

*Full Paper***Cyclic ADP-Ribose Requires FK506-Binding Protein to Regulate Intracellular Ca^{2+} Dynamics and Catecholamine Release in Acetylcholine-Stimulated Bovine Adrenal Chromaffin Cells**Katsuya Morita¹, Tomoya Kitayama¹, Shigeo Kitayama², and Toshihiro Dohi^{1,*}¹Department of Dental Pharmacology, Division of Integrated Medical Science, Hiroshima University Graduate School of Biomedical Sciences, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8553, Japan²Department of Dental Pharmacology, Field of Functional Physiology, Branch of Biophysiological Science, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 2-5-1 Shikada, Okayama 700-8525, Japan

Received December 6, 2005; Accepted March 6, 2006

Abstract. The present study was undertaken to elucidate whether cyclic ADP-ribose (cADPR) mediates the amplification of Ca^{2+} signaling and catecholamine release via the involvement of FK506-binding proteins (FKBPs)/ryanodine receptor (RyR) in bovine adrenal chromaffin cells. cADPR induced Ca^{2+} release in digitonin-permeabilized chromaffin cells and this was blocked by FK506 and rapamycin, ligands for FKBPs; 8Br-cADPR, a competitive antagonist for cADPR; and antibody for FKBP12/12.6, while it was enhanced by cyclosporin A. Ryanodine-induced Ca^{2+} release was not affected by 8Br-cADPR and was remarkably enhanced by FK506, rapamycin, cyclosporin A, and cADPR. FK506 binds to FKBP12.6 and removes it from RyRs, but cADPR did not affect the binding between FKBP12.6 and RyR. In intact chromaffin cells, 8Br-cADPR, FK506, and rapamycin, but not cyclosporin A attenuated the sustained intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) rise induced by acetylcholine (ACh). 8Br-cADPR, FK506, and SK&F 96365 reduced the Mn^{2+} entry stimulated with ACh only when Ca^{2+} was present in the extracellular medium. 8Br-cADPR, FK506, and rapamycin concentration-dependently inhibited the ACh-induced catecholamine (CA) release. Here, we present evidence that FKBP12.6 associated with RyR may be required for Ca^{2+} release induced by cADPR in bovine adrenal chromaffin cells. cADPR-mediated Ca^{2+} release from endoplasmic reticulum in ACh-stimulated chromaffin cells is coupled with Ca^{2+} influx through the plasma membrane which is essential for ACh-stimulated CA release.

Keywords: cyclic ADP-ribose, FK506-binding protein, cytosolic Ca^{2+} dynamics, ryanodine receptor, catecholamine release

Introduction

Although the rise of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is largely dependent on the influx of Ca^{2+} from the extracellular space through nicotinic acetylcholine (ACh) receptor channels and voltage-operated Ca^{2+} channels (VOCC) in bovine adrenal chromaffin cells, the increased Ca^{2+} further stimulates

Ca^{2+} release from intracellular Ca^{2+} stores that are sensitive to ryanodine, called Ca^{2+} -induced Ca^{2+} release (CICR) (1, 2). Cyclic ADP-ribose (cADPR), identified as a novel Ca^{2+} -mobilizing agent, has been shown to activate the cardiac but not the skeletal isoforms of the ryanodine receptor (RyR) channel and proposed it as a candidate for CICR. The presence of cADPR and the enzyme-catalyzing conversion of $\beta\text{-NAD}^+$ into cADPR and the ability of cADPR to release Ca^{2+} through an inositol 1,4,5-trisphosphate (IP_3)-insensitive mechanism have been shown in many tissues and may be a candidate for nonskeletal type RyR endogenous messenger (3, 4).

*Corresponding author. todohi@hiroshima-u.ac.jp

Published online in J-STAGE: April 28, 2006

DOI: 10.1254/jphs.FP0050991

We have previously shown that ACh induced the activation of ADP-ribosyl cyclase, an enzyme-catalyzing conversion of $\beta\text{-NAD}^+$ into cADPR, through Ca^{2+} influx and a cyclic AMP-mediated mechanism, and cADPR-mediated activation of CICR pathway contributes to time-dependent rise of $[\text{Ca}^{2+}]_i$ and to the maximal exocytotic response in chromaffin cells, suggesting cADPR as a second messenger of CICR in the cells (5).

It is currently unknown whether cADPR mediates Ca^{2+} release by direct association with RyR in a manner analogous to IP_3 with its receptor or indirectly, by binding a RyR-associated accessory protein. The function of these intracellular Ca^{2+} -release channels is regulated by different cellular factors. One of these regulators is the group of the FK506-binding proteins (FKBP) belonging to the immunophilin family (6). FKBP12 and its analog, FKBP12.6, are tightly associated with the skeletal RyR1 and cardiac RyR2, respectively (7–9). The interaction between FKBP and RyR1 or RyR2 is well documented and is essential for stabilization, activation, and proper functioning of these channels.

Recent studies suggested that cADPR binds to its specific binding proteins, FKBP12 and FKBP12.6 (9–13). Indeed, a study in pancreatic islets (10) has suggested that cADPR may activate the RyR by relieving the inhibition mediated by FKBP12.6. However, some studies suggest that cADPR directly binds to RyRs to activate them (3, 14–17). Although adrenal chromaffin cells mainly express RyR2, the precise site of action of cADPR in the cells is not known.

The present study was undertaken to elucidate whether cADPR mediates the amplification of Ca^{2+} signaling via stimulation of Ca^{2+} release from endoplasmic reticulum (ER) and Ca^{2+} influx through plasma membrane and whether FKBP are involved in this process in bovine adrenal chromaffin cells.

Materials and Methods

Materials

The following reagents were obtained as indicated: cADPR, cyclosporin A, digitonin, rapamycin, N,N,N',N' -tetramethylethylenediamine (TEMED), and thapsigargin were from Wako Pure Chemicals Industries (Osaka); 8Br-cADPR was from Sigma (St. Louis, MO, USA); SK&F 96365 was from BIOMOL Research Laboratories (Plymouth Meeting, PA, USA); ryanodine was from Calbiochem (La Jolla, CA, USA); fura-2 and fura-2 acetoxymethyl ester were from Dojindo Laboratories (Kumamoto); FK506 was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka); all other chemicals were reagent grade. Thapsigargin was dissolved in

dimethyl sulfoxide to make a stock solution. Cyclosporin A, FK506, rapamycin, and ryanodine were dissolved in ethanol to make stock solutions. Dimethyl sulfoxide (0.1%) or ethanol (0.1%) at the highest final concentrations used had no effects on $[\text{Ca}^{2+}]_i$, Mn^{2+} entry, and catecholamine (CA) release (data not shown). The antibodies used for the present study were obtained from the following companies: mouse monoclonal anti-RyR antibody was from Affinity Bioreagents (Golden, CO, USA); goat polyclonal anti-FKBP12 (C-terminal) antibody was from Sant Cruz Biotechnology (Santa Cruz, CA, USA). Protein A-Sepharose was obtained from Pharmacia (Uppsala, Sweden).

