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Role of cyclic ADP-ribose in Ca^{2+}-induced Ca^{2+} release and vasoconstriction in small renal arteries

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Abstract

Cyclic-ADP-ribose (cADPR) has been reported to serve as a second messenger to mobilize intracellular Ca^{2+} independent of IP_3 in a variety of mammalian cells. This cADPR-mediated Ca^{2+} signaling pathway importantly participates in the regulation of various cell functions. The present study determined the role of endogenous cADPR in mediating ryanodine-sensitive Ca^{2+}-induced Ca^{2+} release (CICR) in vascular myocytes from small renal arteries and vasomotor response of these arteries. In freshly-isolated renal arterial myocytes, addition of $CaCl_2$ (0.01, 0.1, and 1 mM) into the Ca^{2+}-free bath solution produced a rapid Ca^{2+} release response from the sarcoplasmic reticulum (SR), with a maximal increase of 237 ± 25 nM at 1 mM $CaCl_2$. This $CaCl_2$ response was significantly blocked by a cell-membrane permeant cADPR antagonist, 8-bromo-cADP-ribose (8-br-cADPR) (30 μM) or ryanodine (50 μM). Caffeine, a classical CICR or ryanodine receptor activator was found to stimulate the SR Ca^{2+} release ($\Delta[Ca^{2+}]_i$: 253 ± 35 nM), which was also attenuated by 8-br-cADPR or ryanodine. Using isolated and pressurized small renal arteries bathed with Ca^{2+}-free solution, both $CaCl_2$ and caffeine-induced vasoconstrictions were significantly attenuated by either 8-br-cADPR or ryanodine. Biochemical analyses demonstrated that $CaCl_2$ and caffeine did not increase cADPR production in these renal arterial myocytes, but confocal microscopy showed that a dissociation of the accessory protein, FK506 binding protein 12.6 (FKBP12.6) from ryanodine receptors was induced by $CaCl_2$. We conclude that cADPR importantly contributes to CICR and vasomotor responses of small renal arteries through enhanced dissociation of ryanodine receptors from their accessory protein.

Keywords: Nucleotides; Ca^{2+} mobilization; Arterial smooth muscle; Second messenger; Kidney

Introduction

Cyclic-ADP-ribose (cADPR) was first reported to be produced in sea urchin eggs and to possess Ca^{2+} mobilizing activity in sea urchin egg microsomes (Clapper et al., 1987; Lee et al., 1989). Recent studies have reported that cADPR can be detected in a number of mammalian tissues including heart, liver, spleen, and brain tissues as well as red blood cells, pituitary cells, vascular smooth muscle cell, and renal epithelial cells (Koshiyama et al., 1991; Beers et al., 1995). The basal concentrations of cADPR in cardiac muscle, liver, and brain tissues are estimated as 100–200 nM (Galione, 1994; Lee, 1994; Li et al., 2000). As observed in sea urchin eggs, cADPR was also found to cause Ca^{2+} mobilization in these mammalian tissues and cells. Therefore, cADPR has been proposed as a calcium-mobilizing second messenger in various mammalian cells, with the potential to mediate the secretion of hormone such as insulin and catecholamines, the fertilization of

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eggs, the estrogen response in rat uterus, and the effects of nitric oxide (NO), serotonin and acetylcholine in non-muscle tissues (Lee et al., 1989; Lee and Aarhus, 1991; Galione, 1993; Galione et al., 1993; Takasawa et al., 1993; Chini et al., 1997; Morita et al., 1997).

It has been reported that cADPR mobilizes intracellular calcium by a mechanism completely independent of D-*myo*-inositol 1,4,5-tris-phosphate (IP_3), since the IP_3 receptor antagonist, heparin cannot block the effect of cADPR (Lee and Aarhus, 1991; Galione et al., 1993; Lee, 1993; Lee and Aarhus, 1993; Galione, 1994). This signaling nucleotide activates ryanodine receptors by a calmodulin-dependent mechanism, and the inhibitors of calcium-induced calcium release (CICR) such as tetracaine, procaine, and ruthenium red selectively inhibit cADPR-, but not IP_3-sensitive Ca^{2+} release (Galione et al., 1991; Lee, 1993; Lee et al., 1995). Therefore, cADPR has been considered as a Ca^{2+} mobilizing second messenger in different tissues or cells. Recently, we have reported that cADPR is produced and metabolized in renal preglomerular and postglomerular arteries, suggesting that the cADPR-mediated signaling pathway is present in renal arterial myocytes (Li et al., 2000). However, it remains unknown whether endogenous cADPR production acts as an intracellular second messenger to participate in the control of $[Ca^{2+}]_i$ in these renal cells and thereby contributes to vasomotor response in renal arteries.

