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FKBP, THOUGHT TO BE IDENTICAL TO PKCI-2, DOES NOT INHIBIT PROTEIN KINASE C

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Abstract: The FK506 and rapamycin binding protein (FKBP), recently shown to be identical to PKCI-2, and its ligands FK506 and rapamycin either acting alone or complexed to FKBP do not inhibit the kinase activity of isolated protein kinase C or protein kinase C-mediated events in cells.

Much progress has been achieved in efforts to understand signal transmission pathways that are inhibited by the immunosuppressive agents FK506, rapamycin, and cyclosporin A (CsA).[1] These drugs bind to different cytosolic receptors (immunophilins), the FKBPs[2,3,4] and the cyclophilins.[2,5] All known immunophilins have peptidyl prolyl *cis-trans* isomerase (rotamase) activity, which is inhibited following binding of their respective ligands.[2,3] Although inhibition of this enzymatic activity has been shown to be insufficient for mediating the actions of the drugs,[6] biochemical[7] and genetic[8] studies have demonstrated that the immunophilin-drug complexes are responsible for interference of signal transmission. The targets of these inhibitory complexes have yet to be defined. The similar actions and interactions of the immunophilin ligands on distinct signaling pathways leading to either transcription or exocytosis suggest a common, perhaps fundamental, event is being interrupted by these drug-receptor complexes.[9] One possibility is that the immunophilin-drug complex interferes with either a kinase or phosphatase that is responsible for regulating the phosphorylation state of a protein. Thus, a recent publication[10] that notes the virtual amino acid sequence identity of the human FK506 and rapamycin binding protein (FKBP)[11] and the amino acid sequence reported for the bovine protein kinase C inhibitor 2 (PKCI-2)[12] raised the possibility that FK506 and rapamycin modulate the activity of protein kinase C (PKC). Indeed, the sequence reported for PKCI-2 is identical to the amino acid sequence of bovine FKBP purified from thymus;[13] the discrepancy between the two sequences featured in the aforementioned letter can be accounted for by species variation. In addition, electrospray mass spectroscopy of bovine FKBP indicates that the molecular weight of the natural protein is identical to the molecular weight predicted from the amino acid sequence;[13] thus, the natural protein is not post-translationally modified.

Unfortunately, subsequent experiments indicate that the kinase activity of PKC is not modulated by FKBP, FK506, or rapamycin. In vitro PKC enzymatic assays were performed using partially purified PKC from rat brain

Table 1. In vitro protein kinase C assays in the presence of the immunophilins FKBP and cyclophilin (CyP) and the drug-immunophilin complexes FK506-FKBP, rapamycin-FKBP, and CsA-CyP.[18,19]

Activation Method	Agent	Concentration	% Activity of (+) Control
0.5 mM Ca^{2+}	rhFKBP	1 μM	135
40 μg/ml PS[1]	bFKBP	1 μM	105
	FK506	1 μM	100
	rapamycin	1 μM	105
	rhFKBP-FK506	1 μM	129
	bFKBP-FK506	1 μM	98.7
	rhFKBP-rapamycin	1 μM	125
	bFKBP-rapamycin	1 μM	93.1
	rhCyP	1 μM	109
	CsA	10 μM	105
	rhCyP-CsA	1 μM	103
10 μM Ca^{2+}	rhFKBP	15 μM	104
200 μg/ml PS	FK506	17 μM	91.6
5 μM 1,2 diolein[2]	rapamycin	17 μM	95.3
	rhFKBP-FK506	15 μM	88.9
	rhFKBP-rapamycin	15 μM	98.1
	rhCyP	13 μM	105
	CsA	17 μM	104
	rhCyP-CsA	13 μM	108
100 nM TPA	rhFKBP	1 μM	111
10 μg/ml PS[3]	bFKBP	1 μM	93.6
	FK506	1 μM	112
	rapamycin	1 μM	108
	rhFKBP-FK506	1 μM	139
	bFKBP-FK506	1 μM	91.9
	rhFKBP-rapamycin	1 μM	126
	bFKBP-rapamycin	1 μM	101
	rhCyP	1 μM	110
	CsA	10 μM	92.0
	rhCyP-CsA	1 μM	110

[1] Assay conditions: 25 mM Tris-HCl (pH 7.5), 5 mM $Mg(NO_3)_2$, 0.2 mg/ml histone FIIIS (Sigma), 0.5 mM $CaCl_2$, 40 μg/ml PS, 100 μM ATP, 1 μCi γ-[^{32}P]ATP, 0.05 mM EGTA, 10 μl partially purified PKC in 100 μl total volume. The components were incubated for 10 minutes at 30 °C. Each concentration was assayed in triplicate. The dose dependence was also determined (data not shown) at concentrations one and two orders of magnitude below the concentrations listed in this table.