Cell preparation

Chromaffin cells of bovine adrenal glands were isolated enzymatically as described previously (18). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin G (100 U/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), ascorbate (0.1 mM), and HEPES (5 mM) at 37°C in a humidified incubator under 5% CO_2 /95% air atmosphere in suspension culture for 24–72 h for measurement of $[\text{Ca}^{2+}]_i$ or in a 35-mm tissue culture dish (10^6 cells/dish) for 3–6 days for the CA release assay. Cells were washed and suspended in one of the following media before use: normal medium contained; 150 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1.3 mM CaCl_2 , 5 mM glucose, 10 mM HEPES-Tris buffer, and 0.5% bovine serum albumin (BSA), pH 7.4; Ca^{2+} -deficient medium contained 150 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 5 mM glucose, 0.1 mM EGTA, 10 mM HEPES-Tris buffer, and 0.5% BSA, pH 7.4; Ca^{2+} -sucrose medium contained 1.3 mM CaCl_2 , 5 mM glucose, 340 mM sucrose, 10 mM HEPES-Tris buffer, and 0.5% BSA, pH 7.4.

Measurement of Ca^{2+} release

For measurement of Ca^{2+} release from digitonin-permeabilized chromaffin cells, cells were washed and suspended in potassium glutamate buffer (145 mM potassium glutamate, 20 mM PIPES, 1 mM EGTA, pH 6.6) containing an ATP generating system (2 mM Mg^{2+} -ATP, 5 mM creatine phosphate, 40 units/ml creatine phosphokinase) and protease inhibitors (2.5 mM benzamide, 0.5 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 50 $\mu\text{g}/\text{ml}$ trypsin inhibitor) and then the cells were permeabilized by incubating them with digitonin (20 μM) for 5 min at 25°C . The cells were washed and resuspended (1×10^7 cells/ml) in an intracellular medium (KH medium: 140 mM KCl, 10 mM NaCl, 30 mM HEPES, pH 7.0) containing the ATP generating system, protease

inhibitors, mitochondrial inhibitors (10 $\mu\text{g}/\text{ml}$ antimycin A, 10 $\mu\text{g}/\text{ml}$ oligomycin, and 10 mM NaN_3), and 0.025% BSA. The permeabilization was checked by measuring the leakage of lactate dehydrogenase. One milliliter of cell suspension was transferred to a fluorescence cuvette and supplemented with fura-2 (1 μM). Fluorescence was continuously monitored using a fluorometer at an excitation of 340/380 nm and an emission of 510 nm. At the end of each run, sequential additions of 1 nmol of CaCl_2 were used to calibrate the fluorescence signal internally.

Estimation of $[\text{Ca}^{2+}]_i$

$[\text{Ca}^{2+}]_i$ was estimated by the use of the calcium-sensitive dye fura-2, as described previously (5). Briefly, the cells were incubated at 32°C with 1 μM fura-2 acetoxymethyl ester for 30 min for the loading of the dye. Cells were then centrifuged at $10 \times g$ for 10 min and re-suspended to yield 3×10^6 cells. Cells were washed with normal medium, Ca^{2+} -deficient medium with rapid centrifugation, and then re-suspended in the medium immediately before use. Fluorescence was measured with a dual-wavelength fluorescence spectrophotometer with excitation at 340 and 380 nm, and emission at 510 nm. $[\text{Ca}^{2+}]_i$ was calculated from the fluorescence ratio at 340 and 380 nm using the equation of Grynkiewicz et al. (19) and a value of 224 nM for the K_d of fura-2. To monitor the Ca^{2+} entry, some of the experiments were carried out according to the Ca^{2+} -free/ Ca^{2+} reintroduction protocol (20). To this end, fura-2 loaded chromaffin cells were washed with Ca^{2+} -deficient medium with rapid centrifugation before use. After stimulants were added, the first $[\text{Ca}^{2+}]_i$ peak was recorded, and then Ca^{2+} was reintroduced into the medium and the ensuing second $[\text{Ca}^{2+}]_i$ peak was recorded.

Measurement of Mn^{2+} -entry

Divalent cation entry was monitored with the fura-2 Mn^{2+} -quenching technique. Fura-2 has a higher affinity for Mn^{2+} than Ca^{2+} (19). Cells loaded with fura-2 as described above were suspended in Ca^{2+} -deficient medium. Two minutes after the addition of stimulants, Mn^{2+} (0.25 mM, final concentration) was added. Fluorescence was excited at 360 nm. Emission was recorded at 510 nm. Maximal Mn^{2+} quenching values were estimated for each preparation at the end of the recording by permeabilization of the cells with 10 μM digitonin.

Western blotting analysis of FKBP's associated with RyR in adrenal chromaffin cells

Preparation of the CHAPS-solubilized fraction:

Bovine adrenal medullary chromaffin cells were obtained 1 day after seeding. These cells were homogenized in homogenization buffer containing 20 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 10 mM NaF, 10 mM β -glycerophosphate, and 1 $\mu\text{g}/\text{ml}$ of the various protease inhibitors (benzamidine, leupeptin, and anti-pain). The homogenate was centrifuged at $20,000 \times g$ for 5 min at 4°C, followed by the removal of the supernatant. The pellet was resuspended with homogenization buffer. This suspended solution was solubilized in 1% (v/v) CHAPS for 10 min at 4°C and centrifuged at $20,000 \times g$ for 5 min. The supernatant was the CHAPS-solubilized fraction.

Immunoblotting assays: The CHAPS-solubilized fraction was mixed at a volume ratio of 4:1 with sodium dodecylsulfate (SDS) sample buffer containing 10 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% SDS, 0.01% bromophenol blue, and 0.5% β -mercaptoethanol, followed by heating at 100°C for 10 min. These samples were applied to polyacrylamide gel. The polyacrylamide gel concentrations employed were as follows: RyR, 3% stacking gel and 5% separating gel; FKBP, 4.5% stacking gel and 12.5% separating gel. To distinguish the mobilities of the FKBP12 isoforms, gels were polymerized with excess TEMED (21). These gels were run for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. The transferred protein on the membranes were blocked by incubating with the blocking solution, that is, wash buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, and 0.1% Tween 20] containing 5% skim milk at 25°C for 1 h. The membranes were exposed to individual primary antibody against RyR (1:1,000) or FKBP 12 (1:500) diluted in wash buffer containing 1% skim milk overnight at 4°C, washed three times with wash buffer, and then incubated with a biotinylated secondary antibody (1:10,000) at 4°C for 1 h. After being rinsed for 5 min in wash buffer, membranes were placed in solutions containing avidin and biotinylated-peroxidase at 25°C for 1 h. The membrane was again rinsed three times with wash buffer and developed with ECLTM, followed by exposure to X-ray films.

