Cyclic ADP-ribose Binds to FK506-binding Protein 12.6 to Release Ca$^{2+}$ from Islet Microsomes*

(Received for publication, November 11, 1996, and in revised form, December 6, 1996)

Naoya Noguchi, Shin Takasawa, Koji Nata, Akira Tohgo, Ichiro Kato, Fumiko Ikehata, Hitode Yonekura, and Hiroshi Okamoto

From the Department of Biochemistry, Tohoku University School of Medicine, Sendai 980-77, Miyagi, Japan

Cyclic ADP-ribose (cADPR) is a second messenger for Ca$^{2+}$ mobilization via the ryanodine receptor (RyR) from islet microsomes for insulin secretion (Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993) Science 259, 370–373). In the present study, FK506, an immunosuppressant that prolongs allograft survival, as well as cADPR were found to induce the release of Ca$^{2+}$ from islet microsomes. After islet microsomes were treated with FK506, the Ca$^{2+}$ release by cADPR from microsomes was reduced. cADPR as well as FK506 bound to FK506-binding protein 12.6 (FKBP12.6), which we also found occurs naturally in islet microsomes. When islet microsomes were treated with cADPR, FKBP12.6 dissociated from the microsomes and moved to the supernatant, releasing Ca$^{2+}$ from the intracellular stores. The microsomes that were then devoid of FKBP12.6 did not show Ca$^{2+}$ release by cADPR. These results strongly suggest that cADPR may be the ligand for FKBP12.6 in islet RyR and that the binding of cADPR to FKBP12.6 frees the RyR from FKBP12.6, causing it to release Ca$^{2+}$.

Glucose is the primary stimulus of insulin secretion and synthesis in the pancreatic islets of Langerhans (1–3). Cyclic ADP-ribose (cADPR)$^{\dagger}$ is generated in pancreatic islets by glucose stimulation, serving as a second messenger for Ca$^{2+}$ mobilization in the endoplasmic reticulum to secrete insulin (4–6). cADPR activates the ryanodine receptor (RyR) of a variety of cells to release Ca$^{2+}$ from the intracellular stores (4–6, 16).

* This work was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture, Japan and the Kanoe Foundation of Research for New Medicine. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) D86641 and D86642.

‡ Present address: Dept. of Biochemistry, Kanazawa University School of Medicine, Kanazawa 920, Japan.

§ To whom correspondence should be addressed: Dept. of Biochemistry, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-77, Miyagi, Japan. Tel.: 81-22-717-8079; Fax: 81-22-717-8053.

The abbreviations used are: cADPR, cyclic ADP-ribose; RyR, ryanodine receptor; FKBP12, FK506-binding protein 12; FKBP12.6, FK506-binding protein 12.6; MBP, maltose binding protein; RT, reverse transcription; PCR, polymerase chain reaction.

RyRs have been purified from both skeletal and cardiac muscle (17, 18), and FK506-binding protein 12 (FKBP12) and FK506-binding protein 12.6 (FKBP12.6) were copurified with type 1 RyR from striated muscle and with type 2 RyR from cardiac muscle, respectively (19, 20). FKBP12 and FKBP12.6 were shown to bind selectively to type 1 and type 2 RyR, respectively (21). It was reported that the type 1 RyR was activated by dissociation of FKBP12 from the RyR by the addition of FK506 to release Ca$^{2+}$ (22).

In the present study, we show that cADPR binds to FKBP12.6, that FKBP12.6 is present in islet microsomes, and that when cADPR was added to islet microsomes, FKBP12.6 was dissociated from the microsomes to release Ca$^{2+}$.

EXPERIMENTAL PROCEDURES

Materials—Calmodulin was purchased from Calbiochem. Fluo 3 was obtained from Molecular Probes. 17-Alyl-1,14-dihydroxy-12-(2-[4-hydroxy-3-methoxy-cyclohexoxyl]-1-methylvinyl)-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo-[22.3.1.0$^{4,9}$]octacos-18-en-2-3,10,16-tetronone (FK506) was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). A sheep anti-mouse antibody labeled with horseradish peroxidase and ECL reagents were from Amersham Corp., a goat anti-rabbit antibody labeled with horseradish peroxidase was from Zymed (San Francisco, CA), Immobilon-P was from Millipore (Bedford, MA), pMALc2 vector and amylene resin were from New England Biolabs (Beverly, MA), [proyl-$^{3}$H]-dihydro-FK506 and [3H]NAD$^{+}$ were from DuPont NEN, NAD$^{+}$ was from Boeringer, ATP and AR-ribose were from Sigma, nicotinamide was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and v-myo-inositol 1,4,5-trisphosphate and ryanodine were from Biomol Research (Tatick, MA). A monoclonal antibody against human FKBP12 (clone 3F4-70) was kindly provided by Kazuyuki Otaka and Dr. Masakazu Kobayashi (Fujisawa Pharmaceutical Co., Ltd.), and an anti-FKBP12.6 anti-serum, which also reacts with rat FKBP12, was kindly provided by Go Ichien (Eisai Co., Ltd., Tokyo).