[2] Assay conditions: 20 mM Tris-HCl (pH 7.5), 5.8 mM $MgAc_2$, 1 mM EDTA, 193 μM $CaCl_2$, 800 μg/ml histone FVS (Sigma), 18 μM ATP, 1 μCi γ-[^{32}P]ATP, 240 μg/ml PS, 5 mM 1,2-sn-diolein, 12 μl partially purified PKC in a total volume of 300 μl. The mixture was incubated at 30 °C for 5 minutes. Each assay was performed twice.

[3] Assay conditions: 25 mM Tris-HCl (pH 7.5), 5 mM $Mg(NO_3)_2$, 0.2 mg/ml histone FIIIS (Sigma), 10 μg/ml PS, 100 μM ATP, 1 μCi γ-[^{32}P]ATP, 0.05 mM EGTA, 10 μl partially purified PKC in 100 μl total volume. The components were incubated for 10 minutes at 30 °C. Each concentration was assayed in triplicate. The dose dependence was also determined (data not shown) at concentrations one and two orders of magnitude below the concentrations listed in this table.

and three activating conditions: 0.5 mM Ca^{2+} and 40 μg/ml phosphatidylserine (PS);[14] 5 μM 1,2 diolein in the presence of 10 μM Ca^{2+} and 200 μg/ml PS;[15] or 100 nM TPA and 10 μg/ml PS. As shown in Table 1, neither recombinant human FKBP nor natural bovine FKBP inhibits the activity of PKC at concentrations of immunophilins up to 10 μM. In all cases, the concentration of FKBP is at least 100-fold higher than the concentration of PKC estimated from the amount of total protein in a partially purified fraction of PKC and from the specific activity of PKC. Moreover, FKBP bound to either FK506 or rapamycin, and FK506 and rapamycin alone had no effect on the activity of PKC up to concentrations of 1 μM. Since the IC_{50} of FK506 and rapamycin in T cell functional assays is 0.5 nM[1] and FK506 in mast cell functional assays is 2 nM,[9] we infer that FK506 and rapamycin are not acting at pharmacologically relevant concentrations in these cells to block the actions of PKC.

This reasoning is supported by whole cellular assays. 12-O-tetradecanoylphorbol 13-acetate (TPA) activates PKC to phosphorylate a 47 kDa protein in intact platelets.[14] In the presence of concentrations of FK506 and rapamycin up to 1 μM there was no decrease in TPA-induced phosphorylation of this 47 kDa protein (Figure 1A). Similarly, as shown in Figure 1B, FK506 and rapamycin at concentrations up to 1 μM did not inhibit the downregulation of CD3 and CD4 from the surface of human peripheral blood T lymphocytes induced by phorbol dibutyrate (PDBu)[14]. In addition, earlier studies that involved the induction of genes by phorbol ester demonstrated that neither CsA nor FK506 could block PKC-mediated events in T cells.[16]

Given the similarity of the actions of FK506 and CsA in T cells and mast cells and the common enzymatic activity of their receptors, FKBP and cyclophilin, respectively, we also tested whether cyclophilin, CsA, or the cyclophilin-CsA complex modulates isolated PKC enzymatic activity and PKC-mediated events in cells. No inhibition was observed in each of the assays listed above (Table 1, Figure 1).[17]

The experiments reported herein demonstrate that FKBP, found to be identical to the protein named PKCI-2, does not inhibit PKC. Moreover, FKBP and cyclophilin alone or bound to their respective ligands, FK506, rapamycin, and CsA, do not inhibit PKC activity. The finding that FK506 and rapamycin do not alter the effects of FKBP on PKC activity argues against the reported PKC-inhibitory properties being due to an endogenous regulatory ligand not present in the samples of FKBP used in our experiments. Instead, it seems likely that a contaminant is responsible for the inhibition of PKC in the previous work on "PKCI-2."[12] Thus, the mystery of the biochemical mechanisms responsible for FK506's and CsA's actions remains to be solved.

