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Effects of cyclosporin A and FK506 on Fc_ε receptor type I-initiated increases in cytokine mRNA in mouse bone marrow-derived progenitor mast cells: Resistance to FK506 is associated with a deficiency in FK506-binding protein FKBP12

(immunophilin/cyclophilin/calcineurin/cytokine/exocytosis)

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ABSTRACT **The inhibitory effects of cyclosporin A (CsA) and FK506 on Fc_ε receptor type I-initiated increases in cytokine mRNA and the expression of their intracellular binding proteins were studied in interleukin 3 (IL-3)-dependent, mouse bone marrow-derived mast cells (BMMCs). In BMMCs sensitized with IgE anti-trinitrophenyl, CsA inhibited trinitrophenylated bovine serum albumin-induced increases in mRNA for IL-1β, tumor necrosis factor α (TNF-α), and IL-6 in a dose-related manner (IC_{50} values of 4, 65, and 130 nM, respectively). FK506 did not inhibit hapten-specific increases of mRNA for TNF-α or IL-6, and for IL-1β the IC_{50} was >50-fold higher than that of CsA. Neither agent inhibited exocytosis of the endogenous secretory granule mediators β-hexosaminidase and histamine at the IC_{50} values for inhibition of increases in cytokine mRNA. BMMCs expressed cyclophilin, and CsA inhibited the phosphatase activity of cellular calcineurin with an IC_{50} of ≈8 nM. That CsA inhibited IL-1β mRNA accumulation in IgE-activated BMMCs with an IC_{50} similar to that for inhibition of calcineurin activity, whereas the IC_{50} values were ≈20-fold higher for the inhibition of TNF-α and IL-6 mRNA, suggests that the induction of TNF-α and IL-6 is less dependent upon calcineurin activity than is the induction of IL-1β. BMMCs were deficient in the 12-kDa FK506-binding protein FKBP12, but not FKBP13, as assessed by RNA and protein blot analyses. FK506 did not inhibit calcineurin phosphatase activity in BMMCs, even at drug concentrations of 1000 nM. The resistance of BMMCs to inhibition of Fc_ε receptor type I-mediated increases in cytokine mRNA by FK506 is most likely due to their deficiency of FKBP12 and the related inability to inhibit the activity of calcineurin.**

The immunosuppressive drugs cyclosporin A (CsA) and FK506 bind to families of intracellular proteins, termed cyclophilins (CyPs) and FK506-binding proteins (FKBPs), respectively, that have peptidyl-prolyl cis–trans isomerase activity; these molecules are collectively termed immunophilins. CsA and FK506 inhibit calcium-dependent cell responses, such as T-cell receptor-mediated and lectin-mediated transcription of interleukin 2 (IL-2) by human and mouse T lymphocytes (1). The drugs also inhibit stimulus-induced exocytosis of endogenous serine esterase activity from cytotoxic T lymphocytes (2), lactoferrin from human neutrophils (3), and histamine from human basophils (4, 5), but suppression of these functions generally requires higher concentrations of CsA than those which suppress production of IL-2 by T lymphocytes. On a molar basis, FK506 is 10–100 times more potent than CsA in inhibiting graft rejection *in vivo* (6) and IL-2 production *in vitro* by T lymphocytes with subsequent cell proliferation (7). Based upon studies of protein associations (8, 9), it has been proposed that important effects of CsA and FK506 are mediated via binding of drug–immunophilin complexes to the calcium- and calmodulin-dependent protein phosphatase calcineurin. Both CsA and FK506 added individually to T lymphocytes inhibit the calcineurin phosphatase activity in cell extracts (10).

In this study, we have assessed the effects of CsA and FK506 on the IgE-dependent, hapten-specific increases in cytokine mRNA levels and on the release of secretory granule mediators by mouse IL-3-dependent, bone marrow-derived mast cells (BMMCs) (11), which are immature progenitors for the major mast cell subclasses (12, 13). CsA inhibits calcineurin activity and the augmentation of IL-1β mRNA in BMMCs with an IC_{50} (<10 nM) similar to that for the inhibition of IL-2 expression by T lymphocytes (14), whereas the IC_{50} for inhibition of induced tumor necrosis factor α (TNF-α) and IL-6 mRNA requires ≈20-fold more CsA. We have also demonstrated that BMMCs are deficient in the expression of the 12-kDa FKBP (FKBP12) and that concomitantly, FK506 is unable to inhibit calcineurin activity and increases in cytokine mRNA.

