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RAPID COMMUNICATION

REDUCTION OF RYANODINE BINDING AND CYTOSOLIC Ca^{2+} LEVELS IN LIVER BY THE IMMUNOSUPPRESSANT FK506

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ABSTRACT - The mechanism of action of the immunosuppressant FK506 in the liver was studied. The hypothesis was tested that FK506 exerts its effect in the liver by interacting with the ryanodine-binding Ca^{2+} release channel. Two types of experiments were carried out: (1) [^{3}H]-ryanodine binding studies with isolated microsomal fractions, and (2) cytosolic-free Ca^{2+} ($[Ca^{2+}]_i$) measurements with the intracellular Ca^{2+}-indicator fura-2. The inclusion of FK506 in the incubation medium significantly decreased the binding of [^{3}H]-ryanodine to liver microsomes. The B_{max} of binding in control experiments was 405 fmol/mg protein; the presence of FK506 decreased the B_{max} to 157 fmol/mg protein. Measurements of $[Ca^{2+}]_i$ in the presence and absence of FK506 showed a decrease in $[Ca^{2+}]_i$ in the presence of FK506. The data support the notion that FK506 interacts with the ryanodine binding Ca^{2+} channel in the liver and suggest a critical role for the ryanodine-binding Ca^{2+} channel in the hepatic responses to FK506. The interaction between FKBP-12 (FK506 binding protein) and the ryanodine-binding Ca^{2+} channel may be an essential link in the chain of events by which FK506 alters Ca^{2+}-dependent cellular processes.

Key words: FK506; ryanodine-receptor; calcium; immunosuppression; liver

FK506, a product isolated from the fungus *Streptomyces tsukubaensis*, is a potent immunosuppressant that has been shown to prevent the rejection of transplanted organs, including the liver [1,2]. The drug exerts its therapeutic effect by inhibiting T-cell activation, a process that involves increases in $[Ca^{2+}]_i$† levels. FK506 was shown to bind to a 12-kDa binding protein, present in all tissues, which, when complexed with FK506, blocks Interleukin-2 gene transcription in T-cells [3,4]. It was also demonstrated that the FK506 binding protein (FKBP-12) is tightly associated with the ryanodine-binding Ca^{2+} release channel of the skeletal muscle and alters its function [5], but it was not determined whether

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†Abbreviations: $[Ca^{2+}]_i$, cytosolic-free Ca^{2+}.

these changes affect $[Ca^{2+}]_i$ levels. Because the liver also possesses a high-affinity ryanodine binding Ca^{2+} release channel, which is a different protein from the skeletal muscle channel [6,7], studies were carried out to examine whether FK506 exerts an influence on the ryanodine binding Ca^{2+} channel of the liver. Here we report that FK506 inhibited, in a concentration-dependent manner, the binding of [^{3}H]-ryanodine to vesicles isolated from liver smooth endoplasmic reticulum and lowered dramatically $[Ca^{2+}]_i$.

MATERIALS AND METHODS

Animals. Male, Sprague-Dawley rats, weighing between 120 and 160 g, were used in all the experiments. Rats had free access to food and water in the experiments for the preparation of hepatocyte and microsomal fractions.

Preparation of smooth microsomal fraction. Smooth microsomal fractions were prepared by differential centrifugation, followed by sucrose gradient separation as described in previous studies [6,7].

[^{3}H]-Ryanodine binding. Equilibrium binding to the microsomal fraction was determined as described in Refs. 6 and 7. In short, membranes (1 mg/mL) were incubated at 37° for 30 min in a buffer composed of 0.5 M NaCl, 20 mM Tris-HCl (pH 7.4), and 0.5 mM EGTA. [^{3}H]-Ryanodine concentrations between 1 and 80 nM (76 Ci/mmol) were included in the buffers. Non-specific binding was determined in the presence of 100 μM unlabeled ryanodine. The Millipore filtration method was employed to separate bound from unbound ligand, as in previous studies [6,7].

Measurement of $[Ca^{2+}]_i$ with fura-2 AM. $[Ca^{2+}]_i$ was measured in isolated cell preparations as in previous experiments [8], with the following modifications: Hepatocytes were loaded with fura-2 AM on a rocker at room temperature for 30 min and subsequently transferred to a water bath maintained at 32° and incubated for an additional 45 min under a constant supply of 95% O_2, 5% CO_2. After this period, the cells were sedimented by centrifugation and resuspended in a buffer containing 2.5 mM probenecid, in order to block the export of fura-2 from the cells [9]. All subsequent solutions for cell suspensions contained probenecid (2.5 mM). The fluorescence of fura-2 AM loaded cells was monitored at 37° in an LS-5 Perkin-Elmer fluorescence spectrophotometer. The instrument was controlled by a personal computer with the help of a free program developed in-house by Perkin-Elmer personnel (Dean Brown), specifically for instrument control in models LS-5 and LS-3B. The program excited the cells alternatively at 340 and 380 nm, recorded the emission at 510 nm, and calculated the autofluorescence corrected ratio and $[Ca^{2+}]_i$ by a standard formula, as described in Ref. 10. Each experiment was repeated at least three times with similar results.

