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FK506 induces biphasic Ca^{2+} release from microsomal vesicles of rat pancreatic acinar cells

TERUTAKA OZAWA

Department of Physiology, Tohoku University Graduate School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai 980-8575, Japan

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Abstract. The effect of the immunosuppressant drug FK506 on microsomal Ca^{2+} release was investigated in rat pancreatic acinar cells. When FK506 (0.1-200 μM) was added to the microsomal vesicles at a steady state of ATP-dependent $^{45}Ca^{2+}$ uptake, FK506 caused a dose-dependent and a biphasic release of $^{45}Ca^{2+}$. Almost 10% of total $^{45}Ca^{2+}$ uptake was released at FK506 concentrations up to 10 μM (K_m=0.47 μM), and 60% of total $^{45}Ca^{2+}$ uptake was released at FK506 concentrations over 10 μM (K_m=55 μM). Preincubation of the vesicles with cyclic ADP-ribose (cADPR, 0.5 μM) increased the FK506 (≤10 μM)-induced $^{45}Ca^{2+}$ release (Ozawa T, Biochim Biophys Acta 1693: 159-166, 2004). Preincubation with heparin (200 μg/ml) resulted in significant inhibition of the FK506 (30 μM)-induced $^{45}Ca^{2+}$ release. Subsequent addition of inositol 1,4,5-trisphosphate (IP_3, 5 μM) after FK506 (100 μM)-induced $^{45}Ca^{2+}$ release did not cause any release of $^{45}Ca^{2+}$. These results indicate that two types of FK506-induced Ca^{2+} release mechanism operate in the endoplasmic reticulum of rat pancreatic acinar cells: a high-affinity mechanism of Ca^{2+} release, which involves activation of the ryanodine receptor, and a low-affinity mechanism of Ca^{2+} release, which involves activation of the IP_3 receptor.

Introduction

Mobilization of Ca^{2+} from intracellular Ca^{2+} stores plays a crucial role in triggering enzyme secretion and muscle contraction. Inositol 1,4,5-trisphosphate (IP_3), which is produced after receptor stimulation by hormones or neurotransmitters, induces Ca^{2+} release from the IP_3-sensitive Ca^{2+} channel (IP_3 receptor: IP_3R) on the endoplasmic reticulum (ER). The features of the IP_3R in many tissues have been characterized (1,2). The channel protein has been purified (3,4) and cloned (5,6). In addition to the IP_3R, the ryanodine-sensitive Ca^{2+} channel (ryanodine receptor: RyR), which is activated by caffeine, ryanodine or Ca^{2+}, was first identified in excitable tissue. The channel protein has been purified (7,8) and cloned (9,10) in skeletal and cardiac muscles. The mechanism of ryanodine-sensitive Ca^{2+} release in nonexcitable tissue such as the liver and exocrine glands has recently been elucidated (11-15).

The immunosuppressant drug FK506 has been shown to increase (modulate) Ca^{2+} release through the RyR (16,17) or the IP_3R (18). In skeletal muscle sarcoplasmic reticulum (SR), the RyR is known to be tightly associated with a 12-kDa FK506-binding protein (FKBP12) (19,20). In association with the RyR, FKBP12 has been shown to stabilize the closed conformation of the Ca^{2+} release channel (16), and FK506 has been shown to promote dissociation of FKBP12 from the RyR complex (16). In cardiac muscle, FKBP12.6 has been found to be associated with RyR (21,22).

The IP_3R, a structurally related tetramer that has up to 40% sequence identity with RyR (5,6), is also associated with FKBP12 (18). The RyR-FKBP12 complex or the IP_3R-FKBP12 complex can associate with Ca^{2+}-activated phosphatase calcineurin (CN) (23,24). CN has been shown to dephosphorylate the protein kinase C (PKC) phosphorylation site on the IP_3R (23). When FKBP12 is dissociated from the IP_3R complex by FK506, binding of CN to the FK506-FKBP12 complex is stimulated (23). Thereby, the IP_3R is phosphorylated by PKC, and Ca^{2+} flux through the IP_3R is increased (23).

