by an initial increase in [Ca²⁺]ᵢ, (1–3). CsA and FK506 block to their cognate intracellular receptor immunophilins, cyclophilin and FKBP12, respectively (1–3). The complexes of CsA-cyclophilin and FK506-FKBP12 bind to calcineurin, a Ca²⁺/calmodulin-dependent serine/threonine phosphatase, which plays a critical role in signaling pathways necessary for T cell activation. This complex results in inhibition of its phosphatase activity, impairing the translocation of nuclear transcription factors from the cytosol to the nucleus and the formation of a functional transcription complex, which in part regulates the expression of cytokine genes (1–3, 18). The binding site for the NF-AT transcription complex is present within the enhancer sequences of several genes and is required for their regulation by the antigen receptor or by agents that both mobilize Ca²⁺ and activate protein kinase C (1–3, 18). CsA and FK506 also block the production of TNF-α by B cells (19). In mast cells, CsA and FK506 block degranulation as well as transcriptional activation of IL-3 and IL-5 and genes involved in leukotriene synthesis (20–22). In monocyte/macrophages, CsA inhibits LPS-induced expression of tissue factor both at the transcriptional and functional levels, as well as translocation of NF-κB (23).

Colocalization of FKBP12 with calcineurin in brain (1–3, 16), association of FKBP12 with the Ca²⁺ release channels RyR and IP₃R, suggests that immunophilins play a direct role in signal transduction (24–28). Cyclophilin and FKBP12 possess cis/trans-peptidylprolyl isomerase activity that is inhibited by binding of CsA and FK506, respectively (1–3, 29, 30). Cyclophilins aid in protein folding, and they increase folding rates of human carbonic
anhydrazale, ribonuclease Ti, and collagen in vitro (31–33). Cyclo-
philins may also act as chaperons (34), chemotactic agents (35), and stress response proteins (36). CsA binds to the p55 Gag protein of the human immunodeficiency virus (37). In the yeast two hybrid system, FKBPI2 has been found in association with the TGF-βR (38).

We have studied the effects of CsA in murine peritoneal macrophages with respect to the second messengers elicited upon li-
gation of α MSR with the α-M cloned and expressed receptor binding fragment (RBF), of BKR by bradykinin, of EGFR with EGF, and of PDGFR with PDGF Ab. We report here that in macro-
phages, CsA treatment before the addition of agonists does not affect IP3 generation, but it inhibits the binding of [3H]IP3 to IP3 R.

Materials and Methods

Materials

Endotoxin-free RBF was cloned, expressed, and purified as described previously (4, 5). Culture media were purchased from Life Technologies (Grand Island, NY). Fatty acid free BSA, bradykinin, EGF, CsA, and eiconozole were purchased from Sigma (St. Louis, MO). PDGF AB was procured from R&D Systems (Minneapolis, MN). Fura 2/AM and BAPTA/AM were obtained from Molecular Probes (Eugene, OR). Myo-
[2-3H]inositol (specific activity, 10–20 Ci/mmol) and [3H]thymidine (spe-
cific activity, 70 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, MO). All other reagents were the highest purity grade commercially available.

Isolation of peritoneal macrophages

Pathogen-free C57Bl/6 mice (6 wk old) were obtained from Charles River Laboratories (Raleigh, NC). Thiglycollate-elicited macrophages were routinely obtained by peritoneal lavage with HBSS containing 10 mM HEPES (pH 7.2) and 3.5 mM NaHCO3 (HHBSS) (4, 5). The cells were washed once with HBHSS, suspended in RPMI 1640 medium containing 2 mM glutamine, 12.5 U/ml penicillin, 6.25 μg/ml streptomycin, and 5% FCS, and plated at cell density of 5 × 105/cm2 for Ca2+ measurements and 2–4 × 105/well in 6-well plates for other studies. The macrophages were incubated for 2 h at 37°C in a humidified CO2 (5%) incubator. The monolayers were incubated in another volume of the Tris-HCl buffer described above, and the incubations were terminated by aspirating the medium. A volume of cold TCA (5%) was added to each well and the plate left on ice for 30 min. TCA was removed and cells washed once more with TCA (5%) followed by three washings with cold HHBSS. The cells were lysed in 1 N NaOH, and radioactivity was determined by liquid scintillation counting.