Calcium Release Assay—Microsomes were prepared as described previously (4, 6). In brief, 2,000 islets from Wistar male rats (240–280 g) were homogenized with a Pellet mixer (Tefco Electromix, Switzer-land) in 0.2 ml of acetate intracellular medium composed of 250 mM potassium acetate, 250 mM N-methylglucamine, 1 mM MgCl$_2$, and 20 mM Hepes (pH 7.2) supplemented with 0.5 mM ATP, 4 mM phosphocreatine, creatine phosphokinase (2 units/ml), 2.5 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride. After the homogenates had been centrifuged for 45 s at 13,000 × g, the microsomes were prepared by Percoll density gradient centrifugation at 20,000 × g and spin at 10 °C. Release of Ca$^{2+}$ was monitored in 0.6 ml of intracellular medium composed of 250 mM potassium glutamate, 250 mM N-methylglycine, 1 mM MgCl$_2$, and 20 mM Hepes (pH 7.2) supplemented with 1 mM ATP, 4 mM phosphocreatine, creatine phosphokinase (2 units/ml), 2.5 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, 7 μM bovine brain calmodulin (6), and 3 μM Fluo 3 with the addition of 20 μl of the islet microsome fraction (10 μg of protein) (4, 6). Fluo 3 (4, 6) was added into the incubation, and Fluo 3 fluorescence was measured at 490 nm excitation and 535 nm emission with a JASCO CAF-110 intracellular ion analyzer (Tokyo, Japan) at 37 °C (6). Total accumulated Ca$^{2+}$ in islet microsomes was estimated by the increase of Fluo 3 fluorescence caused by the addition of 200 mM inomycin (Sigma) to the Ca$^{2+}$ release medium containing the microsomes, and the ambient free Ca$^{2+}$ concentration ([Ca$^{2+}$]) was calculated using the following equation, as described previously (6): [Ca$^{2+}$] = [K$_0$] + [F - F$_{min}$/F$_{max}$ - F], where K$_0$ = 400 nM. In response to 100 nM cADPR, islet microsomes exhibited 18 ± 2 nM Ca$^{2+}$ release/mg protein, which corresponded to 31 ± 4% of the total accumulated Ca$^{2+}$.

DNA Cloning—1 μl of rat islet cDNA library (2 × 10$^{6}$ plaque-forming unit) (23, 24) was used as a template for PCR (23–25). The sequences of sense and antisense primers were 5’-GGATTCCTGCGTCTTTTCCCTCCTCCTCTCCTCCT-3’ and 5’-GGATTCTTGGATTGATTTAGCATATAGTTT-3’ for the isolation of rat FKBP12 cDNA, which corresponded to nucleotide sequences 48–68 and 636–658 of mouse FKBP12 mRNA.
measurement was a peak value estimated from Fluo3 fluorescence (6).

The monoclonal antibody was diluted at 0.2 μg/ml, and the antiserum (anti-FKBP12.6) was incubated with the indicated concentrations of FK506 and then challenged with the addition of 3 μl of 20.1 μM cADPR to the medium (the final cADPR concentration was 100 nM).

Fig. 1. Effects of FK506 on Ca2+ release from islet microsomes. A, Ca2+ release by FK506 from islet microsomes. Ca2+ release was induced by FK506 at the indicated concentrations. The Ca2+ release measurement was a peak value estimated from Fluo3 fluorescence (6). B, Ca2+ release by cADPR from islet microsomes after the FK506-induced Ca2+ release. Islet microsomes were incubated in 0.6 ml of Ca2+ release medium for 10 min with the indicated concentrations of FK506 and then challenged with the addition of 3 μl of 20.1 μM cADPR to the medium (the final cADPR concentration was 100 nM).