In rat pancreatic acinar cells, the presence of an IP_3-sensitive Ca^{2+} release mechanism (25,26) and the presence of a ryanodine (caffeine)-sensitive Ca^{2+} release mechanism (14,27) have been shown. Recently, it has also been shown that the presence of FK506 modulates Ca^{2+} release through a ryanodine-sensitive mechanism (28). However, details of the FK506-dependent mechanism, including the presence of FKBP, are unclear.

In the present study, the properties of microsomal Ca^{2+} release induced by the addition of FK506 were investigated in

Correspondence to: Dr Teruaka Ozawa, Department of Physiology, Tohoku University Graduate School of Medicine, Seiryo-machi 2-1, Aoba-ku, Sendai 980-8575, Japan
E-mail: teozawa@mail.tains.tohoku.ac.jp

Abbreviations: cADPR, cyclic ADP-ribose; CN, calcineurin; ER, endoplasmic reticulum; FKBP, FK506-binding protein; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; PKC, protein kinase C; RyR, ryanodine receptor; SR, sarcoplasmic reticulum

Key words: FK506, FK506-binding protein, IP_3 receptor, ryanodine receptor, Ca^{2+} release, pancreatic acinar cells

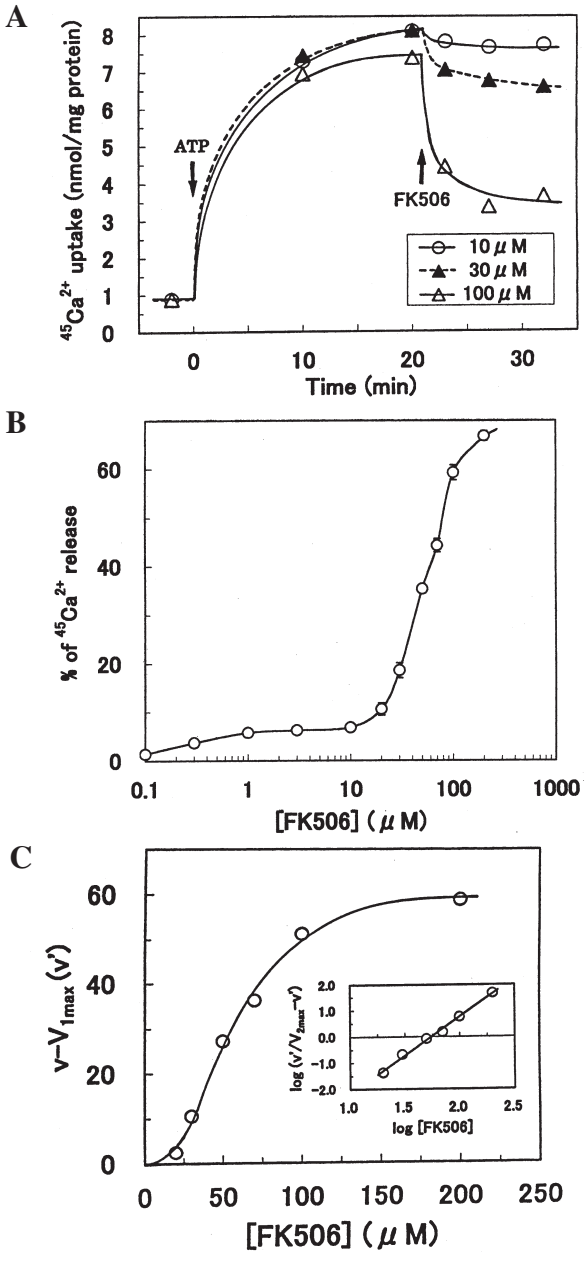

Figure 1. Effect of FK506 on $^{45}Ca^{2+}$ release from microsomal vesicles of rat pancreatic acinar cells. (A) Typical curves for FK506 (10 μM, ○; 30 μM, ▲; 100 μM, △)-induced $^{45}Ca^{2+}$ release from the vesicles. The vesicles (1 mg/ml) were preincubated in a KCl buffer containing $^{45}Ca^{2+}$ (1 μCi/ml) for 15 min. $^{45}Ca^{2+}$ uptake was initiated by the addition of 2 mM K_2ATP (at 0 min). FK506 was added to the vesicles as indicated. (B) Dose-response curve of FK506 (0.1-200 μM)-induced $^{45}Ca^{2+}$ release from the vesicles. The vertical line represents the ratio of released $^{45}Ca^{2+}$ over a 6-min period after the addition of FK506 to the $^{45}Ca^{2+}$ that had been taken up by ATP. Each point is the mean ± S.E. from 3 to 11 experiments of the type shown in (A). For values at concentrations up to 10 μM, the percentages reported in a previous study (28) were plotted. Note that the effect of FK506 on $^{45}Ca^{2+}$ release from the vesicles is biphasic. (C) Dose-response curve of