Immunoprecipitation: A monoclonal anti-RyR antibody (15 μg) was added to 300 μl of the CHAPS-solubilized fraction and incubated overnight at 4°C. Protein A-Sepharose was then used to precipitate the antibodies for 2 h at 4°C. The reaction mixture was centrifuged at $1,500 \times g$ for 5 min. The immunoprecipitates were washed with 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl, 10 mM NaF, and 10 mM β -glycerophosphate, followed by centrifugation at $1,500 \times g$ for 5 min. Precipitates thus obtained were

suspended in 100 μl of 100 mM glycine-HCl buffer (pH 3.5), followed by centrifugation at $1,500 \times g$ for 5 min. These elution samples were reacted with SDS sample buffer, as described above. The immunoprecipitates with anti-RyR antibody were subjected to Western blot analysis using anti-RyR or anti-FKBP12 antibodies.

CA assay

For the measurement of the release of CA, incubations were carried out as described previously (18). The incubation medium was separated from the cells, mixed with perchloric acid (5% of final concentration), and centrifuged at $4,500 \times g$ for 15 min. The total CA content in the resultant supernatant was measured fluorometrically according to the method of von Euler and Lishajko (22) with adrenaline as a standard.

Statistical analyses

The significance of the differences in mean values between groups was examined using analysis of variance, followed by ANOVA. Student's *t*-test was used to evaluate statistical significance within groups.

Results

Ca^{2+} release in digitonin-permeabilized cells

To explore the role of FKBP on the action of cADPR, the effect of immunosuppressants such as FK506 and rapamycin, which bind to FKBP and remove them from RyR, were examined in digitonin-permeabilized chromaffin cells. As shown in Fig. 1A, cADPR caused the rapid increase of Ca^{2+} release and the increase was sharply attenuated close to the basal level within 60 s. This transient effect of cADPR may be due to rapid metabolism, as cADPR hydrolase activity is very high in chromaffin cells (5). The possibility of the desensitization to cADPR could be ruled out because the repeated addition of cADPR produced the similar responses (data not shown). The cADPR-induced increase of Ca^{2+} release was inhibited by FK506 and rapamycin. On the other hand, cyclosporin A, which does not interact with FKBP but binds to cyclophilin and inhibits calcineurin, produced a large and sustained enhancement of Ca^{2+} release induced by cADPR. 8Br-cADPR, which is a competitive antagonist for cADPR and antibody for FKBP12/12.6, as well as FK506 and rapamycin, effectively blocked the Ca^{2+} release induced by submaximum (0.3 μM) and maximum (10 μM) concentrations of cADPR (Fig. 1B). Cyclosporin A enhanced the Ca^{2+} release induced by 0.3 μM , but caused no further increase of the Ca^{2+} release induced by 10 μM of cADPR. Thus, FKBP associated with RyR are sug-

gested to be required for Ca^{2+} release by cADPR. The enhancement by cyclosporin A of the cADPR-induced Ca^{2+} release is in agreement with the requirement of FKBP for the action of cADPR, because cyclosporin A does not dissociate FKBP from RyR, and decreases the attenuation of the activity of RyR by inhibiting de-phosphorylation by calcineurin. FK506, rapamycin, and cyclosporin A by themselves had no effect on the basal Ca^{2+} release (data not shown). Ryanodine is well documented to activate RyR. The effects of 8Br-cADPR and the immunosuppressants on ryanodine-induced Ca^{2+} release were examined (Fig. 1C). Ryanodine caused a gradual increase in Ca^{2+} release. Ryanodine-induced Ca^{2+} release was not affected by 8Br-cADPR and was remarkably enhanced by FK506, rapamycin and cyclosporin A. Ca^{2+} release induced by ryanodine was also enhanced by cADPR, and in turn, cADPR-induced Ca^{2+} release was enhanced by ryanodine (Fig. 1D). These results may support that ryanodine by a mechanism independent from cADPR and cADPR through a FKBP-dependent mechanism enhance synergically RyR function.

Immunoblotting analysis

In pancreas β -cell microsomes, cADPR has been reported to release FKBP12.6 from RyR2. We have studied the possible release by cADPR of the FKBP from RyR in adrenal medullary chromaffin cells. The CHAPS-solubilized fraction was prepared from adrenal medullary chromaffin cells, followed by immunoprecipitation with the anti-RyR antibody. SDS-PAGE and immunoblotting used polyclonal anti-FKBP12 antibody, which recognized both FKBP12 and FKBP12.6 isoforms. Immunoreactivity was detected at the molecular weight position of RyR (565 kDa) in the immunoprecipitates of the CHAPS-solubilized fraction by immunoblotting using an antibody against RyR (Fig. 2A). In control conditions, one 12.6-kDa protein (FKBP12.6) was recognized by the FKBP12 antibody in the immunoprecipitates, and a 12-kDa protein (FKBP12) was recognized in the supernatant fraction. These results indicate that FKBP12.6, but not FKBP12 is associated with RyR in bovine adrenal chromaffin cells.

In the Western blot analysis experiments, we did not observe that cADPR released endogenous FKBP12.6 from the immunoprecipitates with anti-RyR antibody (Fig. 2C). As a positive control, the addition of FK506 (5 μM) released FKBP12.6 into the supernatant. Concomitantly, the corresponding FKBP band is greatly diminished in the precipitates (Fig. 2B). As in the control conditions, the FKBP12.6 remained attached to the immunoprecipitates after incubation with 5 μM