With respect to the signaling mechanisms mediating vasomotor response of renal arteries, there is considerable evidence that a variety of agonists such as angiotensin II, vasopressin, norepinephrine, ATP, and UTP mobilize Ca^{2+} from the sarcoplasmic reticulum (SR) through IP_3-mediated signaling pathway in renal arterial myocytes (Salomonsson and Arendshorst, 1999; Inscho et al., 1999a,b). However, the signaling pathway by which other stimuli or agonists such as the membrane depolarization, Ca^{2+} influx, and acetylcholine-induced Ca^{2+} mobilization in these cells remains unknown. Given the role of cADPR in mediating the SR Ca^{2+} release and its presence in renal arterial myocytes, we hypothesized that cADPR may serve as another important second messenger to mobilize intracellular Ca^{2+} in renal arterial myocytes, in particular as an activator of Ca^{2+}-induced Ca^{2+} release (CICR) and in this way participates in the control of $[Ca^{2+}]_i$ in these cell, mediating vasoconstrictor response associated with CICR. The present study was designed to test this hypothesis by using fluorescence microscopic spectrometry, cADPR cycling assay, high-performance liquid chromatography (HPLC) technique, and confocal microscopic detection in freshly dissociated renal arterial myocytes and isolated and pressurized small renal arteries. Our results demonstrated that cADPR-activated CICR via ryanodine receptors is an important mechanism mediating Ca^{2+} mobilization and vasoconstriction independent of the IP_3 signaling pathway in small renal arteries.

Materials and methods

Dissection of small renal arteries and dissociation of arterial myocytes

Male Sprague–Dawley rats weighing 250–300 g were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and the kidneys were removed and placed in an ice-cold saline solution. Two tissue slices containing renal arteries were made from 1–1.5 mm of the outer edges of each kidney. The slices were then transferred to a microdissection dish filled with ice-cold low calcium physiological saline solution (PSS) containing 145 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 0.05 mM $CaCl_2$, and 10 mM glucose (pH 7.4). The dissection was performed under an MZ18 stereomicroscope (Li et al., 2000). The excess tubular structures were stripped away, and preglomerular renal arteries (<200 μm) were isolated and removed from the kidney slices with a sharpened microdissection scissors. Renal arterial myocytes were enzymatically dissociated as we described previously (Zou et al., 1996a,b). Briefly, 7 to 10 dissected arterial segments were transferred into low calcium PSS and incubated for 10 min at room temperature. The supernatant was then discarded, and fresh low calcium PSS containing 0.5 mg/ml papain was added. After 20-min incubation at 37°C, the supernatant was discarded. Finally, the digestion solution containing collagenase of 1.5 mg/ml, trypsin inhibitor of 1 mg/ml, and elastase of 0.5 mg/ml in low calcium PSS was added and allowed to incubate at 37°C. The supernatant of the incubation was discarded at the first 15 min and then collected every 10 min. After each collection of the supernatant, fresh digestion solution was added, and the incubation continued until all arterial tissues were digested (about 5 collections). The cells in the supernatant were washed twice by centrifugations at 1500 rpm for 3 min, and resuspended with ice-cold low calcium PSS until used.