FK506 (>10μM)-induced $^{45}Ca^{2+}$ release from the vesicles. The V_{max} value (V_{1max}: 8.1%) of the first phase was subtracted from the values at concentrations over 10 μM shown in (B), and the corrected values were plotted. Hill plot analysis (inset) yielded a Hill coefficient (n) of 3.0, an apparent K_m value of 55 μM and V_{2max} value of 60%.

rat pancreatic acinar cells. It was found that there are two different types of FK506-induced Ca^{2+} release mechanism in the ER of rat pancreatic acinar cells: one mechanism, which has a high sensitivity to FK506 (K_m=0.5 μM), involves activation of the ryanodine-sensitive Ca^{2+} channel and the other, which has a lower sensitivity to FK506 (K_m=55 μM), involves activation of the IP_3-sensitive Ca^{2+} channel.

Materials and methods

Materials. Creatine kinase and trypsin inhibitor were obtained from Boehringer Mannheim (Mannheim, Germany). Adenosine trisphosphate dipotassium salt (K_2ATP), creatine phosphate disodium salt, benzamidine, D-myo-inositol 1,4,5-trisphosphate (IP_3) and heparin were purchased from Sigma Chemical (St. Louis, MO, USA). FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Cyclic ADP-ribose (cADPR) was obtained from Wako Pure Chemical (Osaka, Japan). Ryanodine was obtained from Calbiochem (La Jolla, CA, USA) and $^{45}CaCl_2$ (14-19 C_i/g) was purchased from New England Nuclear (Boston, MA, USA).

Preparation of microsomal vesicles. Pancreatic microsomal vesicles were prepared as described previously (26,27,29). Briefly, isolated acinar cells from male Wistar rats (180-200 g) were homogenized in an ice-cold 'mannitol buffer' containing 290 mM mannitol, 10 mM KCl, 5 mM HEPES, 1 mM $MgCl_2$, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml of trypsin inhibitor, adjusted with Tris to pH 7.0. After centrifugation of the cell homogenate at 11,000 x g for 15 min, the 'fluffy layer' on top of the pellet, which is enriched by approximately twofold in the ER (26), was collected. The microsomal vesicles were kept frozen in liquid nitrogen until use. The protein concentration was measured by the method of Bradford (30) using bovine serum albumin as a standard.