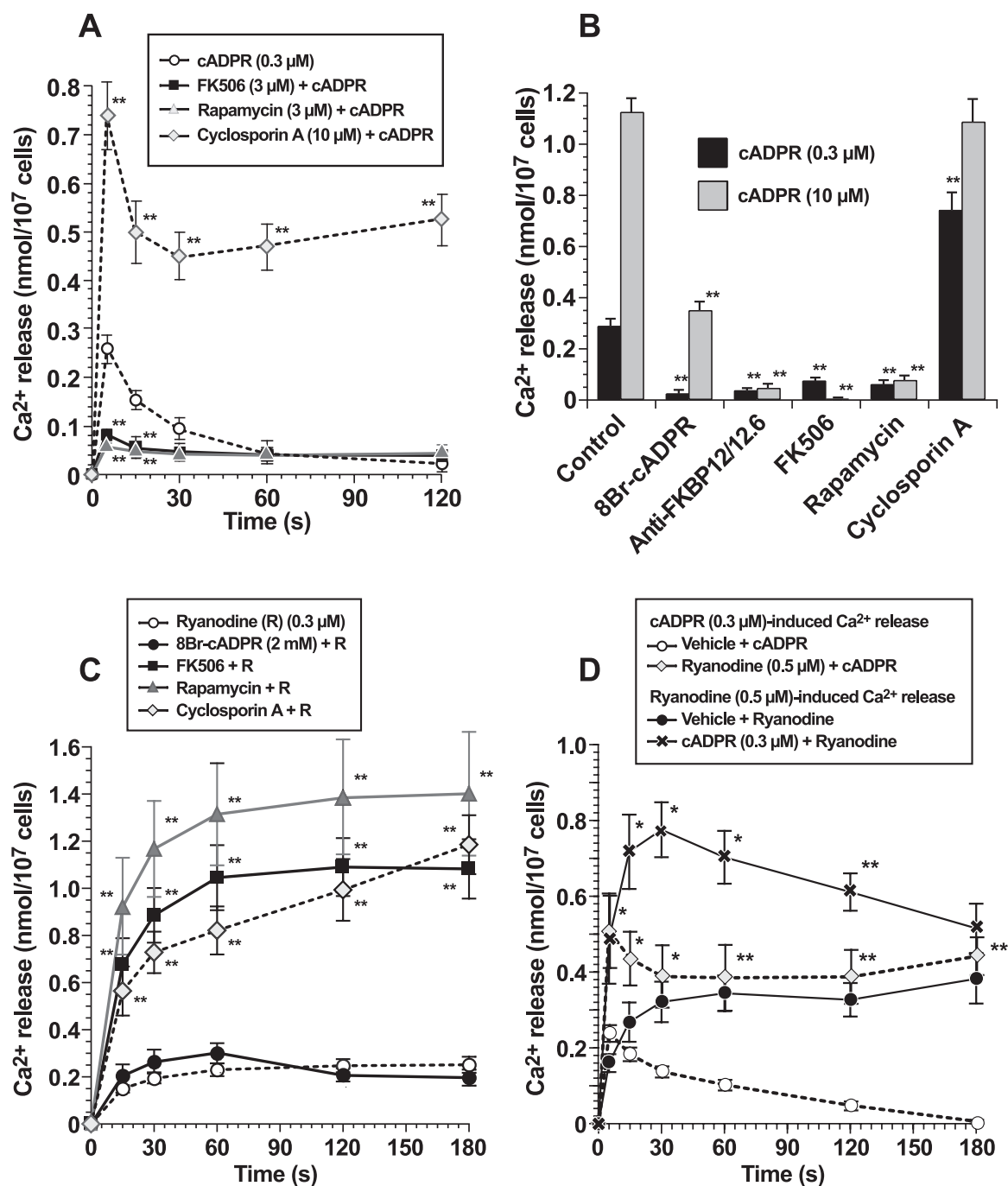


Fig. 1. cADPR- and ryanodine-induced Ca²⁺ release from digitonin-permeabilized bovine adrenal chromaffin cells and effects of various treatments. **A** and **C**: The patterns of Ca²⁺ release induced by cADPR (**A**) and ryanodine (**C**), and the effects of 8Br-cADPR, FK506, rapamycin, and cyclosporin A on Ca²⁺ release. 8Br-cADPR (20 μ M), FK506 (3 μ M), rapamycin (3 μ M), and cyclosporin A (10 μ M) were added 1–3 min before the addition of cADPR (0.3 μ M) or ryanodine (0.3 μ M). Fluorescence was continuously monitored using a fluorometer at an excitation of 340/380 nm and an emission of 510 nm. Ca²⁺ released from the permeabilized chromaffin cells was calibrated by the addition of known amounts of Ca²⁺. Values are the mean \pm S.E.M. of the Ca²⁺ release at the time indicated ($n = 4$ to 7). **B**: Effects of 8Br-cADPR, anti-FKBP12 antibody, FK506, rapamycin, and cyclosporin A on the Ca²⁺ release induced by submaximum (0.3 μ M) and maximum (10 μ M) concentrations of cADPR. Anti-FKBP12 antibody (10 μ g/ml) was added 30 min before the addition of cADPR. Anti-FKBP12 antibody was dialyzed before use for 3 h at 4°C against the KH solution. Values are the mean \pm S.E.M. of the peak rise of Ca²⁺ release ($n = 3$ to 5). **D**: Effects of ryanodine and cADPR on the Ca²⁺ release induced by cADPR and ryanodine. Ryanodine (0.5 μ M) and cADPR (0.3 μ M) were added 1 min before the addition of cADPR (0.3 μ M) or ryanodine (0.5 μ M), respectively. Values are the mean \pm S.E.M. of the Ca²⁺ release at the time indicated ($n = 4$ to 7). *, significantly different from the corresponding control at $P < 0.05$. **, significantly different from the corresponding control at $P < 0.01$.

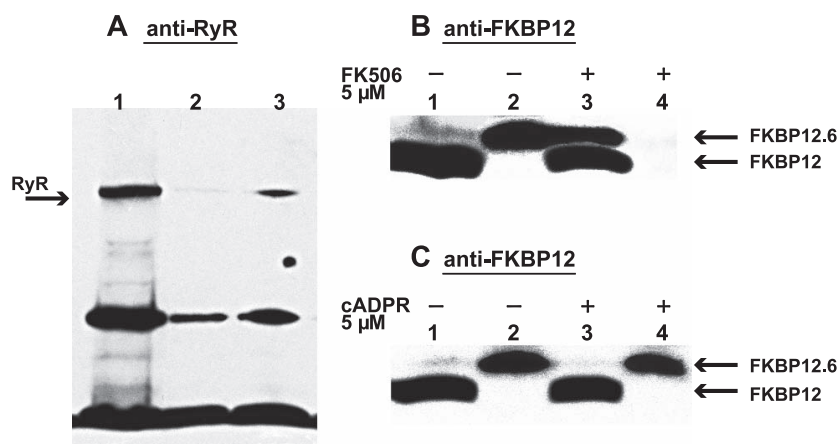


Fig. 2. Association of FKBP12.6 with the RyR in the CHAPS-solubilized adrenal chromaffin cells membrane fraction. Interaction of FKBP-RyR was examined by immunoprecipitation with anti-RyR antibody, followed by detection with the antibody raised against the N-terminus of FKBP12 of human origin, which could detect both FKBP12 and FKBP12.6 isoforms. The CHAPS-solubilized fraction was treated with $5\ \mu\text{M}$ FK506 or $5\ \mu\text{M}$ cADPR for 10 min at 25°C and subjected to immunoprecipitation with anti-RyR antibody. The resultant immunoprecipitant and supernatant were analyzed by immunoblotting. Detection of RyR or FKBP12 or FKBP12.6 bands was conducted by Western blot analysis with anti-RyR (A) or anti-FKBP12 (B and C) antibodies, respectively. A: Lane 1, CHAPS-solubilized fraction; lane 2, supernatant of immunoprecipitation with antibody for

