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FK506 and cyclosporin each block antigen-induced T cell receptor signalling that is dependent on CD4 co-receptor and operates in the *absence* of detectable cytoplasmic calcium fluxes

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Abstract The T cell hybridoma "171", which lacks CD4 but expresses T cell receptor (TCR) for hen egg white lysozyme, requires introduction of wild-type CD4 for antigen-mediated induction and secretion of interleukin-2 (IL-2). Mutant CD4, which fails to associate with the tyrosine kinase p56lck does not support IL-2 secretion, suggesting that a role of CD4 is to bring cytoplasmic p56lck into alignment for signal transduction to the IL-2 promotor. Using 171, 171-CD4 (wild-type) and 171-CD4 (mutant), we found that IL-2 secretion was inhibited by FK506 and cyclosporin but not by rapamycin. However, this inhibition was *not* associated with calcium fluxes since no change in cytoplasmic free calcium levels ($[Ca]_i$; resting level 80 n*M*) was detectable during antigen stimulation of the 171 or 171-CD4 cells. Thus, although FK506 and cyclosporin inhibited calcium-dependent signalling to the IL-2 promoter via inhibition of the protein phosphatase calcineurin, it is possible that IL-2 induction via TCR/CD4 requires an FK506 (and cyclosporin) sensitive step which is independent of cytoplasmic calcium changes.

Key words TCR/CD4 · FK506 Induction of interleukin-2

Introduction

Encounter with antigen at the T cell surface is communicated through the cytoplasm to the nucleus to generate an immune response. Free soluble antigen fails to invoke a response but does so when complexed with class II molecules on the surface of antigen-presenting cells [APC]. This antigen-class II complex of the APC has affinity for the T cell receptor (binding to antigen) plus CD4 (binding to class II) on the surface of the $CD4^+$ T lymphocyte that results in a relatively stable intercellular bridge. This initiates signalling within the T cell including activation of p56lck, which associates with the cytoplasmic domain of CD4. Signal transduction normally proceeds through the cell by a series of "on/off" switches provided by reversible phosphorylation of critical target proteins that control cell communication networks. Cytoplasmic kinases (to add phosphate) or phosphatases (to remove phosphate) are, in turn, regulated by their own state of phosphorylation together with ions such as calcium or magnesium. Calcineurin is a calcium-calmodulin dependent phosphatase (PP2B) and is required for interleukin-2 (IL-2) induction in $CD4^+$ T cells. Evidence suggests that the drugs cyclosporin A (CyA) and FK506 are powerful, specific immunosuppressive molecules because they block a dephosphorylation step mediated by calcineurin that is mandatory for signal progression to the IL-2 gene.

Here we showed that FK 506 and cyclosporin, but not rapamycin, blocked IL-2 induction initiated by T cell receptor (TCR)-CD4 complexing with antigen-class II. We were, however, unable to detect any changes in $[Ca^{++}]_i$ levels associated with IL-2 induction.

Materials and methods

Cells and antigen

The generation of three T cell lines that express the T cell receptor for hen egg white lysozyme [171, 171-CD4 (wild-type), 171-CD4 (mutant)] and of APC for hen egg white lysozyme is detailed elsewhere [1, 2]. The 15 amino acid analogue peptide (NLANIPASALLSSD; amino acids 74–88) of hen egg white lysozyme was kindly donated by Glaxo, Paris. The drugs CsA, FK 506 and rapamycin were generous gifts from Sandoz, Basel, Fujisawa Pharmaceuticals Company and Wyeth Ayerst Research, respectively.

T cell activation and IL-2 assays

Antigen-presenting cells were incubated with 20 μM HEL peptide in flat-bottomed microtitre wells together with the 171 T cells. Each of the three 171 cell lines was made up in growth medium containing dilutions of FK 506, CsA or rapamycin. All cultures were in triplicate and culture supernatants were harvested at 21 h. The amount of IL-2 in the experimental supernatants was assayed by the ability to support HT-2 cell proliferation for 24 h as measured by tritiated thymidine incorporation over the final 4 h. Any direct effect of residual drug in the experimental supernatants on the HT-2 cells was controlled for in parallel assays containing an IL-2 standard.

Cytoplasmic calcium changes

Cells were loaded with the calcium-sensitive dyes fura-2 or indo-1. Cytoplasmic calcium levels were recorded for bulk cell populations by spectrophotometry and in single cell assays using FACS analysis.

Results

IL-2 secretion

Antigen presentation to 171-CD4 (wild-type) cells resulted in 30–50 U/ml IL-2 secretion by 24 h: FK 506 and CsA each inhibited this IL-2 secretion in a dose-ependent manner with complete inhibition at 1 nM and 100 nM, respectively. The 171 cells gave no IL-2 response, whilst the mutant 171-CD4 cells secreted around 10 units/ml, with the same sensitivity to inhibition by the two drugs as the 171-CD4 [wild-type] cells. The results for 171-CD4 (wild-type) are shown in Fig. 1.