RESULTS

It has been established in previous studies that the liver possesses high-affinity ryanodine-binding sites that represent a new, yet unidentified protein, which functions as a Ca^{2+} channel [6-8].

To evaluate whether FK506 interacts with the hepatic ryanodine-binding protein, first the effect of the drug on the binding of [^{3}H]-ryanodine was tested. It was assumed that an interaction of the drug with the ryanodine-binding protein would manifest itself in changes in [^{3}H]-ryanodine binding.

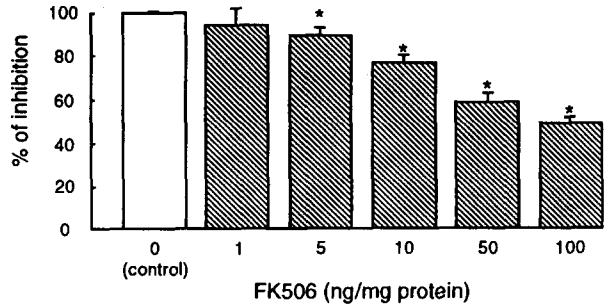

Fig. 1. Inhibition of [^{3}H]-ryanodine binding by FK506. Smooth microsomal fractions were prepared. The microsomal vesicles were preincubated with the desired concentrations of FK506 for 30 min on ice. Control vesicles were preincubated under identical conditions with ethanol equal to the amount in which FK506 was dissolved. Equilibrium binding of [^{3}H]-ryanodine (20 nM) was determined by the Millipore filtration technique, as described in Refs. 6 and 7. Key: (*) statistically significant vs control ($P < 0.01$; t-test).

For testing the effect of FK506 on [^{3}H]-ryanodine binding in the liver, smooth microsomal fractions were prepared and incubated with increasing concentrations of the drug, as described in Fig. 1. The dosage employed was selected to be in the range commonly employed in the protocol of liver transplant operations [1]. As shown in Fig. 1, a concentration-dependent decrease in ryanodine binding was evident. Scatchard plot analysis (Fig. 2) revealed a greater than 60% decrease in B_{max} for [^{3}H]-ryanodine binding, but no changes in the K_d. Cyclosporin, an immunosuppressant that exerts its effect by binding to a different receptor protein [3], had no effect on ryanodine binding (results not shown). Thus, the binding studies demonstrated a specific interaction between the ryanodine-binding site and FK506.

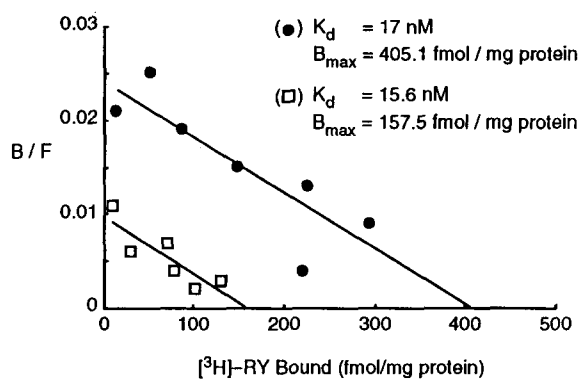

Fig. 2. Scatchard plot analysis of the effect of FK506 on [^{3}H]-ryanodine binding. [^{3}H]-Ryanodine binding was measured as in Fig. 1 at concentrations of ryanodine ranging between 1 and 80 nM (76 Ci/mmol) in the presence (□) and absence (●) of FK506 (100 ng/mg protein).

Because ryanodine changes $[Ca^{2+}]_i$ in the liver, the possibility that FK506 affects $[Ca^{2+}]_i$ was examined. To test the effects of FK506 on $[Ca^{2+}]_i$, isolated hepatocytes loaded with the Ca^{2+} indicator dye fura-2 AM were employed, as described in Materials and Methods. As shown in Fig. 3A, addition of FK506 to the cell preparation caused an immediate and sharp drop in $[Ca^{2+}]_i$. The decrease was sustained during the 30-min measurement time. For comparison, the effect of ryanodine addition is also shown (Fig. 3B). As reported previously [8], ryanodine addition to cells caused a small, steady increase in $[Ca^{2+}]_i$. In additional experiments, isolated cells were preincubated with the same amount of FK506 at 32° for 30 min and $[Ca^{2+}]_i$ was measured subsequently. In these experiments, FK506 lowered $[Ca^{2+}]_i$ from 503 to 228 nM ($P = 0.04$). The drop in $[Ca^{2+}]_i$ following the addition of FK506 may be due to increased extrusion of intracellular Ca^{2+}, increased sequestration of intracellular Ca^{2+} into cellular organelles, or

both. The experiments presented here do not allow one to distinguish between these possibilities. If, however, one assumes that the effects of FK506 are due to the interaction between its receptor protein and the ryanodine binding Ca^{2+} channel, then the latter possibility is likely. The ryanodine binding Ca^{2+} channel is localized at the endoplasmic reticulum, and binding of FK506 to FKBP-12 may alter the activity of the channel in such a way that more Ca^{2+} is retained in that compartment.