$[Ca^{2+}]_i$ assay in renal arterial myocytes

A calcium-sensitive fluorophore, fura 2-AM (Molecular Probes, Eugene, OR) was used for monitoring $[Ca^{2+}]_i$ (Tsien, 1989). Freshly-dissociated renal arterial myocytes were transferred on poly-D-lysine-coated glass coverslips and incubated at 37°C for 30 min. These coverslips were washed using Hanks' buffer which contains 130 mM NaCl, 5.4 mM KCl, 5.5 mM HEPES, 10 mM Glucose, 1.25 mM $CaCl_2$, and 1 mM $MgCl_2$ and then incubated with 5 μM fura 2-AM in Hanks' buffer at 37°C for 30 min to load this fluorescent dye into the cells. For fluorescent microscopic spectrometry of $[Ca^{2+}]_i$, the coverslip with fura-2 loaded renal arterial myocytes was rinsed twice with Hanks' buffer, mounted on a perfusion chamber and then on the stage of an inverted microscope (Nikon Diaphot), and an isolated cell was

localized under the objective. A 20-min continuous incubation with Hanks' buffer was allowed for complete desertification of intracellular fura-2. After determination of fura-2 loading efficiency by scanning the fura-2 spectrum, the ratio of fura-2 emissions when excited at 340 and 380 nm (F_{340}/F_{380}) was monitored by using a fluorescence microscopic spectrometry system (PTI). $[Ca^{2+}]_i$ was calculated using following formula:

$$\left[Ca^{2+}\right]_i(\mathrm{nM}) = K_d(F_O/F_S)(R - R_{min})/(R_{max} - R)$$

Where R is the ratio of F_{340}/F_{380}. K_d is a dissociation constant. R_{min} and R_{max} are minimal and maximal ratio of F_{340}/F_{380}, respectively; F_O and F_S represent the maximal and minimal signal intensity at 380 nm, respectively. K_d was 224 nM. R_{max} was calculated from the fluorescence intensity after permeabilizing the cells with 5 μM ionomycin and represented maximal $[Ca^{2+}]_i$. R_{min} was obtained by addition of 2 mM EGTA and represented the minimal $[Ca^{2+}]_i$ (Tsien, 1989; Cornfield et al., 1994).

To examine the role of endogenous cADP-ribose in regulating Ca^{2+} influx-induced Ca^{2+} release and thereby in the control of $[Ca^{2+}]_i$ in renal arterial myocytes, the cells were preincubated in a Ca^{2+}-free Hanks' buffer for 20 min, and the increase in $[Ca^{2+}]_i$ calculated based on the ratio of F_{340}/F_{380} was produced by addition of $CaCl_2$ (0.1, 0.5, and 1 mM). In addition, a direct activation of ryanodine receptors was induced by addition of caffeine (0.01–1 mM) into the Ca^{2+}-free bath solution. The increase in extracellular calcium by $CaCl_2$ causes an influx of Ca^{2+}, which produces global increase in $[Ca^{2+}]_i$ via CICR in vascular smooth muscle cells. To determine the role of cADPR or ryanodine receptor in CICR, the renal arterial myocytes were incubated with 8-br-cADPR (30 μM), a cell permeant cADPR antagonist, or ryanodine receptor blocker, ryanodine (50 μM), and then $CaCl_2$- or caffeine-induced Ca^{2+} response was redetermined.

Vascular reactivity in isolated and pressurized small renal arteries

Small arcuate or interlobular renal arteries (100–200 μm diameter) were dissected from Male Sprague–Dawley rats and cannulated with two glass micropipette tips in a water-jacketed perfusion chamber as we have described previously. The arteries were perfused and bathed in a physiological saline solution (PSS) that was bubbled with 95%O_2–5%CO_2 mixture, pressurized to 70 mm Hg, and equilibrated at 37°C. The internal diameters were recorded using a video recording system. After a 1-h equilibration period, a pocket of air (0.3 ml) was perfused through the lumen of the artery to remove the endothelium. After an equilibration time period of 15 min, the bath solution was changed to a Ca^{2+}-free PSS solution, and the artery was allowed to re-equilibrate for an additional 20 min before addition of $CaCl_2$ or caffeine. A dose-dependent response curve to $CaCl_2$ (0.01–4 mM) was determined in the presence or absence of either 8-br-cADPR (30 μM) or ryanodine (50 μM).

In order to determine the role of cADPR in caffeine-induced contractions of renal arteries, the sarcoplasmic reticulum (SR) of arteries was first recharged with 40 mM KCl in normal Ca^{2+} PSS. Then a vasoconstrictor response to caffeine (1–3 mM) was observed in the presence or absence of either 8-br-cADPR (30 μM) or ryanodine (50 μM).