MATERIALS AND METHODS

Cell Cultures. Mouse BMMCs were obtained by culturing bone marrow cells from the femurs and tibias of 6- to 16-week-old BALB/c mice (The Jackson Laboratory) for 3–8 weeks in the presence of 50% WEHI-3 [American Type Culture Collection (ATCC)] cell-conditioned medium (WCM) and 50% enriched medium as described (15). After 3 weeks, >96% of the nonadherent cells in culture were mast cells as assessed by metachromatic staining with toluidine blue (16).

Jurkat J77 cells were obtained from K. Smith, Dartmouth Medical School, and the rat basophilic leukemia cell line RBL-1 was obtained from ATCC.

Activation of BMMCs. For IgE-dependent activation, BMMCs (10^7 per ml) were incubated for 1 hr at 37°C with a

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Abbreviations: CsA, cyclosporin A; CyP, cyclophilin; FKBP, FK506-binding protein; IL, interleukin; BMMC, mouse bone marrow-derived mast cell; TNF-α, tumor necrosis factor α; WCM, 50% WEHI-3 cell-conditioned medium; TNP, trinitrophenyl; BSA, bovine serum albumin.

saturating concentration (50 μg/ml) of mouse IGEL a2 monoclonal IgE anti-trinitrophenyl (TNP) (17) (ATCC) in 50% WCM. The cells were washed twice in 50% WCM by centrifugation, suspended in WCM at a concentration of 1.25 × 10^7 per ml, and incubated for 10 min at 37°C. Incremental concentrations of CsA (Merck), FK506 (Sandoz), or vehicle control (50% methanol diluted in 50% WCM) were added at 30-sec intervals to tubes in duplicate. After each 10-min interval from the start of the drug/vehicle additions, 50% WCM or an optimal concentration (100 ng/ml) of TNP-bovine serum albumin (BSA) was added to parallel tubes in duplicate. After incubation for 10 min more at 37°C, samples of the cells were removed, diluted with equal volumes of 0.15 M ethylenediaminetetraacetic acid (EDTA), and sedimented by centrifugation at 250 × *g* for 5 min at 4°C. The supernatants were decanted and retained, and the pellets were suspended to their original volumes with a 1:1 (vol/vol) mixture of 50% WCM/0.15 M EDTA and sonicated on ice.

β-Hexosaminidase was quantitated by spectrophotometric analysis of the hydrolysis of *p*-nitrophenyl β-D-2-acetamido-2-deoxyglucopyranoside (18). Histamine was determined with a commercial RIA kit (AMAC, Westbrook, ME). The percent release values were calculated by the formula $[S/(S + P)] \times 100$, where S and P are the respective mediator contents of the samples of each supernatant and cell pellet. The net percent release values were obtained by subtracting the percent release of replicate sensitized cells incubated in medium alone from that of cells challenged with TNP-BSA. The percent inhibition was defined as $[(V - D)/V] \times 100$, where V is the net percent antigen-induced release in buffer containing vehicle and D is the net percent release in the presence of drug.

mRNA Analysis. For assessment of levels of mast cell cytokine mRNA, sensitized BMMCs were sedimented at 250 × *g* for 5 min at 4°C one hour after the addition of either medium or TNP-BSA, and their total cellular RNA was isolated by the acid guanidinium thiocyanate–phenol/chloroform extraction method (19). RNA was precipitated with 2-propanol, washed with 80% ethanol, and quantitated by absorbance at 260 nm. For RNA blots, portions (10–20 μg) of each RNA sample were electrophoresed in a 1.3% agarose/6% formaldehyde gel and transferred to a charged nylon membrane (Zeta-Probe, Bio-Rad) by capillary transfer overnight in 20× standard saline citrate (SSC). The membranes were baked at 80°C for 2 hr, washed in 0.2× SSC/0.2% SDS at 65°C for 30 min, and prehybridized in glass bottles at 43°C overnight in 50% formamide/5× SSC/0.1% SDS/1 mM EDTA/10 mM sodium phosphate/5× Denhardt's solution containing denatured herring sperm DNA (100 μg/ml; Sigma). Blots were hybridized with random primer-labeled (Boehringer Mannheim) cDNA probes [mouse IL-1β (20), TNF-α (21), IL-6 (22), and actin (23)] at 43°C overnight in 50% formamide/5× SSC/0.1% SDS/1 mM EDTA/10 mM sodium phosphate/1× Denhardt's solution (Sigma) containing denatured herring sperm DNA (100 μg/ml) and 10% dextran sulfate. The RNA blots were washed twice in 1× SSC/0.2% SDS/10 mM sodium phosphate at room temperature and then twice in 0.2× SSC/0.2% SDS/10 mM sodium phosphate for 15–20 min at 65°C. The blots were wrapped in plastic wrap, and autoradiography was performed on Kodak XAR-5 film. The radioactivity associated with each band was quantitated with a Betascope 603 blot analyzer (Betagen, Waltham, MA). The counts for each cytokine mRNA were divided by the counts for β-actin mRNA in the same lane. The actin-adjusted values for cytokine mRNA from sensitized cells maintained in buffer were subtracted from the actin-adjusted values of replicates that were activated with TNP-BSA, to provide an actin-adjusted, agonist-induced net increment for each cytokine-specific transcript for each experimental treatment. The effects of each drug treatment were calculated as percent inhibition of the value obtained for cells exposed to vehicle.