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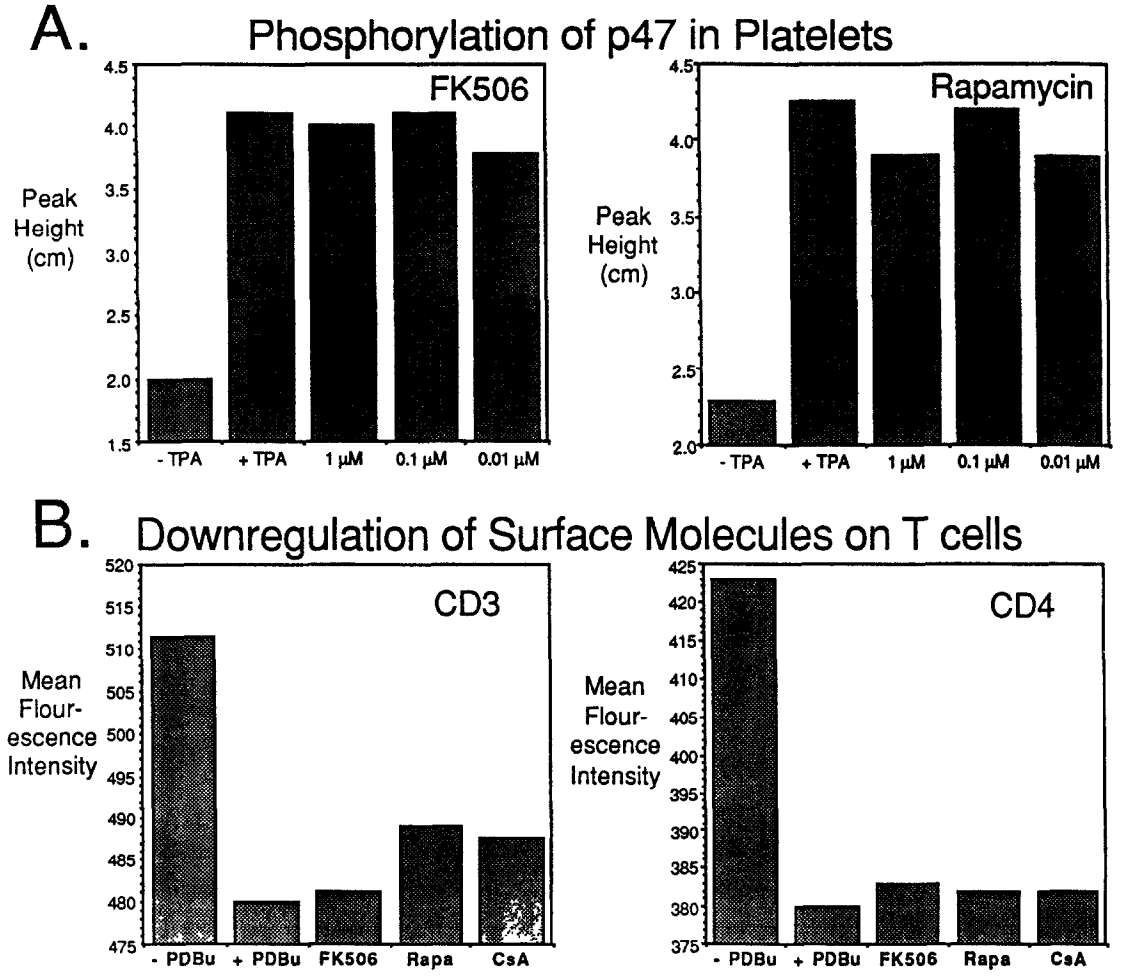

Figure 1: Effects of FK506, rapamycin, and CsA on PKC-mediated events in whole cells. (A) TPA induced phosphorylation of a 47 kDa protein in human platelets. The left panel shows the effects of FK506 and the right panel shows the effects of rapamycin. The first two bars represent the control experiments, with and without TPA. The next three bars represent assays in the presence of TPA and the indicated concentration of drug. The autoradiographs were quantitated by densitometric analysis and the peak height of each sample is graphed. (B) The effects of FK506, rapamycin, and CsA on phorbol dibutyrate (PDBu)-mediated downregulation of surface CD3 and CD4 on lymphocytes isolated from the blood of a human volunteer. The cells were stained with antibodies directly conjugated to either phycoerythrin (anti-CD3) or FITC (anti-CD4) and analyzed by a flourescence activated cell sorter. Each drug was assayed at three concentrations, but only the highest concentration (1 μM) is graphed. As above, the first two bars represent the negative and positive controls, and the next three bars represent assays performed in the presence of PDBu and the indicated drug.

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18. PKC was prepared from rat brain as described.[14,15] The drugs, proteins, and drug-protein complexes were tested at three or more concentrations, but only the highest concentration is shown. PKC was activated by three different conditions that involve different mechanisms of activation. Thus, the drugs, proteins or drug-receptor complexes are not competitively interacting at the active site or the nucleotide binding site on the catalytic subunit or at the phospholipid or the diacylglycerol-phorbol ester binding site on the regulatory subunit of PKC. PKC assays were also performed under the second set of assay conditions except that a 4:1 ratio of PC: PS was used in place of pure PS to mimic more closely the phospholipid composition of the plasma membrane; these conditions gave essentially the same results (data not shown). In addition, since we observed some activation with FKBP, an activation assay was performed, i.e., in the absence of phorbol ester or added calcium, and neither FKBP nor FKBP bound to FK506 or rapamycin activated PKC (data not shown).
19. Abbreviations: PS = phosphatidylserine, PC = phosphatidylcholine, TPA = 12-O-tetradecanoylphorbol 13-acetate, rhFKBP = recombinant human FK506 binding protein, bFKBP = natural bovine FK506 binding protein, rhCyP = recombinant human cyclophilin, CsA = cyclosporin A.