Results

CsA and generation of IP3

Treatment of macrophages with CsA alone showed very little ef-
fect on the generation of IP3 (Fig. 1A). CsA also did not inhibit the generation of IP3 in macrophages stimulated with RBF, bradyki-
in, EGF, and PDGF (Fig. 1). These results show that CsA does not affect receptor-stimulated second messenger events at the cell surface. Receptor-activated increase in IP3 rapidly declines due to its further metabolism either by a phosphatase or kinase or both (42). In cells, however, to which CsA was added before RBF, bradykinin, EGF, and PDGF, respectively, the levels of IP3 re-
mained significantly elevated compared with CsA untreated cells.

These results suggest that CsA impairs the metabolism of IP3. The difference in the elevation caused by bradykinin in cells treated with CsA as compared with untreated cells is less significant. The reason for this difference is unclear. From the mechanistic standpoint RBF, like EGF and PDGF, functions like a growth factor (4, 5), which is not the case with bradykinin. Thus RBF, EGF, and PDGF may activate different signaling mechanisms downstream from IP3 generation.

Morphogenesis of macrophages

The effect of CsA on increased [Ca2+], levels observed in macro-
phages after stimulation with RBF, bradykinin, EGF, and PDGF is shown in Fig. 2. CsA itself showed very little effect on [Ca2+], levels (Fig. 2A). RBF transiently increased [Ca2+], levels by about 4-fold within 5–20 sec after stimulation over the basal value in 85–90% cells studied (150–160 cells in four independent experi-
ments; Fig. 2A). Depletion of IP3-sensitive intracellular Ca2+ stores triggers capacitative Ca2+ entry across the plasma mem-
brane, which sustains elevated [Ca2+], levels (13, 14). We studied this Ca2+ entry under our experimental conditions by adding 1 mM Ca2+ to the medium (Fig. 2A). Consistent with our previous
studies (43), addition of Ca\(^{2+}\) 5 min after RBF stimulation further increased [Ca\(^{2+}\)]\(_i\) levels by 2- to 3-fold in 85–90% of the cells (Fig. 2A). Incubation of macrophages with CsA for 15 min at 37°C before the addition of RBF completely inhibited both IP\(_3\)-mediated release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores and capacitative Ca\(^{2+}\) entry in 80–85% of cells studied (120 –130 cells in four independent experiments; Fig. 2A).

Ligation of BKR on macrophages with bradykinin transiently increased [Ca\(^{2+}\)]\(_i\) levels by about 3-fold over the basal value within 5–20 sec after stimulation in 75–80% of the cells (100–120 cells in four individual experiments). Addition of 1 mM Ca\(^{2+}\), 4 min after bradykinin, caused a further sustained increase in [Ca\(^{2+}\)]\(_i\) levels in 80 – 85% cells studied (Fig. 2B). Incubation of macrophages with CsA, drastically inhibited both the IP\(_3\)-mediated increase in [Ca\(^{2+}\)]\(_i\) as well as Ca\(^{2+}\) entry in 75–80% of the cells (100–110 cells in four experiments; Fig. 2B). We also studied the effects of CsA on IP\(_3\) induction mediated by EGF and PDGF as well as capacitative entry of [Ca\(^{2+}\)]\(_i\). Both these growth factors

FIGURE 1. CsA and IP\(_3\) generation in macrophages. Details of measurements of IP\(_3\) in \[^{3}H\]myoinositol-labeled macrophages (2 × 10\(^6\) cells/well) stimulated with various agonists preincubated with or without CsA (500 ng/ml) for 15 min at 37°C have been described previously (4, 5). Values are mean ± SEM from two independent experiments performed in duplicate and are expressed as % change over basal value at zero time. A, Changes in IP\(_3\) levels induced by RBF (50 pM) (●), CsA (△), and CsA + RBF (○). B, Bradykinin (50 nM)-induced IP\(_3\) generation (●), CsA(△), CsA + bradykinin (○). C, Changes in IP\(_3\) levels induced by EGF (200 ng/ml) (●), CsA (△), CsA + EGF (○). D, PDGF (50 ng/ml)-induced changes in IP\(_3\) levels (●), CsA (△), and CsA + PDGF (○).