RyR (supernatant); lane 3, immunoprecipitates with an antibody specific for RyR (precipitates). The arrow indicates the position of RyR. B and C: Lane 1, supernatant derived from CHAPS-solubilized fraction treated without FK506 (B) or cADPR (C); lane 2, precipitates derived from CHAPS-solubilized fraction treated without FK506 (B) or cADPR (C); lane 3, supernatant derived from CHAPS-solubilized fraction treated with FK506 (B) or cADPR (C); lane 4, precipitates derived from CHAPS-solubilized fraction treated with FK506 (B) or cADPR (C). The arrows indicate the position of FKBP12 and FKBP12.6. The FKBP12.6 coimmunoprecipitation is blocked by the inclusion of FK506, but not by cADPR, whereas a constant amount of RyR is present in each immunoprecipitate. Data shown are representative of three similar experiments.

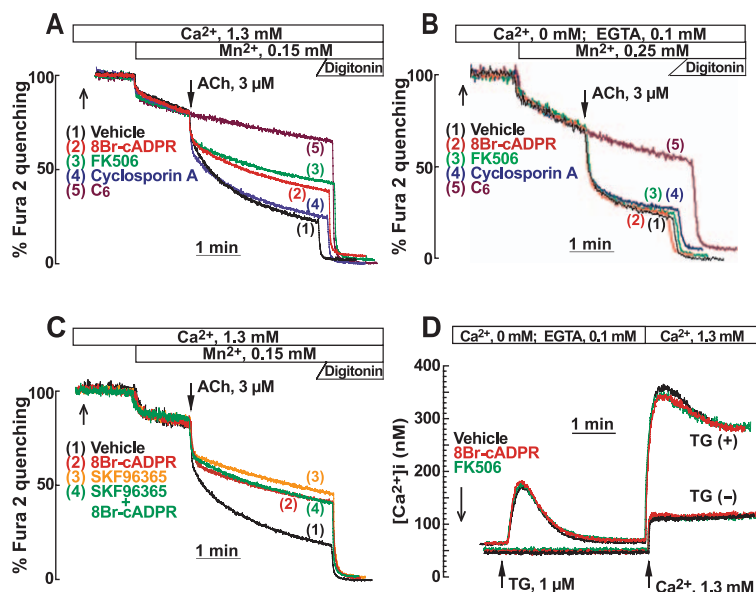
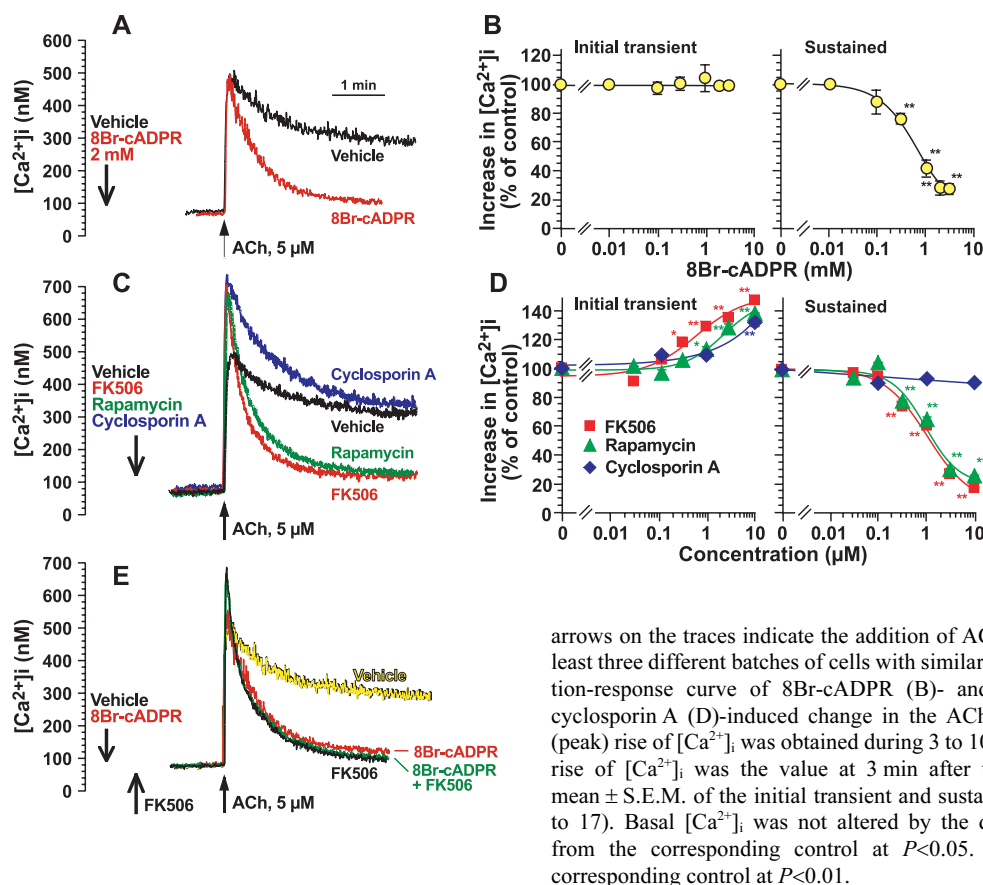
cADPR. In summary, we found no evidence that cADPR released FKBP12.6 from the RyR of adrenal chromaffin cells, in contrast with studies from pancreas islet microsomes, which showed FKBP12.6 release from the microsomal fraction after incubation with cADPR.

$[\text{Ca}^{2+}]_i$ rise in intact cells

As it is suggested that cADPR causes Ca^{2+} release through RyR via interaction with FKBP in digitonin-permeabilized chromaffin cells, whether this mechanism is involved in $[\text{Ca}^{2+}]_i$ rise induced by ACh in intact chromaffin cells was examined. The effects of 8Br-cADPR and immunosuppressants on ACh-induced $[\text{Ca}^{2+}]_i$ rise are shown in Fig. 3. ACh induced a sharp initial $[\text{Ca}^{2+}]_i$ rise, and the following sustained rise and the pretreatment of 8Br-cADPR attenuated the sustained $[\text{Ca}^{2+}]_i$ rise without affecting the initial peak $[\text{Ca}^{2+}]_i$ rise as shown in the typical time course (Fig. 3A) and in the concentration relationship (Fig. 3B). FK506 and rapamycin enhanced the initial transient $[\text{Ca}^{2+}]_i$ rise induced by ACh, while it reduced the sustained $[\text{Ca}^{2+}]_i$ rise (Fig. 3C) and these effects were concentration-dependent from 0.3 to $10\ \mu\text{M}$ (Fig. 3D). Cyclosporin A enhanced the initial transient $[\text{Ca}^{2+}]_i$ rise and did not reduce the sustained rise induced by ACh (Fig. 3: C and D). When FK506 was administered for 30 min after the treating with 8Br-cADPR, no further decrease in $[\text{Ca}^{2+}]_i$ than that in cells treated with each of 8Br-cADPR or FK506 alone was observed (Fig. 3E). These results suggest that cADPR and FKBP participate in the sustained phase of $[\text{Ca}^{2+}]_i$ rise in ACh-stimulated cells.