RESULTS AND DISCUSSION

We have previously shown that islet microsomes release Ca2+ in response to cADPR (4). In the present study, we found that FK506, one of the most widely used immunosuppressive agents, induced the release of Ca2+ from islet microsomes. The dose-response curve of FK506 on the Ca2+ release from islet microsomes was concentration-dependent, with half-maximal release occurring at 2 μM, and a maximal Cu2+ release occurring at 10 μM (Fig. 1A). In addition, as shown in Fig. 1B, after the islet microsomes were treated with FK506, the Ca2+ release by ADP-ribose, nicotinamide, d-xylo-inositol 1,4,5-trisphosphate, and ryanodine as competitors.

Fig. 2. Amino acid sequence of rat FKBP12/12.6, the mRNA expression in rat tissues and existence of FKBP12.6 in islet microsomes. A, deduced amino acid sequence of rat FKBP12 and alignment with human (31, 32), mouse (26), bovine (33), and rabbit (19) FKBP12 and rat, human, (27, 28), and bovine (33) FKBP12.6. Identical residues are indicated by dots. B, RT-PCR detection of rat FKBP12 and FKBP12.6 mRNAs. Lane 1, liver; lane 2, spleen; lane 3, thymus; lane 4, alveolar macrophage; lane 5, islets; lane 6, streptozotocin-nicotinamide-induced insulinomas; lane 7, RIN-5F cells; lane 8, cerebellum; lane 9, cerebrum; lane 10, heart; lane 11, salivary gland; lane 12, skeletal muscle; lane 13, kidney. C, immunoblot analysis of FKBP12. Lane 1, 50 ng of FKBP12; lane 2, 10 ng of FKBP12; lane 3, 50 ng of FKBP12.6; lane 4, islet microsomes (100 μg of protein); lane 5, cerebellar microsomes (100 μg of protein); lane 6, islet microsomes (50 μg of protein). G3PDH, glyceraldehyde-3-phosphate dehydrogenase.

**Table 1.** Amino acid sequence of rat FKBP12/12.6, the mRNA expression in rat tissues and existence of FKBP12.6 in islet microsomes.

| Tissue       | FKBP12 | FKBP12.6 |
|--------------|--------|----------|
| Liver        | 50 ng  | 100 μg   |
| Spleen       | 10 ng  | 50 μg    |
| Thymus       | 50 ng  | 50 μg    |
| Alveolar Macrophage | 10 ng  | 10 μg    |
| Islets       | 100 μg | 100 μg   |
| Streptozotocin-Nicotinamide-Induced Insulinomas | 50 μg | 50 μg |
| RIN-5F Cells | 100 μg | 100 μg   |
| Cerebellum   | 50 μg  | 50 μg    |
| Cerebrum     | 100 μg | 100 μg   |
| Heart        | 50 μg  | 50 μg    |
| Salivary Gland | 100 μg | 100 μg   |
| Skeletal Muscle | 100 μg | 100 μg   |
| Kidney       | 50 μg  | 50 μg    |

ADP-ribose, nicotinamide, d-xylo-inositol 1,4,5-trisphosphate, and ryanodine as competitors.
cADPR from the microsomes was reduced depending on the concentration of FK506; the maximal reduction was seen at 5–25 μM FK506. Because cADPR and FK506 appear to induce the release of Ca\(^{2+}\) by a common mediator, we next tried to determine if this occurs by a targeting of the same ligand. The cellular target for FK506 is thought to be FKBP12 and FKBP12.6. Therefore, we isolated FKBP12 and FKBP12.6 cDNAs from a rat islet cDNA library. As shown in Fig. 2A, rat FKBP12 is composed of 108 amino acids and highly conserved with human (31, 32), mouse (26), bovine (33), and rabbit (19) FKBP12. Rat FKBP12.6 is also a 108-amino acid protein and completely conserved with human (27, 28) and bovine (33), FKBP12 is composed of 108 amino acids and highly conserved with human (31, 32), mouse (26), bovine (33), and rabbit (19) FKBP12. Rat FKBP12.6 is also a 108-amino acid protein and completely conserved with human (27, 28) and bovine (33). RT-PCR analyses revealed that FKBP12 and FKBP12.6 mRNAs were ubiquitously expressed in rat tissues with pancreatic islets and streptozotocin/nicotinamide-induced insulinomas (40, 41); a second messenger for Ca\(^{2+}\) mobilization in glucose-induced insulin secretion in islets (4, 5), is expressed in islets and streptozotocin/nicotinamide-induced insulinomas but not in RINm5F cells (23, 24). We then isolated microsomes from rat islets and carried out immunoblot analyses. As shown in Fig. 2 (C and D), although islet microsomes did not contain FKBP12 (Fig. 2C, lane 4), the microsomes contained FKBP12.6 (Fig. 2D, lanes 5 and 6), suggesting that FKBP12.6 is the target for FK506 and/or cADPR to release Ca\(^{2+}\) from islet microsomes.