Measurement of $^{45}Ca^{2+}$ uptake. Isolated membrane vesicles were preincubated for 15 min at a protein concentration of 1.0 mg/ml in 0.6 ml of an incubation buffer containing 155 mM KCl, 5 mM HEPES, 0.15 mM $CaCl_2$ (corresponding to 2 μM free Ca^{2+} concentration), 1.0 mM EDTA, 3.57 mM $MgCl_2$ (corresponding to 1.0 mM free Mg^{2+} concentration), 10 mM NaN_3, 5 μM oligomycin, 5 μg/ml antimycin A, 10 mM creatine phosphate, 8 U/ml creatine kinase, and 1 μC_i/ml of $^{45}CaCl_2$, adjusted with Tris/HCl to pH 7.0 at 25°C. $^{45}Ca^{2+}$ uptake into the vesicles was initiated by the addition of K_2ATP at a final concentration of 2 mM. After a steady state of $^{45}Ca^{2+}$ uptake had been reached, FK506 was added to the medium. FK506 was dissolved in DMSO and was added from the stock solution in a volume not exceeding 0.5% (vol/vol). At indicated times, $^{45}Ca^{2+}$ content of membrane vesicles was determined by a rapid filtration technique, as described previously (13). Radioactivity was determined by a liquid scintillation counter (LS6500, Beckman).

Values are presented as means ± S.E. Statistical analysis was performed using Student's t-test.

Results

A steady state of $^{45}Ca^{2+}$ uptake into pancreatic microsomal vesicles was reached ~20 min after the addition of ATP (Fig. 1A). The amount of $^{45}Ca^{2+}$ taken up into the microsomal

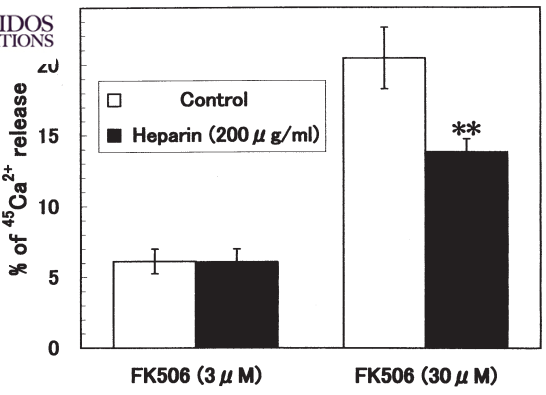

Figure 2. Effect of heparin on FK506-induced $^{45}Ca^{2+}$ release from pancreatic microsomal vesicles. The experimental procedures were the same as those described in the legend to Fig. 1A. Heparin was present from the beginning of the incubation. FK506 (3 or 30 μM)-induced $^{45}Ca^{2+}$ release in the absence (Control) and that in the presence of heparin (200 μg/ml) were compared. The vertical line represents the ratio of released $^{45}Ca^{2+}$ as described in the legend to Fig. 1B. Each value is the mean ± S.E. from 5 to 9 experiments. Note that heparin significantly inhibited the $^{45}Ca^{2+}$ release induced by 30 μM of FK506 but not that induced by 3 μM of FK506. ** indicate the level of significant difference compared to the control without heparin (P<0.01).

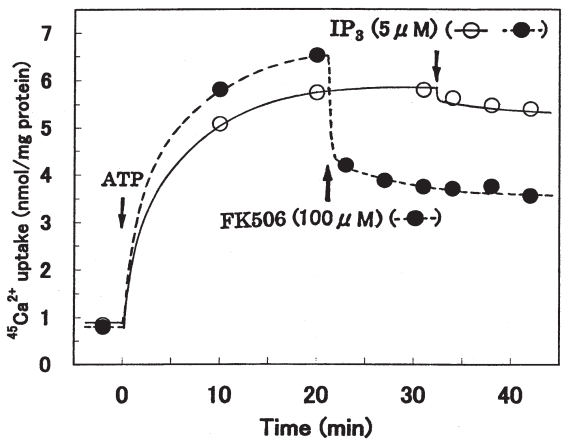

Figure 3. Effect of subsequent addition of IP_3 (5 μM) after FK506 (100 μM)-induced $^{45}Ca^{2+}$ release from pancreatic microsomes. The experimental procedures were the same as those described in the legend to Fig. 1A. FK506 (100 μM) was added to the medium 21 min after the addition of ATP (●). IP_3 (5 μM) was added where indicated in the absence (○) and presence of FK506 (●). Note that subsequent addition of IP_3 after the FK506 (100 μM)-induced $^{45}Ca^{2+}$ release did not cause any release of $^{45}Ca^{2+}$. Curves are representative of similar experiments.