Cycling assay for endogenous cADPR levels in renal arterial myocytes

The cADPR basal levels in cultured renal arterial myocytes were determined as described previously (Graeff and Lee, 2002). 6×10^6 cultured renal arterial myocytes per sample were used. After being exposed to $CaCl_2$ (1 mM) or caffeine (1 mM) at 37°C for 5 min, these cells were scraped by a plastic scraper and spined down by centrifugation. The pellets were extracted with 0.5 ml of 0.6 M perchloric acid at 4°C. Perchloric acid was removed by mixing the aqueous sample with a solution containing 3 vol. of 1,1,2-trichlorotrifluoroethane to 1 vol. of tri-*n*-octylamine. Following a centrifugation step for 10 min at 1500 × *g*, the aqueous layer containing cADPR was removed, then incubated overnight at 37°C with an enzyme mixture containing: 0.44 unit/ml nucleotide pyrophosphatase, 12.5 units/ml alkaline phosphatase, 0.0625 unit/ml NADase, 2.5 mM $MgCl_2$, and 20 mM sodium phosphate (pH 8.0), all nucleotides except cADPR in samples were hydrolyzed.

Reactions were conducted in 96-well plates. To 0.1 ml of cADPR or other nucleotide samples, 50 μl of reagent was added containing 0.3 μg/ml ADP-ribosyl cyclase, 30 mM nicotinamide, and 100 mM sodium phosphate (pH 8.0). This initiated the conversion of cADPR in the samples to NAD^+. The conversion was allowed to proceed for 15 min at room temperature. The cycling reagent (0.1 ml) was then added, which contained 2% ethanol, 100 μg/ml alcohol dehydrogenase, 20 μM resazurin, 10 μg/ml diaphorase, 10 μM FMN, 10 mM nicotinamide, 0.1 mg/ml BSA, and 100 mM sodium phosphate (pH 8.0). The cycling reaction was allowed to proceed for 2–4 h and the increase in the resorufin fluorescence (with excitation at 544 nm and emission at 590 nm) was measured periodically using a fluorescence microplate reader. With known concentrations of standard curve, quantitative measurements were performed. The results shown are means ± SE from at least three independent measurements.

HPLC analysis of ADP-ribosyl cyclase activity in renal arterial myocytes

Cultured renal arterial myocytes at confluence were rinsed 3 times with 10 ml of chilled PBS and collected

using a cell scraper at 4°C. The cells were divided into 4 different Eppendorf tubes (1 ml each) and preincubated at 37°C for 10 min. To examine the effects of $CaCl_2$ and caffeine on ADP-ribosyl cyclase activity, renal arterial myocytes were treated with $CaCl_2$ (1 mM) or caffeine (1 mM), respectively. After incubation at 37°C for 15 min, the cells were washed with Hanks' solution. The pellets of cells were suspended in HEPES buffer (pH 7.0) containing (in mM): HEPES, 10; NaCl, 148; KCl, 5; $CaCl_2$, 1.8; $MgCl_2$, 0.3; and glucose, 5.5. They were then sonicated 6 times (each time for 20 s) with a sonifier cell disrupter (Branson, Model 185) at 4°C. After centrifugation at 3500×*g* for 10 min, the supernatant was collected. To determine ADP-ribosyl cyclase activity, the supernatant of 100 μg of protein was incubated with 100 μM of β-nicotinamide guanine dinucleotide (β-NGD^+) at 37°C for 60 min. The reaction mixtures were centrifuged at 4°C through an Amicon microultrafilter at 13,800 × *g* to remove proteins, and then analyzed by HPLC with a fluorescence detector (Hewlett-Packard 1090 HPLC system and 1046 Å spectrofluorometer). The excitation wavelength of 300 nm and the emission wavelength of 410 nm were used to detect the fluorescent products. All HPLC data were collected and analyzed by a Hewlett-Packard Chemstation. Nucleotides were resolved on a 3-μm Supelcosil LC-18 column (4.6 × 150 mm) with a 5-μm Supelcosil LC-18 guard column (4.6 × 20 mm; Supelco, Bellefonte, Pa., USA). The injection volume was 20 μl. The mobile phase consisted of 150 mM ammonium acetate (pH 5.5) containing 5% methanol (solvent A) and 50% methanol (solvent B). The solvent system was a linear gradient of 5% solvent B in A to 30% solvent B in A over 1 min, held for 25 min, and then increased to 50% solvent B over 1 min. The flow rate was 0.8 ml/min. Peak identities were confirmed by comigration with known standards. Quantitative measurements were performed by comparison of known concentrations of standards (Geiger et al., 2000; Li et al., 2000; Ge et al., 2003).