FIGURE 2. CsA and intracellular Ca\(^{2+}\) levels. Details of measurements of Ca\(^{2+}\) in Fura-2/AM loaded macrophages have been described previously (4, 5). Change shown in [Ca\(^{2+}\)]\(_i\) under the experimental conditions is representative of four independent experiments for each agonist with or without preincubation with CsA (500 ng/ml for 15 min), in low Ca\(^{2+}\) (75 μM) HBSS containing 10 mM HEPES, pH 7.2 and 3.5 mM NaHCO\(_3\), using about 110–160 cell in each case. A, Changes in [Ca\(^{2+}\)], induced by RBF (50 pM) (●), CsA + RBF (○), or CsA (500 ng/ml) alone (△). B, Changes in [Ca\(^{2+}\)], induced by bradykinin (50 nM) (●), and CsA + bradykinin (○). C, Changes in [Ca\(^{2+}\)], induced by EGF (200 ng/ml) (●), and CsA + EGF (○). D, Changes in [Ca\(^{2+}\)], induced by PDGF (50 ng/ml) (●), and CsA + PDGF (○). Arrows indicate the time of additions.
increased IP₃-mediated and capacitative entry-mediated increase in [Ca²⁺]ᵢ in 80–85% of the cells (120–130 cells in four separate experiments in each case; Fig. 2, C and D). Incubation of macrophages with CsA before the addition of EGF or PDGF drastically inhibited both the IP₃-mediated and capacitative entry-mediated increase in [Ca²⁺]ᵢ in 70–80% of the cells (100–120 cells in four independent experiments; Fig. 2, C and D).

CsA and [³H]IP₃ binding to IP₃R
The data presented above indicate that CsA does not prevent the generation of IP₃ in macrophages on ligation of α₂MSR, BKR, EGFR, and PDGFR (Fig. 1); however, it does nearly abolish IP₃-mediated transient release of Ca²⁺ from intracellular Ca²⁺ stores (Fig. 2). One possible explanation for these observations is that CsA may prevent the binding of IP₃ to IP₃R and the opening of Ca²⁺ channels. Therefore, we next studied the binding of [³H]IP₃ to IP₃R in membrane preparations from macrophages treated with either buffer or CsA. We have shown previously that the binding of [³H]IP₃ in macrophage microsomal preparations is concentration-dependent, and [³H]IP₃ binds to a single class of IP₃ binding sites with a Kᵦ of 4.3 ± 0.6 pM (39). The binding of [³H]IP₃ to IP₃R in membranes prepared from CsA-treated cells was reduced by 70–80% compared with buffer-treated controls (Fig. 3).

CsA and agonist-induced DNA synthesis
In view of the results described above and the known regulatory role of elevated [Ca²⁺]ᵢ in gene expression, we studied the effects of CsA on DNA synthesis (Fig. 4). Stimulation of macrophages with RBF, EGF, and PDGF caused a 2- to 3-fold increase in DNA synthesis (Fig. 4). Incubation of macrophages with CsA before the addition of the respective ligands inhibited DNA synthesis by 50–90%, depending on the ligand (Fig. 4).

Modulations of [Ca²⁺]ᵢ levels and DNA synthesis
The effects of modulating [Ca²⁺]ᵢ levels in macrophages was studied in three ways: 1) with thapsigargin a depletor of Ca²⁺ from intracellular IP₃-sensitive and IP₃-insensitive Ca²⁺ stores; 2) BAPTA/AM, a chelator of intracellular Ca²⁺; and 3) eiconozole, an inhibitor of Ca²⁺ entry from the medium (44). In each study, these agents were added to the cells before addition of CsA followed by RBF and incubation for 20 h at 37°C (Fig. 5). Thapsigargin by itself slightly increased macrophage DNA synthesis; however, when thapsigargin was added 20 min before CsA and RBF to cells, it resulted in a further reduction of DNA synthesis as compared with the decrease observed in cells treated with CsA and RBF (Fig. 5). Likewise, modulation of [Ca²⁺]ᵢ levels with BAPTA/AM or eiconozole before adding CsA and RBF further reduced DNA synthesis observed in cells treated with CsA plus RBF (Fig. 5).
Discussion