vesicles over a period of 20 min after the addition of ATP was 5.3±0.2 nmol/mg protein (n=94). The addition of FK506 (0.1-200 μM) to the vesicles at a steady state of ATP-dependent $^{45}Ca^{2+}$ uptake caused a dose-dependent release of $^{45}Ca^{2+}$ (Fig. 1A). The ratio of $^{45}Ca^{2+}$ released after the addition of FK506 to the $^{45}Ca^{2+}$ that had been taken up by an ATP-dependent mechanism showed a biphasic change (Fig. 1B). Approximately 10% of the $^{45}Ca^{2+}$ that had been taken up by ATP was released in the first phase at FK506 concentrations of less than 10 μM (Fig. 1B). Lineweaver-Burk plot evaluation of the first phase showed a K_m value of 0.47 μM and V_{max} value of 8.1% (28). Approximately 60% of the $^{45}Ca^{2+}$ that had been taken up by ATP was further released in the second phase at FK506 concentrations of more than 10 μM (Fig. 1B). To estimate the real percentage of the second phase, the maximal value (8.1%) of the first phase was subtracted from the percentage at concentrations over 10 μM shown in Fig. 1B. The corrected values are plotted in Fig. 1C. Hill plot evaluation of the second phase (Fig. 1C, inset) showed a Hill coefficient (n) of 3.0, a K_m value of 55 μM and V_{max} value of 60%.

To determine the involvement of the RyR in the FK506-induced $^{45}Ca^{2+}$ release from the vesicles, the effect of cADPR, which is known to modulate the ryanodine-sensitive Ca^{2+} release mechanism (13,28,31), on the FK506-induced $^{45}Ca^{2+}$ release was investigated. Preincubation of the vesicles with cADPR (0.5 μM) shifted the dose-response curve of the FK506 (≤10μM)-induced $^{45}Ca^{2+}$ release upward (28). It was shown from Lineweaver-Burk plot evaluation of the release that the presence of cADPR (0.5 μM) increased the V_{max} value from 8.1% to 14.4% without affecting the K_m value (28). The extent of stimulation of FK506 (30 μM)-induced $^{45}Ca^{2+}$ release by cADPR (0.5 μM) was nearly the same as that of the V_{max} value of the first phase by cADPR (0.5 μM) [ratio of released $^{45}Ca^{2+}$ over a 6-min period after the addition of FK506 (30 μM) to the $^{45}Ca^{2+}$ that had been taken up: 18.3±1.6% (n=5) without cADPR vs. 23.2±2.1% (n=6) with cADPR (0.5 μM)]. These data indicate that a high-affinity mechanism of the FK506-induced Ca^{2+} release activates the RyR but that a low-affinity mechanism does not.

To determine the involvement of the IP_3R in the FK506-induced $^{45}Ca^{2+}$ release from the vesicles, the effect of heparin, an inhibitor of the IP_3R (2,3,32), on the release was investigated. Preincubation of the vesicles with heparin (200 μg/ml) resulted in significant (P<0.01) inhibition of $^{45}Ca^{2+}$ release induced by 30 μM of FK506 but not that induced by 3 μM of FK506 (Fig. 2). This result indicates that a low-affinity mechanism of the FK506-induced Ca^{2+} release activates the IP_3R but that a high-affinity mechanism of that does not.

To determine whether a high concentration of FK506 induces Ca^{2+} release from the IP_3-sensitive Ca^{2+} pool, IP_3 (5 μM) was added to the vesicles after 100 μM of FK506 released $^{45}Ca^{2+}$ from the vesicles. Fig. 3 shows that the addition of IP_3 after FK506 (100 μM)-induced $^{45}Ca^{2+}$ release did not cause any release of $^{45}Ca^{2+}$. This result indicates that a high concentration of FK506 emptied the IP_3-sensitive Ca^{2+} pool.