For assessment of FKBP mRNA content, samples (10 μg) of cytoplasmic RNA were electrophoresed through a 1% agarose/6% formaldehyde gel, transferred to a nitrocellulose filter by capillary blotting, crosslinked by UV to the filter, and hybridized as described (24). Full-length cDNA probes to human FKBP12 (350 base pairs) (25) and FKBP13 (600 base pairs) (26) were labeled with ^{32}P by random priming and found to hybridize predominantly with species of 1.8 kilobases and 0.6 kilobase, respectively, on RNA blots of Jurkat cells. Hybridization was carried out at 42°C overnight in 50% formamide/5× SSC/2.5 mM sodium phosphate, pH 6.5/5× Denhardt's solution/0.2% SDS containing denatured salmon sperm DNA at 500 μg/ml and 1 μCi (37 kBq) of ^{32}P-labeled probe per lane. Blots were washed at a final stringency of 0.1× SSC/0.1% SDS at 65°C and exposed to Kodak X-Omat film at −70°C with intensifying screens.

Protein Blots. Detergent extracts of cells were prepared as described (10) and protein concentrations were determined using Bradford reagent (Bio-Rad) with BSA as a standard. Proteins were separated by SDS/15% PAGE and electroblotted to poly(vinylidene difluoride) (Immobilon, Millipore) membranes with a Bio-Rad minigel system. Membranes were blocked for either 1 hr or overnight in Tris-buffered saline (TBS) containing 1.5% BSA, 1.5% ovalbumin, and 0.02% sodium azide. Membranes were then incubated for 2–3 hr with rabbit IgG specific for a human FKBP12 peptide (gift of Matthew Harding, Vertex Pharmaceuticals, Cambridge, MA) or a human FKBP13 peptide-specific rabbit IgG (generated by Berkeley Antibody, Richmond, CA) diluted in TBS containing 0.15% BSA and 0.15% ovalbumin, washed three times in TBS containing 0.05% Tween 20, and developed with an alkaline phosphatase-conjugated anti-rabbit IgG system (Promega).

For analysis of CyPs, BMMCs were pelleted and resuspended in SDS/PAGE sample buffer (27) containing 0.5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml), chymostatin (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), and BSA (0.5 mg/ml). Samples were boiled for 5 min, flash frozen in liquid nitrogen, and stored at −70°C. Proteins were resolved by SDS/15% PAGE (27) and electroblotted to nitrocellulose at 150 mA in 50 mM Tris/600 mM glycine/20% methanol over 6–8 hr. The membranes were treated with blocking solution (20 mM Tris·HCl, pH 7.5/150 mM NaCl/5% Carnation nonfat dry milk) for 2 hr at room temperature. The blocking solution was removed, and the membranes were incubated overnight at 4°C with antisera (diluted 1:100 in blocking solution) from rabbits immunized with recombinant human CyPA or human CyPB (gifts from Kim McIntyre, Hoffmann–La Roche). Membranes were washed four times (once for 1 min, twice for 20 min, and once for 1 min) with the blocking solution and treated with 20 μCi of ^{125}I-labeled donkey anti-rabbit IgG (Amersham) for 1 hr at room temperature. Membranes were washed once for 5 min with blocking solution and four times for 5 min with phosphate-buffered saline (pH 7.2), dried on blotting paper, and exposed to x-ray film for 5–20 hr at −70°C with intensifying screens.