Mn^{2+} entry and Ca^{2+} entry

Store-operated Ca^{2+} channels (SOC) have been shown to be an important pathway permitting Ca^{2+} entry through the plasma membrane, contributing to the sustained $[\text{Ca}^{2+}]_i$ rise. This pathway is activated by mobilizing Ca^{2+} from intracellular stores in various cells (23). The Mn^{2+} -quench technique was used to evaluate the Ca^{2+} entry. This cation has been used as a surrogate for Ca^{2+} entry, given its quenching effect of fura-2 (19). ACh caused an initial sharp and following sustained quenching of the fura-2 fluorescence in the presence of Mn^{2+} , and the effect of ACh was completely blocked by pretreatment with a nicotinic receptor antagonist, hexamethonium (Fig. 4A). The results reflect the Mn^{2+} entry with 2 phases, the initial and sustained entry evoked by nicotinic ACh receptor stimulation. When cells were treated with 8Br-cADPR or FK506, the initial sharp quenching of fura-2 fluorescence induced by ACh was not altered, but the sustained quenching of fura-2 fluorescence was reduced. Therefore, the sustained but not the initial Mn^{2+} entry involves the pathway activated by cADPR and FKBP. Cyclosporin A did not affect both ACh-induced phases of quenching of fura-2 fluorescence, suggesting that the Mn^{2+} entry is not altered by the agent. We have previously demonstrated that ACh stimulated cADPR synthesis via the Ca^{2+} -dependent activation of the adenylate cyclase pathway (5). To test the involvement of cADPR formed by ACh-stimulation in ACh-induced Mn^{2+} quenching, the effect of 8Br-cADPR and immunosuppressants on the Mn^{2+} quenching in Ca^{2+} -deprived medium were examined.



When Ca^{2+} was depleted in the medium, the ACh-induced Mn^{2+} quenching was not affected by 8Br-cADPR, FK506, or cyclosporin A (Fig. 4B). These results that 8Br-cADPR, FK506, and cyclosporin A reduced the Mn^{2+} quenching only when Ca^{2+} was present in the extracellular medium may suggest that cADPR formed in response to ACh-stimulation activated SOC. Actually, the SOC inhibitor SK&F 96365 reduced the sustained but not initial Mn^{2+} entry, and 8Br-cADPR in the presence of SK&F 96365 produced no further reduction of Mn^{2+} entry than that induced by 8Br-cADPR alone (Fig. 4C). To test whether 8Br-cADPR and FK506 directly block SOC, the Ca^{2+} entry was evaluated by measuring the changes in the $[\text{Ca}^{2+}]_i$ rise by the introduction of Ca^{2+} into the medium after the treatment of cells with Ca^{2+} mobilization in Ca^{2+} -deprived medium. It has been shown that the mobilization of Ca^{2+} from Ca^{2+} stores by the inhibition of Ca^{2+} -ATPase in the stores by thapsigargin can activate SOC without the activation of any receptors (24). Thapsigargin caused a small rise in $[\text{Ca}^{2+}]_i$. When Ca^{2+} was introduced into the medium, a larger rise in $[\text{Ca}^{2+}]_i$ was produced than in the control. Both 8Br-cADPR and FK506 had no effect on the $[\text{Ca}^{2+}]_i$ rise induced by thapsigargin and Ca^{2+} introduction after thapsigargin (Fig. 4D). 8Br-cADPR and FK506 did not act on SOC itself.

CA release

Effects of the cADPR antagonist 8Br-cADPR and immunophilin ligands, FK506, rapamycin and cyclosporin A, on CA release in bovine adrenal chromaffin cells were studied (Fig. 5). 8Br-cADPR, FK506, and rapamycin concentration-dependently inhibited ACh-induced CA release. Cyclosporin A did not affect the release. None of these agents affected the basal release. The concentrations required for 50% inhibition of the CA release of 8Br-cADPR, FK506, and rapamycin were 0.57 mM, 1.25 μM , and 1.90 μM , respectively, which were in correspondence with those for the inhibition of the ACh-induced $[\text{Ca}^{2+}]_i$ rise. SK&F 96365, an inhibitor of SOC significantly decreased ACh-induced CA release. 8Br-cADPR and FK506 produced no further decrease in CA release in the presence of SK&F 96365. ω -Agatoxin IVA, an inhibitor of P/Q type VOCC further decreased ACh-induced CA release in the presence of FK506 (Fig. 5A) or SK&F 96365 (Fig. 5B), respectively. The similar result was obtained with rapamycin (data not shown). The lack of the additional inhibition of SK&F 96365 and 8Br-cADPR or FK506 was not due to a saturating effect because ω -agatoxin IVA further inhibited ACh-induced CA release in the presence of SK&F 96365 or FK506.