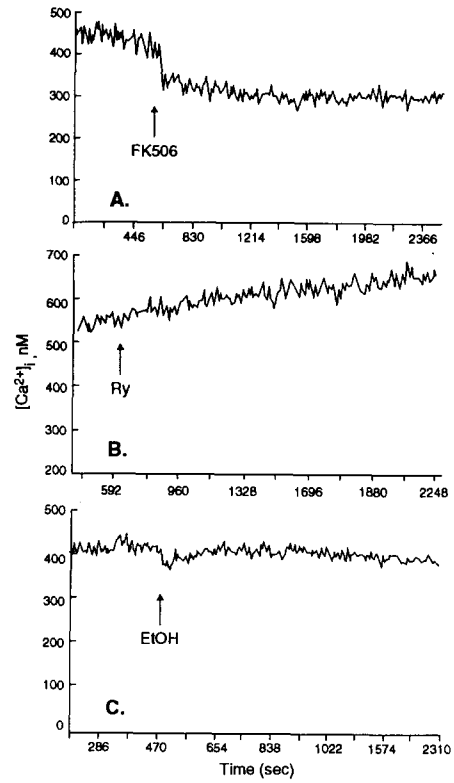

Fig. 3. Effects of FK506 and ryanodine on $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured in isolated liver cell preparations with the fluorescent Ca^{2+} indicator fura-2 AM [8-10]. Steady-state levels of $[Ca^{2+}]_i$ varied in different cell preparations and ranged between 130 and 700 nM in the four preparations tested. The effects of the drugs were similar in each preparation. A representative of each experiment is presented in the figure. Control values were obtained in the presence of ethanol equal to the amount used to dissolve the drugs (panel 3C). The concentrations of FK506 and ryanodine were 500 ng/mL and 10 μM, respectively.

DISCUSSION

The data presented demonstrate a profound effect of FK506 on the ryanodine binding Ca^{2+} channel and $[Ca^{2+}]_i$ in the liver. These effects are likely to be exerted through the binding protein for FK506, FKBP-12, which is present in the liver [3]. Because in skeletal muscle this protein was shown to be associated with the ryanodine binding Ca^{2+} release channel and binding of the drug results in modulation of the channel [5], a similar situation might exist in the liver; namely, that the drug-receptor protein complex modulates the channel protein, and this change is manifested in the decrease in ryanodine binding, which would lead to changes in cellular Ca^{2+} levels. Thus far, three types of ryanodine-receptor proteins have been structurally identified and characterized. These proteins are present in skeletal and cardiac muscles, and a smaller version of the molecule, representing the 3'-terminal region of the skeletal muscle receptor, is present in brain and smooth muscle [11,12]. The presence of these three forms of receptor in the liver has been tested. Northern blot analysis of mRNA from liver with cDNA probes from skeletal and cardiac muscles gave negative results [13]. Similar studies using cDNA probe for the brain version of the receptor also gave negative results with liver mRNA. Thus, the hepatic receptor clearly represents a novel molecular form of the receptor. However, the data presented here, which show an interaction between FKBP-12 and the hepatic ryanodine receptor, indicate that structural similarities do exist between the hepatic and the skeletal forms of the ryanodine receptor, because both interact with

FKBP-12. This interaction may be the underlying mechanism by which FK506 stimulates liver regeneration [14,15].

The immunosuppressant effects of FK506 are due to its inhibitory effect on lymphocyte activation, a Ca^{2+}-dependent process. The activation of T-lymphocytes by various agents involves increases in cytosolic free Ca^{2+} levels. Two components contribute to this increase: the release of Ca^{2+} from intracellular storage sites [16] and the influx of Ca^{2+} through plasma membrane Ca^{2+} channels [17,18]. Calcineurin, a Ca^{2+}/calmodulin regulated phosphatase, was suggested to be the key signalling enzyme responsible for T-cell inhibition [3,4]. The exact mechanism by which FK506 alters the T-cell Ca^{2+} signalling mechanism is not known [3]. It has been suggested that T-cells may possess a ryanodine-binding Ca^{2+} release channel [19], but this has not been tested so far. The demonstration of a modulatory effect of FK506 on the hepatic ryanodine binding Ca^{2+} channel raises the possibility that this effect of the drug may be widespread and could be the mechanism by which it affects cytosolic Ca^{2+} levels.

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