Binding and Elution of Immunophilins to Solid-Phase CsA. BMMCs were washed three times by centrifugation in phosphate-buffered saline at 4°C. The cell pellet was suspended at a concentration of 10^7 cells per ml in lysis buffer [50 mM Tris·HCl, pH 7.5/1% Nonidet P-40/150 mM NaCl/1 mM EDTA/50 mM NaF/300 μM sodium pyrophosphate/5 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride/leupeptin (10 μg/ml)/pepstatin (10 μg/ml)], and the suspension was incubated for 20 min at 4°C and homogenized in a Dounce homogenizer with a type B pestle. The homogenate was centrifuged at 25,000 × *g* for 30 min at 4°C, and the

supernatant was centrifuged at 100,000 × g for 1 hr at 4°C. The protein concentration of the resultant supernatant was determined by the Bradford assay.

Fifty-microliter columns of CsA- and ethanolamine-capped Affi-Gel 10 (Bio-Rad) (28) were equilibrated with lysis buffer, and 5-ml portions of BMMC extract were passed over each resin two times at a flow rate of 12 ml/hr at 4°C. The columns were washed twice with 1 ml of lysis buffer, once with 1 ml of lysis buffer containing 650 mM NaCl, and twice with 1 ml of 20 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40. Each resin was transferred to a separate Eppendorf tube with ≈1 ml of 50 mM Tris·HCl (pH 7.5), and the tubes were centrifuged at 4000 × g for 2 min at 4°C. The supernatants were withdrawn and discarded, and 250 μl of 50 mM Tris·HCl (pH 7.5) and 25 μl of 6 mM CsA in methanol were added to each resin. The samples were mixed for 12 hr at 4°C and centrifuged at 4000 × g for 2 min at 4°C. The eluates were lyophilized, resuspended in water, and analyzed by SDS/PAGE with silver staining (29).

Calcineurin Assay. Cells were cultured with immunosuppressive agents or vehicle for 1 hr, hypotonic lysates were prepared, and calcineurin activity was measured as described (10). Assays were performed in duplicate, and the cpm measured in assay tubes lacking the source of calcineurin were subtracted from the cpm in samples obtained in the presence of the enzyme to obtain a net value.

RESULTS

Effects of CsA and FK506 on IgE-Mediated, Hapten-Specific Increases in Cytokine mRNA and Exocytosis of Secretory Granule Mediators. BMMCs sensitized with IgE were incubated with vehicle, CsA, or FK506 for 10 min and activated with antigen in the continued presence of the drugs. Samples were taken at 10 min for measurement of β-hexosaminidase and histamine release and at 1 hr for assessment of mRNA levels for three cytokines by RNA blot analysis. The mean IC_{50} values for CsA-induced inhibition of the increased expression of IL-1β, TNF-α, and IL-6 mRNA and for the release of β-hexosaminidase (Fig. 1) and histamine (data not shown) were 4, 65, 130, >2000, and >2000 nM, respectively. Preincubation of IgE-sensitized BMMCs with CsA for up to 60 min before antigen challenge did not decrease the IC_{50} values for inhibition of β-hexosaminidase release or cytokine mRNA levels (data not shown).

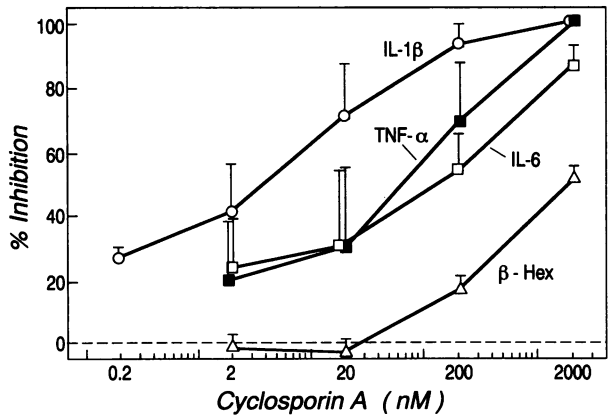

FIG. 1. Dose–response analysis of the effects of CsA on mRNA levels for IL-1β (○), TNF-α (■), and IL-6 (□) and on the release of β-hexosaminidase (△) by BMMCs. Cells were sensitized with mouse monoclonal IgE anti-TNP, incubated for 10 min with vehicle or drugs, and incubated with medium or TNP-BSA for 10 min (β-hexosaminidase) or 1 hr (cytokines). Data are expressed as mean ± SD, $n = 3$ for IL-1β and β-hexosaminidase, and $n = 5$ for TNF-α and IL-6.