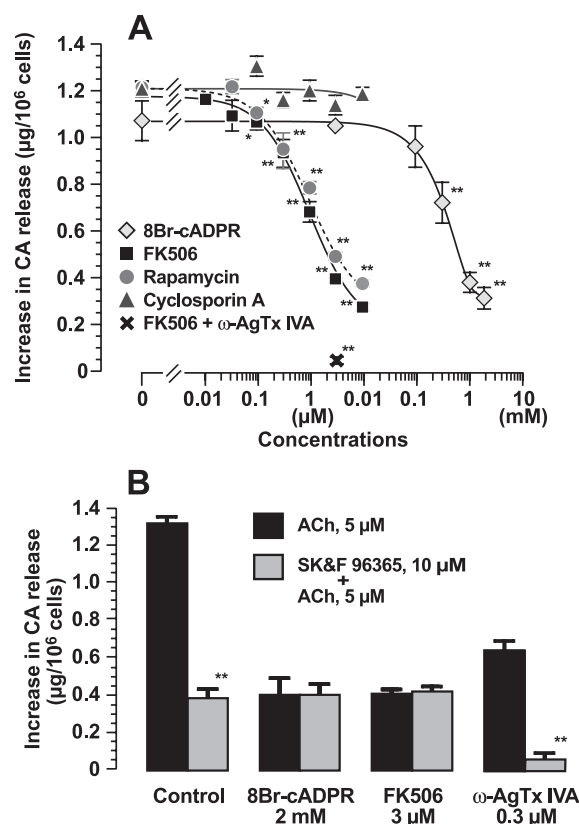


Fig. 5. Effects of 8Br-cADPR and immunophilin ligands on CA release induced by ACh. Cells were preincubated with medium containing various concentrations of 8Br-cADPR for 30 min prior to a 6-min stimulation with ACh (5 μM). FK506, rapamycin, and cyclosporin A were treated 3 min before the stimulation with ACh. In the experiments with SK&F 96365, the additional incubation with SK&F 96365 for 2 min after the incubation with 8Br-cADPR, FK506, or rapamycin was performed. ω -Agatoxin IVA (ω -AgTx IVA, 0.3 μM) was added 3 min before the addition of SK&F 96365 or FK506. Values are the mean \pm S.E.M. of the net increase in CA release of 6 to 9 experiments assayed in triplicate. Basal CA release was not altered by the drugs tested. *, significantly different from the corresponding control at $P < 0.05$. **, significantly different from the corresponding control at $P < 0.01$.

Here we present evidence that cADPR is important for a long-lasting $[\text{Ca}^{2+}]_i$ rise, which is essential for CA release.

Discussion

RyR1 and RyR2 are highly expressed in skeletal and cardiac muscle cells, respectively (25); and RyR3 is more ubiquitously expressed, but at much lower levels (26). Among the three isoforms of the ryanodine receptors known, RyR2 is mainly expressed in the adrenal chromaffin cells (27). It has been reported that cADPR has a significant stimulatory effect on the apparent association velocity but not the capacity of

[^3H]ryanodine binding to Jurkat membranes (28). The similar enhancement of [^3H]ryanodine binding has been shown in cardiac sarcoplasmic reticulum (SR) vesicles (3), rat brain microsomes (29), and in T-cells (30). This evidence indicates a direct modulatory effect on RyR channel opening by cADPR, as [^3H]ryanodine has been suggested to bind the RyR in the open conformation. However, some studies suggest the indirect mode of action of cADPR via accessory proteins such as FKBP. FKBP binds tightly to RyRs, resulting in the inhibition of Ca^{2+} release (7, 8, 31–36). FK506 or rapamycin, ligands of FKBP, dissociate FKBP12 from the RyR-FKBP12 complex (7, 8, 34, 37, 38), and the Ca^{2+} -release channel activity of the RyR is thereby increased, resulting in the release of Ca^{2+} in myocardial or skeletal muscle cells (8, 32, 37–39). Recent data have clarified the role of FKBP12.6 in the regulation of RyR2 and its physiological importance in several tissues. cADPR, like immunophilin ligands, binds to FKBP12.6 in the RyR and causes the dissociation of FKBP12.6 from the RyR to form the FKBP 12.6-cADPR complex and enhances Ca^{2+} release through the RyR in pancreatic islet cells (10) and in reconstituted RyRs from arterial smooth muscle (11). These results suggest that cADPR induces Ca^{2+} release from the SR by stimulation of FKBP12.6 dissociation from the RyRs. However, the dissociation of FKBP from RyR by cADPR is not an exclusive mechanism underlying cADPR-induced Ca^{2+} release depending on the tissues. In sea urchin eggs and rat pancreatic acinar cells, the presence of FK506 did not inhibit or further stimulated cADPR-induced Ca^{2+} release (40, 41), suggesting that cADPR-binding proteins are not FKBP. Calmodulin has also been shown to be obligatory for cADPR to increase the sensitivity of CICR for Ca^{2+} (17, 42). Another possibility, although not yet fixed, was proposed that RyRs are not involved in the cADPR-induced Ca^{2+} release but separate cADPR-sensitive channels or that the Ca^{2+} pump may be the target molecule for cADPR (43–45).

The evidence in the present study that cADPR lost its action in the presence of FK506 and rapamycin in digitonin-permeabilized chromaffin cells suggests that FKBP is required for cADPR to work on Ca^{2+} release channel in ER. The Ca^{2+} - and calmodulin-dependent phosphatase, calcineurin, was reported to interact with the RyR via FKBP as in the case of the IP_3 receptor (IP_3R) (21, 46, 47). It has been reported that cyclosporin A, which could not dissociate FKBP/calcineurin from RyR, but inhibits calcineurin by interacting with cyclophilin, a calcineurin associated protein, evokes Ca^{2+} oscillation as well as FK506 or rapamycin did in cardiomyocytes, suggesting that calcineurin-mediated

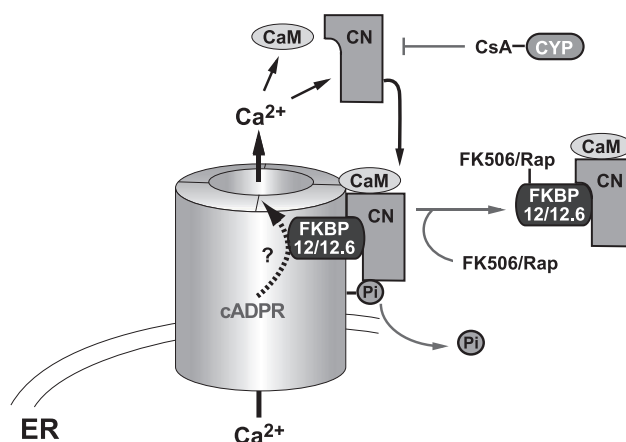


Fig. 6. A schematic model of cADPR to stimulate Ca^{2+} release from endoplasmic reticulum (ER) in bovine adrenal chromaffin cells. CaM: calmodulin, CN: calcineurin, CsA: cyclosporin A, CYP: cyclophilin, FKBP: FK506-binding protein, RaP: rapamycin.

dephosphorylation is involved in the regulation of the RyR channel activity (21, 48). The observation that cyclosporin A that does not dissociate FKBP enhanced cADPR-induced Ca^{2+} release in contrast to the inhibitory effect of FK506 and rapamycin that dissociate FKBP from RyR further supports the requirement of FKBP for the action of cADPR. The evidence that cADPR by itself produced a transient increase in Ca^{2+} release and produced not only the enhanced but also the sustained release of Ca^{2+} in the presence of cyclosporin A suggests the important negative regulation by calcineurin-dependent de-phosphorylation of the RyR channel. Because the presence of FKBP is unnecessary for the action of ryanodine, the enhancement of ryanodine-induced Ca^{2+} release by FK506, rapamycin, and cyclosporin A could be due to their interference of the action of calcineurin, subsequent inhibition of de-phosphorylation of RyR, and decrease of the inactivation of RyR channel. Ca^{2+} release by a low concentration of ryanodine in digitonin-permeabilized chromaffin cells was enhanced by cADPR, and the effect of cADPR was enhanced by ryanodine. We have previously reported that an imperatoxin inhibitor that has inhibitory action on the RyR channel, although it is not a specific inhibitor of RyR channel, blocked Ca^{2+} release induced by both ryanodine and cADPR in chromaffin cells without affecting on IP_3 -induced Ca^{2+} release (5). Taken together, these results suggest the involvement of the enhancement of RyR channel in cADPR-induced activation of Ca^{2+} release (Fig. 6).