Next, we examined the binding of FKBP12.6 to cADPR. The recombinant rat FKBP12.6 bound to FK506 at a \(K_d\) value of 32 nM. As shown in Fig. 3A, cADPR was found to bind to FKBP12.6 at a \(K_d\) value of 35 nM. The cADPR binding was inhibited by FK506 and neither structurally nor functionally related analogues of cADPR inhibited the cADPR binding to FKBP12.6 (Fig. 3B). These results indicate that FKBP12.6 acts as a cADPR-binding protein and strongly suggest that cADPR is the actual ligand for FKBP12.6 because FK506 does not normally exist in mammalian cells.

It was reported that FKBP12.6 bound to RyR tightly and that by the addition of FK506, FKBP12.6 was dissociated from RyR to form FK506-FKBP12.6 complexes (19, 22, 33).
In addition, the open probability of the type 1 RyR Ca$^{2+}$ channel was greatly increased when FKBP12 was released from the RyR by the addition of FK506 (22). As shown in Fig. 4, after treatment of islet microsomes with cADPR, FKBP12.6 was not detected in the microsomes but was recovered in the supernatant (Fig. 4A), and Ca$^{2+}$ release from the microsome treated by cADPR or FK506 was reduced. FK506 as well as cADPR then had almost no effect in releasing Ca$^{2+}$ from the 1 μM cADPR-pretreated microsomes (Fig. 4B). Our recent experiment indicated that type 2 RyR is expressed in rat islets. From these results, it is strongly suggested that when cADPR binds to FKBP12.6 in islet microsome RyR and causes the dissociation of FKBP12.6 from the endoplasm reticulum. As described previously (6), the RyR can also be activated by Ca$^{2+}$/calmodulin-dependent protein kinase II. The interaction between the dissociation of FKBP12.6 from RyR and the phosphorylation of RyR by Ca$^{2+}$/calmodulin-dependent protein kinase II remains to be elucidated.

Acknowledgments—We are grateful to Kazuyuki Otsuka and Masakazu Kohayashi (Fujisawa Pharmaceutical Co., Ltd.) for providing the antibody against FKBP12, Hideo Kumagai for technical assistance, and Brent Bell for valuable assistance in preparing the manuscript for publication.

REFERENCES

1. Hedeskov, C. J. (1980) Physiol. Res. 69, 442–509
2. Itoh, N., and Okamoto, H. (1980) Nature 283, 100–102
3. Okamoto, H. (1981) Mol. Cell. Biochem. 37, 43–61
4. Takasawa, S., Nata, K., Yonekura, H., and Okamoto, H. (1993) Science 259, 370–373
5. Okamoto, H., Takasawa, S., and Tohgo, A. (1995) Biochimie 77, 356–363
6. Takasawa, S., Ishida, A., Nata, K., Nakagawa, K., Noguchi, N., Tohgo, A., Kato, I., Yonekura, H., Fujisawa, H., and Okamoto, H. (1995) J. Biol. Chem. 270, 3257–3259
7. Galione, A., Lee, H. C., and Busa, W. B. (1991) Science 253, 1143–1146
8. Koshiyama, H., Lee, H. C., and Tashjian, A. H., Jr. (1991) J. Biol. Chem. 266, 16985–16988
9. Meszaros, L. G., Bak, J., and Chu, A. (1993) Nature 364, 76–79
10. Hua, S.-Y., Tokimasa, T., Takasawa, S., Furuya, Y., Nohmi, M., Okamoto, H., and Kuba, K. (1994) Neuron 12, 1073–1079
11. Thorn, P., Gerasimenko, O., and Petersen, O. H. (1994) EMBO J. 13, 2038–2045

2 K. Nata, M. Kuroki, T. Kumagai, S. Takasawa, and H. Okamoto, manuscript in preparation.