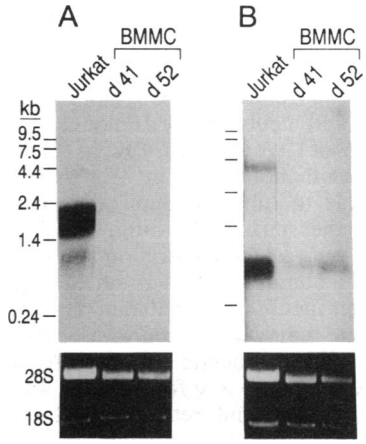

FIG. 2. mRNA levels for FKBP12 (*A*) and FKBP13 (*B*) in the Jurkat human T-lymphocyte line and at two ages of a single BMMC line [day (d) 41 and 52]. Total cytosolic RNA was isolated, and 10 μg of RNA was loaded per lane. The 18S and 28S RNAs were visualized by ethidium staining (*Lower*). kb, Kilobase(s).

At 200 nM, FK506 did not inhibit the release of β-hexosaminidase (49 ± 4% net percent release in vehicle versus 47 ± 5% with drug; mean ± SD, $n = 5$). At 200 nM, FK506 inhibited the hapten-specific induced mRNA level for IL-1β by 51 ± 4% (mean ± half-range, $n = 2$), but at 2000 nM it did not inhibit the increased expression of mRNA for IL-6 and TNF-α ($n = 2$). Thus, the IC_{50} of FK506 for IL-1β was ≈50-fold higher than that of CsA.

Expression of FKBPs by BMMCs. Because of the resistance of BMMCs to inhibition by FK506, RNA blots were prepared from BMMCs and the human T-cell line Jurkat (10 μg of RNA each) and probed with specific cDNAs for FKBP12 and FKBP13. Whereas mRNA for FKBP12 was readily detected in Jurkat cells, the signal was barely detected from BMMC RNA (Fig. 2*A*). When a parallel blot was probed with an FKBP13 cDNA, mRNA was readily detected from both Jurkat cells and BMMCs (Fig. 2*B*).

To determine the levels of immunoreactive FKBPs in BMMCs, detergent cell extracts were resolved by SDS/PAGE and immunoblotted with specific rabbit anti-FKBP12 peptide IgG (Fig. 3). BMMC extracts with the same amount of total protein contained less FKBP12 than extracts of RBL-1 and Jurkat cells, and the signal decreased with the age of the BMMCs. By contrast, FKBP13 levels were similar in

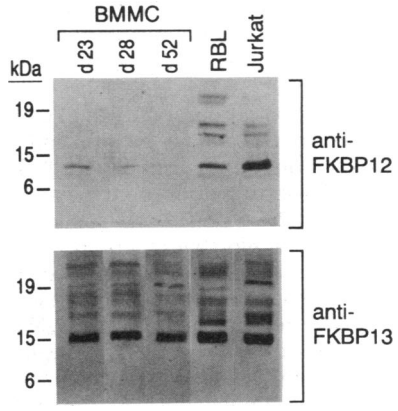

FIG. 3. Immunoblot analysis of FKBP12 (*Upper*) and FKBP13 (*Lower*) expression from 23-, 28-, and 52-day (d) BMMCs (28- and 52-day samples from the same BMMC line), the rat RBL-1 mucosal mast cell-like line, and the Jurkat human T-lymphocyte line. FKBP13 migrates at ≈15 kDa (28).

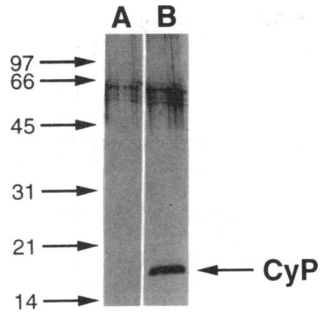

FIG. 4. CyP isolated from a BMMC extract by affinity chromatography on an immobilized CsA column (lane B); eluate from a control resin is shown in lane A. Eluates were analyzed by SDS/PAGE with silver staining.

the three types of cells and did not decrease with age in BMMCs (Fig. 3).