Confocal microscopic detection of FKBP12.6 dissociation from ryanodine receptors in renal arterial myocytes

For fluorescence microscopy, renal arterial myocytes were immobilized on glass coverslips coated with 1% poly-L-lysine for 15 min, stimulated with $CaCl_2$ (1 mM) with or without pretreatment of 8-br-cADPR (30 μM) for 15 min, and then fixed for 10 min in 1% paraformaldehyde (PFA) in PBS. The fixed cells were washed in 0.05% Tween 20/PBS, blocked with 0.1% bovine serum albumin in PBS and permeabilized by a 10-min incubation in 0.1% Triton X-100. Then, the cells were stained for 45 min each with a polyclonal rabbit anti-FKBP12.6 (1:200, ABR), followed by incubation with 0.5 μg/ml Texas Red (TR)-conjugated F(ab)2-fragments of anti-rabbit antibody (Molecular Probes), respectively, following by washing staining with BODIPY-labeled ryanodine (1 μM, Molecular Probes) for 30 min. Under a Leica TCS SP2 scanning confocal microscope, fluorescence imagings were observed and recorded. A negative control staining was performed with irrelevant monoclonal mouse antibodies. The colocalization of ryanodine receptors and FKBP 12.6 was analyzed offline overlay both fluorescence images of Texas Red (Excitation and Emission wavelength is 490/510 nm) and BODIPY (Excitation and Emission wavelength is 590/620 nm).

Statistics

Data are presented as means ± SE. The significance of differences within and between groups for multiple groups of data was evaluated using two-way ANOVA followed by a post test (Duncan's multiple range test) and for two groups of data using Student's *t* test

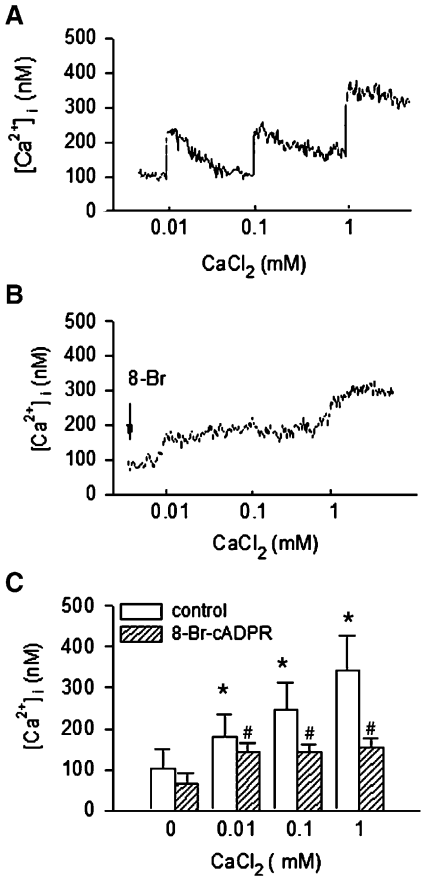

Fig. 1. Effects of cADPR antagonist, 8-br-cADPR (8-Br) on $CaCl_2$-induced increase in $[Ca^{2+}]_i$ in renal arterial myocytes. (A, B) Representative recordings of Ca^{2+} release from the SR in response to $CaCl_2$ (0.01–1 mM) and the effect of 8-br-cADPR (30 μM) on $CaCl_2$-induced Ca^{2+} release response. (C) Summarized data showing integrated peak transient $[Ca^{2+}]_i$ response to $CaCl_2$ in the absence or presence of 8-br-cADPR (30 μM). *Indicates significant difference ($P < 0.05$) compared with control. #Indicates significant difference compared to the values in the absence of 8-br-cADPR (n = 13 cells from 7 rat kidneys).