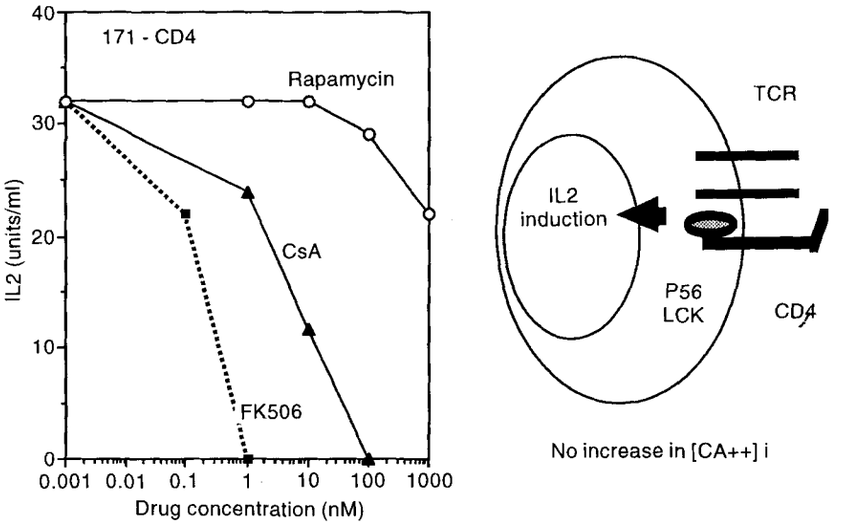

Fig. 1 FK 506 and cyclosporin A (CsA) each inhibit IL-2 secretion by 171-CD4 (wild-type) cells stimulated by antigen presentation. We cultured 171-CD4 (wild-type) cells in the presence of HEL peptide presented by class II of APC. Cultures included different concentrations of FK 506, CsA and rapamycin. Neither FK 506 nor CsA inhibited the IL-2-dependent HT-2 cells used to assay IL-2 levels: rapamycin had some inhibitory effect on HT-2 cells and the results shown have been corrected for this. The high (1 μM) dose of rapamycin also caused some inhibition of the 171 cells, which might account for the observed effect at high dose. In contrast FK 506 and CsA each were potent, specific inhibitors of IL-2 secretion. The cartoon illustrates the 171-CD4 cell where CD4 is able to associate with p56lck to boost signal transduction from the T cell receptor (TCR) to the nucleus for IL-2 induction. Assays of cytoplasmic calcium levels during this signalling failed to detect a calcium signal

Cytoplasmic calcium $[Ca^{++}]_i$ levels

$[Ca^{++}]_i$ in the 171-CD4 cells was 80 n*M*, which is normal for resting cells. In both bulk culture and single cell assays no increase in $[Ca^{++}]_i$ was detectable upon coincubation with APC plus HEL or with 20 μg/ml conA, that is, under conditions known to induce IL-2 secretion. The positive control cells [Jurkat] gave a good calcium signal under these experimental conditions.

Is calcineurin constitutively active in 171 cells?

Since there was no detectable change in cytoplasmic calcium in the 171 cells and intracellular calcium levels were in the normal range, we considered the possibility that the calcineurin might be constitutively active. The findings of O'Keefe et al. [3] suggest that mutant calcineurin that is constitutively active synergises with PKC (using PMA treatment) to activate the IL-2 promoter. Thus, we incubated 171 cells with PMA and looked for IL-2 secretion as evidence for an active form of calcineurin. No IL-2 was detected.

Discussion

CD4 acts as a co-receptor for antigen-mediated signal transduction in two ways, by stabilising the interaction between presented antigen and the T cell receptor, and by delivering positive regulatory signals mediated by its associated with p56lck. Since we found that FK506 and CsA were equivalent in their inhibition of IL-2 secretion in both the 171-CD4 (wild-type) and 171-CD4 (mutant) cells, we suggest that p56lck activity boosts the TCR signal at a site that preceeds the site of drug action.

Although FK506 and CsA each act on calcium-dependent signalling, they do not inhibit calcium changes per se [4] but bind to and inhibit calcineurin when complexed to their respective binding proteins (FKBP or cyclophilin) [5]. Calcineurin is thought to play a pivotal role in the IL-2 gene activation pathway, causing dephosphorylation of the cytosolic NF-AT subunit to promote its translocation to the nucleus [discussed in ref 6].

It is possible that IL-2 secretion in the 171 cells is able to bypass the need for calcium, but, apart from the lack of an early calcium signal, we found no evidence of calcium-elated abnormalities that might reduce or negate a central role of calcium in activation of calcineurin. The resting level of $[Ca^{++}]_i$ was normal at around 80 n*M* and, thus, well below that required to activate wild-type calcineurin (around 1 μ*M* [21]). PMA failed to synergise for the secretion of IL-2, arguing against the presence of calcium-independent calcineurin activity, or indeed of a putative calcium-independent translocation of cytosolic NF-AT to the nucleus. An abnormally high concentration of calcineurin might minimise any calcium requirement for signalling, but this would be expected to give resistance to FK506 [3, 7] which was not found.

If calcium signalling does occur in the 171 cells then it is possible that TCR/CD4 engagement with antigen/MHC provides sufficient calcium to activate calcineurin in a highly localised manner. In conclusion, we showed that antigen-mediated signalling that requires CD4 co-receptor function is specifically inhibited by FK506 and CsA, both when CD4 is uncoupled from p56lck [171-CD4 (mutant); low level IL-2 secretion] and when CD4 is able to associate with p56lck [171-CD4 (wild-type); high level IL-2 secretion] and that this occurs in the absence of detectable calcium fluxes.

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