Expression of Cyclophilins by BMMCs. When extracts of BMMCs were bound to and specifically eluted from a CsA-affinity matrix, a CyP species of $M_r \approx 18,000$ was recovered from the CsA affinity matrix, but not from a control matrix, suggesting that BMMCs express CyPA (Fig. 4). As assessed by immunoblotting of BMMC extracts with anti-CyPA and anti-CyPB polyclonal antibodies (each of which also weakly crossreacted with the other CyP), BMMCs contained two immunoreactive species of M_r 17,000 and 20,000 (data not shown), indicative of CyPA and CyPB, respectively (30).

Calcineurin Phosphatase Activity. To determine whether the level of immunophilin expression in BMMCs related to the ability of CsA and FK506 to inhibit calcineurin activity, intact cells were exposed to the drugs for 1 hr, and cell lysates were then assessed for calcineurin activity in the presence of 500 nM okadaic acid, which inhibits phosphatases 1 and 2A (10, 31). CsA inhibited BMMC calcineurin phosphatase activity with an IC_{50} of $\approx$8 nM (n = 2), whereas FK506 at concentrations up to 1000 nM gave no appreciable inhibition (Fig. 5). Decreasing the incubation time of BMMCs with drugs to 10 min did not change the IC_{50} values (data not shown).

DISCUSSION

The addition of CsA to BMMCs sensitized with IgE anti-TNP before Fc_ε receptor type I perturbation with TNP-BSA resulted in a dose-related decrease in the augmentation of mRNA levels for IL-1β, TNF-α, and IL-6, with IC_{50} values

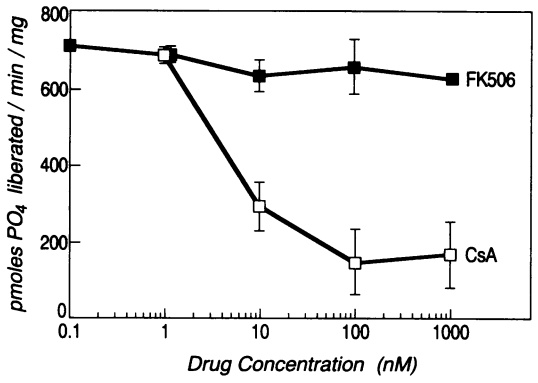

FIG. 5. Dose–response analysis of the effects of CsA (□) and FK506 (■) on calcineurin-mediated phosphatase activity. Cells were incubated with the indicated concentrations of drugs or vehicle, cytoplasmic extracts were prepared and incubated with a [^{32}P]phosphorylated peptide substrate, and liberated phosphate was measured. Cells exposed to vehicle released 743 ± 49 pmol of phosphate per min per mg of protein. Data are mean ± half-range, n = 2.

of 4, 65, and 130 nM, respectively (Fig. 1). The IC_{50} for inhibition of IL-1β is similar to that obtained for the inhibition of calcineurin phosphatase activity in BMMCs by CsA (Fig. 5), suggesting that calcineurin plays a critical role in the signal transduction pathway leading to increased IL-1β mRNA after cell activation with IgE and antigen. The higher IC_{50} values for TNF-α and IL-6 suggest that calcineurin inhibition may be insufficient to explain the effects of CsA on these cytokines in BMMCs and that there may be additional intracellular signals that are inhibited at a higher drug concentration. Inhibition of IL-6 mRNA stimulated by IgE plus antigen in an IL-3-dependent, BMMC-like continuous mouse mast cell line has been reported to be incomplete at $\approx$1700 nM CsA (32). In human T lymphocytes activated by concanavalin A plus phorbol myristate acetate, the IC_{50} for CsA inhibition of TNF-α mRNA augmentation is $\approx$100 nM, whereas the IC_{50} for inhibition of IL-2 mRNA augmentation is $\approx$5 nM, reflecting a 20-fold difference (14). Thus, cytokines in both mast cells and T lymphocytes differ in their sensitivity to inhibition by CsA.