Discussion

In the present study, according to the different sensitivities for FK506, it was demonstrated that two different types of FK506-induced Ca^{2+} release mechanism operate in the ER of rat pancreatic acinar cells; i.e, FK506 can activate the RyR at concentrations up to 10 μM (K_m=0.5 μM) and can activate the IP_3R at concentrations over 10 μM (K_m=55 μM). It is unlikely that a low concentration (≤10 μM) of FK506 acts on the IP_3R since the component of the first phase of FK506-induced $^{45}Ca^{2+}$ release was not inhibited by heparin (Fig. 2). It is also unlikely that a high concentration (>10 μM) of FK506 acts on the RyR since the extent of stimulation of 30 μM FK506-induced $^{45}Ca^{2+}$ release by cADPR (0.5 μM) was nearly the same as that of

$^{45}Ca^{2+}$ release induced by FK506 at concentrations of less than 10 μM (see Results).

FK506 is known to modulate the RyR by dissociating FKBP from the receptor (16,17). It has been shown that the presence of FK506 decreases the threshold of caffeine or ryanodine required for Ca^{2+} release in skeletal muscle cells (16) and pancreatic acinar cells (28). cADPR dissociates FKBP12.6 from the RyR in pancreatic islets (33) and coronary arterial smooth muscle cells (34). In a previous study, it was shown that the presence of cADPR decreased the threshold of caffeine or ryanodine required for Ca^{2+} release in rat pancreatic acinar cells by the same extent as that in the case of FK506 (28). This finding suggests that cADPR modulates the RyR in rat pancreatic acinar cells by the same mechanism as that by which FK506 modulates the RyR.

The FK506 ($\leq$10 μM)-induced $^{45}Ca^{2+}$ release from pancreatic microsomal vesicles was stimulated by the presence of cADPR (see Results). If FKBP is removed from the RyR by cADPR before the addition of FK506, it is unlikely that the FK506-induced $^{45}Ca^{2+}$ release following pretreatment of the vesicles with cADPR is due to the dissociation of FKBP. FK506 is a compound with a macrocyclic lactone ring structure. It has been shown that rapamycin and ivermectin, macrocyclic lactone derivatives, increased the open probability of FKBP12-stripped RyRs in skeletal muscle (35,36). This finding suggests that the compounds activate the RyR by a mechanism other than dissociation of FKBP. The first phase of the FK506-induced $^{45}Ca^{2+}$ release from the vesicles shown in Fig. 1 may be explained by a direct activation of the RyR by FK506.

In the rat cerebellum, FK506 dissociates FKBP12 from the IP_3R and decreases the threshold of IP_3 required for Ca^{2+} release (18). The EC_{50} value for the dissociation of FKBP12 from the IP_3R complex has been shown to be 10-100 nM FK506 (23). This range is even lower than the K_m value (55 μM) for the second phase of the FK506-induced $^{45}Ca^{2+}$ release from pancreatic microsomal vesicles. The second phase of the FK506-induced $^{45}Ca^{2+}$ release may be due to a direct activation of the IP_3R, since heparin inhibited the FK506 (30 μM)-induced $^{45}Ca^{2+}$ release from the vesicles (Fig. 2) and the IP_3-sensitive Ca^{2+} pool of the vesicles was emptied by the addition of FK506 (100 μM) (Fig. 3). The finding that the second phase of the FK506-induced $^{45}Ca^{2+}$ release from the vesicles represents a sigmoid curve (Fig. 1C) suggests allosteric changes in the IP_3R by the binding of FK506. There is a possibility that the allosteric effect induces greater release from the receptor by FK506 [the maximum value (60%) of the second phase of the FK506-induced $^{45}Ca^{2+}$ release vs. the maximum percentage (~25%) of the IP_3-induced $^{45}Ca^{2+}$ release (37)].

Further studies using an FKBP antibody are needed to clarify whether the Ca^{2+} release from the ER induced by FK506 in rat pancreatic acinar cells is due to mechanisms without dissociation of the FKBP.

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