The primary findings of this study with peritoneal macrophages are: 1) CsA at nontoxic doses does not inhibit IP₃ generation consequent to ligation of α₂MSR, BKR, EGF, and PDGF; 2) CsA inhibits the binding of [³H]IP₃ to IP₃Rs; 3) CsA inhibits both the initial IP₃-mediated release of Ca²⁺ from internal stores as well as capacitatively Ca²⁺ entry; 4) CsA inhibits DNA synthesis observed in macrophages when these cells are stimulated with RBF, EGF, and PDGF, respectively; and 5) modulation of [Ca²⁺]i levels with thapsigargin, BAPTA/AM, or eiconozole further reduces DNA synthesis observed in cells treated with CsA before stimulation with RBF. These results show that signal transduction events generated upon cell surface receptor ligation are impaired by CsA in macrophages as well as in T-cells (2). They are the first observations suggesting that the action of CsA may involve a direct effect on IP₃R.

There is a growing body of literature suggesting that capacitative Ca²⁺ entry into cells, as well as intracellular mobilization of Ca²⁺, affects many cellular functions requiring protein and DNA synthesis (14). Ca²⁺ mobilization has been implicated in lymphocyte and fibroblast mitogenesis, posttranslational processing and trafficking of newly synthesized lysosomal and membrane proteins, translocation of transcription factors from the cytoplasm to the nucleus, expression of cytokine genes, modulation of specific cell cycle events, and reinitiation of DNA synthesis (14). Addition of thapsigargin and di-β-pyridylhydroquinone to Swiss 3T3 cells at lower concentrations causes reinitiation of DNA synthesis in synergy with either phorbol 12,13-dibutyrate or bombesin (14, 18, 44–49). At higher concentrations, these agents inhibit DNA and protein synthesis in smooth muscle cells, where cell growth has been linked to intracellular Ca²⁺ pool contents (44–48). Elevated [Ca²⁺], pools have been reported to modulate DNA synthesis in rheumatoid synovial fibroblasts and murine macrophages on stimulation with RBF (41). In Jurkat T lymphocytes, which demonstrate defective capacitative Ca²⁺ entry, maintenance of elevated [Ca²⁺] levels during early T cell activation is required for IL-2 gene induction (50). In several T cell lines and T lymphocytes, interruption of Ca²⁺ signaling by chelating extracellular Ca²⁺ in the absence of protein synthesis results in the export of nuclear transcription factors to the cytoplasm within 5 min (18).

IP₃-R mediates intracellular Ca²⁺ release elicited by hormones and neurotransmitters that bind to cell surface receptors to activated phosphatidylinositol-specific phospholipase C and generate IP₃ (13, 42). While most of IP₃-R is associated with the endoplasmic reticulum, some may occur on plasma membranes and mediate Ca²⁺ entry (50, 51). Intracellular Ca²⁺ release is predominantly mediated by RyR and IP₃-R Ca²⁺ release channels (16, 24–28). FKBP12 is physiologically associated with the intracellular Ca²⁺ release channels, RyR and IP₃-R (28). Both of these channels are tetramers of four identical subunits each of 80 kDa and possess 40% homology in the transmembrane region (13, 52, 53). In preparations of IP₃-R, FKBP12 binds to it very tightly and FK506 and rapamycin dissociate this complex, making it more leaky and reduce Ca²⁺ accumulation (24). Recent studies demonstrate that calcineurin is physiologically associated in a physical and functional complex with IP₃-R and FKBP12 (27). This association is disrupted by FK506 and rapamycin but not by CsA, suggesting that calcineurin is bound on IP₃-R at a site different from FKBP12 (27). In a IP₃-R complex, calcineurin retains its catalytic activity toward known substrates such as mitogen-activating protein-2 and it regulates the phosphorylation status of IP₃-R induced by Ca²⁺-activated kinases and phosphatases (27). Inhibition of calcineurin by CsA is a consequence of altering the phosphorylation status of IP₃-R and it will affect Ca²⁺ conductance. It would also inhibit calcineurin activity toward other cellular substrates such as NF-AT and thus alter Ca²⁺ fluxes and the resultant physiologic responses (27).

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