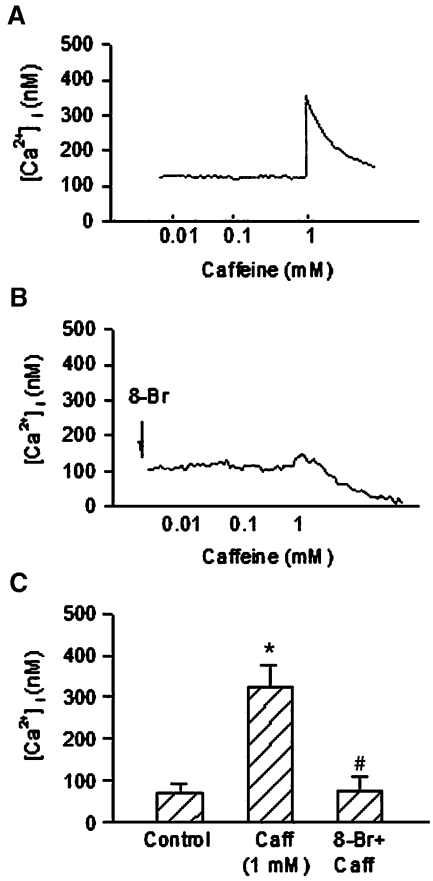

Fig. 2. Effects of 8-br-cADPR on caffeine-induced increase in $[Ca^{2+}]_i$ in renal arterial myocytes. (A, B) Representative traces showing the caffeine (0.01–1 mM)-induced increase in $[Ca^{2+}]_i$ and the effect of 8-br-cADPR (30 μM) on caffeine-induced Ca^{2+} release response. (C) Summarized data showing integrated peak transient $[Ca^{2+}]_i$ response to caffeine in the absence or presence of 8-br-cADPR (30 μM). Caff represents caffeine in the bath and 8-Br means pretreatment of the cells with 8-br-cADPR. *Indicates significant difference ($P < 0.05$) compared with control. #Indicates significant difference compared to the values in the absence of 8-br-cADPR ($n = 8$ cells from 6 rat kidneys).

(SigmaStat, San Rafael, CA). $P < 0.05$ was considered statistically significant.

Results

Effects of cADPR antagonist, 8-br-cADPR on $CaCl_2$-induced increase in $[Ca^{2+}]_i$ in renal arterial myocytes

Fig. 1A presents a typical fluorescent microscopic spectrometric recording depicting $CaCl_2$-induced Ca^{2+} release response from the SR of renal arterial myocytes. Fig. 1B shows the effect of 8-br-cADPR on $CaCl_2$-induced Ca^{2+} release response. $CaCl_2$ (0.01–1 mM) produced a Ca^{2+} transient in a concentration-dependent manner. The initial peak of the Ca^{2+} transient primarily represented the SR Ca^{2+} release as described in previous studies (Salomonsson and Arendshorst, 1999; Inscho et al., 1999a,b), which was followed by a sustained plateau due to Ca^{2+} influx. Therefore, the peak increase in this Ca^{2+} transient was calculated to determine the SR Ca^{2+} release. In the presence of 8-br-cADPR (30 μM), the effect of $CaCl_2$ on the SR Ca^{2+} release was substantially blocked (Fig. 1B), but a sustained increase in $[Ca^{2+}]_i$ was still observed. Fig. 1C summarized the effects of 8-br-cADPR on $CaCl_2$-induced Ca^{2+}release in these cells (n = 13 cells from 7 rat kidneys). $CaCl_2$ at the lowest concentration studied (0.01 mM) significantly stimulated the SR Ca^{2+} release by 37 nM, and maximal Ca^{2+} release response resulted in a 237 nM increase in $[Ca^{2+}]_i$ at 1 mM $CaCl_2$. 8-br-cADPR not only decreased basal $[Ca^{2+}]_i$, but also blocked $CaCl_2$-induced SR Ca^{2+} release.