In contrast to its effect on cytokine mRNA levels, CsA in concentrations up to 2000 nM inhibited the release of the secretory granule mediators β-hexosaminidase (Fig. 1) and histamine by <50%, and the refractoriness of the BMMCs was not reduced by incubating them for up to 60 min with CsA before challenge (data not shown). The resistance of BMMCs to the inhibition of exocytosis by CsA is a characteristic shared by human blood basophils, which exhibit an IC_{50} of 800–1000 nM for the inhibition of histamine release mediated by IgE plus antigen (4, 5). By contrast, the IgE-mediated release of exogenously incorporated [^{3}H]serotonin into RBL-2H3, which is a transformed cell line with several secretory granule characteristics of rat mucosal mast cells (33–35), is inhibited by CsA with an IC_{50} of 200 nM (36, 37). However, there is uncertainty whether exogenously added serotonin is released from the same intracellular compartment as endogenous secretory granule components such as β-hexosaminidase and histamine (38, 39). The IC_{50} for CsA-mediated inhibition of esterase exocytosis from clones of mouse cytotoxic T lymphocytes activated with anti-T cell receptor or phorbol myristate acetate and calcium ionophore is 50–150 nM (2). Neutrophil exocytosis elicited by complement component C5a, f-Met-Leu-Phe, or phorbol myristate acetate is not inhibited by CsA (3), and for exocytosis elicited with calcium ionophore, the IC_{50} of CsA is about 35 nM, with a maximum inhibition of 70% at 100 nM. In a separate report (40), calcium ionophore-stimulated exocytosis from human neutrophils was reduced by only 50% with 4000 nM CsA. Thus, exocytosis of endogenous preformed mediators by multiple cell types is more resistant to inhibition by CsA than is cytokine gene transcription and the resultant production of bioactive protein by T cells. This distinction is now presented in a single cell type activated with a physiologic stimulus (Fig. 1).

In contrast to the CsA-mediated inhibition of BMMC cytokine mRNA levels, no significant inhibition for TNF-α or IL-6 was obtained with FK506, and the IC_{50} of FK506 for inhibition of IL-1β (200 nM) was 50-fold higher than that for CsA, and considerably higher than conventional concentrations. By contrast, FK506 inhibits the transcription of IL-2 and TNF-α in human T lymphocytes with IC_{50} values of 0.06 and $\approx$10 nM, respectively (14). Because IL-1β is particularly sensitive to inhibition by CsA in BMMCs, with an IC_{50} (Fig. 1) identical to that for inhibition of calcineurin activity by CsA (Fig. 5), the ability of high concentrations of FK506 to inhibit IL-1β in BMMCs may reflect the interaction with minimal amounts of FKBP12 in some BMMCs (Fig. 3). In one experiment with 7-week-old BMMCs, 2000 nM FK506 failed to inhibit the IgE-dependent increment in IL-1β mRNA (data not shown).

RNA (Fig. 2) and protein (Fig. 3) blot analyses revealed that BMMCs express little FKBP12 compared with Jurkat and RBL-1 cells. Of 12 cell lines examined, including members of the T-cell, B-cell, monocyte, and mast cell lineages, BMMCs contained the lowest amount of mRNA for FKBP12 (data not shown). At the protein level, the FKBP12 deficiency was greatest in BMMCs cultured for ≈50 days. FKBP13 (Figs. 2 and 3) and FKBP25 (preliminary data) are detected in BMMCs. That the inability of FK506 to inhibit the activation response of BMMCs is associated with minimal expression of FKBP12 suggests that FKBP12 plays a critical role in mediating FK506 inhibition of cytokine transcription. *In vitro*, complexes of CsA–CyPA (8), CsA–CyPB (41), CsA–CyPC (8, 9), and FK506–FKBP12 (8), but not FK506–FKBP13 (P. K. Martin and S.L.S., unpublished results), bind to calcineurin. The addition of CsA or FK506 to T lymphocytes inhibits the activity of cellular calcineurin (10). Calcineurin-mediated phosphatase activity in drug-treated BMMCs was inhibited by CsA (IC_{50} = 8 nM), but FK506 at concentrations up to 1000 nM had no significant effect (Fig. 5). By contrast, CsA and FK506 inhibited calcineurin phosphatase activity in Jurkat cells, with IC_{50} values of 7 and 0.4 nM, respectively (10). Thus, the minimal expression of FKBP12 in BMMCs may explain the failure of FK506 to inhibit both calcineurin activity and activation-dependent cytokine induction.

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