A study of immunoprecipitation with an antibody against FKBP12, which also reacts with FKBP12.6, showed that a large amount of FKBP12 present in the cytosol and expressed FKBP12.6 was associated with

RyR in bovine adrenal chromaffin cells. FK506 actually dissociated FKBP12.6 from RyR, while cADPR did not dissociate FKBP12.6 from RyR under the same conditions. Moreover, the FK506-induced dissociation of FKBP12.6 from RyR by itself did not cause Ca^{2+} release in adrenal chromaffin cells. Therefore, it is suggested that FKBP12.6 is required for cADPR action at the RyR. As yet, it remains to be determined whether FKBP12.6 serves as a target molecule for cADPR binding or as scaffolding for cADPR to interact with RyR. The mechanism of action of cADPR/FKBP12.6 is different from those in the pancreatic islets where the dissociation itself of FKBP12.6 from RyR by FK506 or cADPR results in the activation of RyR (10).

In intact cells, the sustained phase of the ACh-induced $[\text{Ca}^{2+}]_i$ rise was remarkably attenuated by 8Br-cADPR, an antagonist of cADPR, without affecting the initial phase of the ACh-induced $[\text{Ca}^{2+}]_i$ rise. We have previously reported that ACh activates ADP-ribosyl cyclase via the influx of extracellular Ca^{2+} and the following activation of adenylate cyclase and cyclic AMP-dependent protein kinase in chromaffin cells (5). Therefore, it is suggested that cADPR formed in response to ACh stimulation of chromaffin cells mediates the ACh-induced sustained rise of $[\text{Ca}^{2+}]_i$. This is supported by the observation that FK506 and rapamycin, but not cyclosporin A, reduced the sustained rise of $[\text{Ca}^{2+}]_i$ induced by ACh with an increased initial rise of $[\text{Ca}^{2+}]_i$. These results are in contrast to the effect of VOCC antagonists that effectively reduced the initial phase, with less effect on the sustained phase (49). Therefore, the initial rise of $[\text{Ca}^{2+}]_i$ induced by ACh is largely dependent on the Ca^{2+} influx through VOCC. 8Br-cADPR and FK506 in combination produced the similar inhibition of the sustained ACh-induced $[\text{Ca}^{2+}]_i$ rise by each alone, suggesting both agents act with the similar mechanism of action, that is, interference with the action of cADPR. However, the initial rise of $[\text{Ca}^{2+}]_i$ is enhanced by FK506 even in the presence of 8Br-cADPR and thus a cADPR-dependent mechanism is not involved in this stage of $[\text{Ca}^{2+}]_i$ rise. Considering that FK506, rapamycin, and cyclosporin A all enhanced the ACh-induced initial rise of $[\text{Ca}^{2+}]_i$, the inhibition of dephosphorylation of RyR by interfering with the action of calcineurin may keep RyR in an activated state. Accumulating evidence suggests that RyRs form a structural and functional coupling to membrane VOCC (50–52). This coupling could be enhanced by the inhibition of calcineurin-mediated dephosphorylation of the RyR channel.

Another coupling between the release of Ca^{2+} from its store and the activation of an un-identified plasma membrane Ca^{2+} channel (SOC) has been proposed (23,

53, 54). Although, the mechanisms for the activation of SOC are not known, there is a hypothesis that emptying Ca^{2+} stores produces a Ca^{2+} influx factor (CIF), which mediates the activation of SOC (55, 56), or that Ca^{2+} stores form a structural and functional coupling to membrane SOC (53, 57–59). Actually, ACh caused Mn^{2+} entry with 2 phases, initial entry and the following sustained entry; and the former was not altered but the latter was reduced by 8Br-cADPR, FK506, and the SOC inhibitor SK&F 96365. Therefore, the sustained Mn^{2+} entry involves the SOC pathway activated by cADPR. That the inhibitory effect of 8Br-cADPR and FK506 was not due to the inhibition of SOC itself was confirmed because these agents did not affect the thapsigargin-induced activation of Ca^{2+} influx. Taken together that ACh stimulates the synthesis of cADPR depending on the Ca^{2+} influx following activation of adenylate cyclase in intact bovine adrenal chromaffin cells (5), the present evidence that 8Br-cADPR and FK506 reduced Mn^{2+} entry only when Ca^{2+} was present in the medium further suggests that cADPR was formed in response to ACh-stimulation in the physiological condition and activated SOC through stimulating Ca^{2+} release from the RyR channel. Cyclosporin A produced a large and lasting Ca^{2+} rise in digitonin-permeabilized cells, while it did not enhance the sustained $[\text{Ca}^{2+}]_i$ rise induced by ACh in intact cells. The different results between leaky cells and intact cells may suggest the involvement of unknown mechanisms for regulation of the sustained $[\text{Ca}^{2+}]_i$ rise. It has been reported that among the human TRP family, the role of TRPM2 in Ca^{2+} influx became evident in nonexcitable cell neutrophils. Heiner et al. (60) suggested that ADPR, a metabolite of cADPR, activates TRPM2 channels gating by the binding to the Nudix box domain in the C-terminal tail of the channel in neutrophil granulocytes. Although the possibility that cADPR or its metabolite may directly activate some channels for Ca^{2+} influx other than SOC could not be entirely ruled out, the evidence that FK506 and rapamycin inhibit cADPR-induced stimulation of Mn^{2+} entry suggest the important role for SOC.

This pathway plays an important role for CA release because 8Br-cADPR, FK506, rapamycin, and SK&F 96365, but not by cyclosporin A reduced CA release induced by ACh; and these agents produced no further reduction in the presence of SK&F 96365, a SOC inhibitor.

Here we present evidence that cADPR requiring the presence of FKBP12.6 activates Ca^{2+} release channels in ER; RyR channels as the candidate for an endogenous messenger of CICR in ACh-stimulated bovine adrenal chromaffin cells. This pathway coupled with Ca^{2+} entry through the plasma membrane is critical for the stimula-

tion of exocytosis.

Acknowledgments

We are grateful to Fujisawa Pharmaceutical Co., Ltd. (Osaka) for providing the FK506. This work was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

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