Effects of 8-br-cADPR on caffeine-induced Ca^{2+} release response in renal arterial myocytes

Fig. 2A presents a typical fluorescent microscopic spectrometric recording depicting Ca^{2+} release response induced by caffeine (0.01–1 mM) from the SR of renal arterial myocytes. Caffeine produced a large rapid increase in $[Ca^{2+}]_i$ at 1 mM, but it had no significant effect on $[Ca^{2+}]_i$ at 0.01 and 0.1 mM. When these renal arterial myocytes were pretreated with 8-br-cADPR (30 μM) after reloading Ca^{2+} into the SR, caffeine-induced Ca^{2+} release was substantially blocked (Fig. 2B). As summarized in Fig. 2C, caffeine induced Ca^{2+} release by 253 nM in the absence of 8-br-cADPR, but only by 6 nM with the presence of 8-br-cADPR (n = 8 cells from 6 rat kidneys).

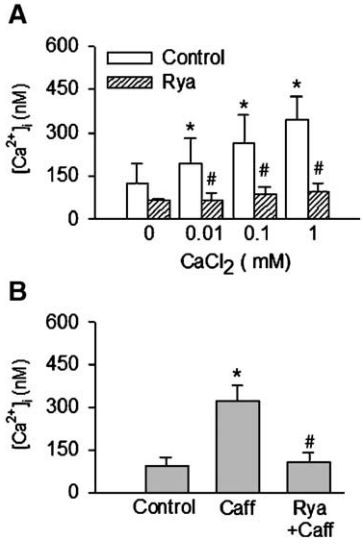

Fig. 3. Effects of ryanodine (Rya, 50 μM) on $CaCl_2$ (0.01–1 mM) or caffeine (Caff, 1 mM)-induced increase in $[Ca^{2+}]_i$ in renal arterial myocytes. (A) $CaCl_2$-induced response (n = 9 cells from 7 rat kidneys); (B) caffeine-induced response (n = 8 cells from 7 rat kidneys). *Indicates significant difference ($P < 0.05$) compared with control. #Indicates significant difference compared to the values in the absence of ryanodine.

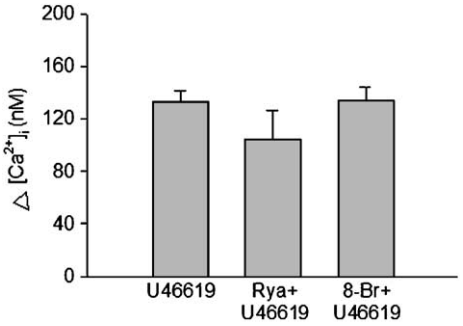

Fig. 4. Effects of 8-br-cADPR (8-Br) or ryanodine (Rya) on U46619-induced Ca^{2+} release in renal arterial myocytes (n = 8 cells from 7 rat kidneys).

Effects of ryanodine on $CaCl_2$-induced increase in $[Ca^{2+}]_i$ in renal arterial myocytes

The summarized results of these experiments are presented in Fig. 3A. Addition of $CaCl_2$ produced a concentration-dependent Ca^{2+} release in renal arterial myocytes. Pretreatment of the renal arterial myocytes with ryanodine (50 μM) for 20 min significantly attenuated the Ca^{2+} release induced by $CaCl_2$ (n = 9 cells from 7 rat kidneys). In the presence of ryanodine, $CaCl_2$ at 1 mM increased $[Ca^{2+}]_i$ in these cells by 26 nM, which was significantly lower than the increase of 226 nM obtained in the absence of ryanodine.

Effects of ryanodine on caffeine-induced increase in $[Ca^{2+}]_i$ in renal arterial myocytes

The summarized results of these experiments are presented in Fig. 3B. Caffeine (1 mM) produced a large Ca^{2+} release response in renal arterial myocytes. Pretreatment of the renal arterial myocytes with ryanodine (50 μM) for 20 min significantly attenuated the Ca^{2+} release induced by caffeine. Compared to an increase of 219 nM in the absence of ryanodine, caffeine increased $[Ca^{2+}]_i$ in these cells only by 12 nM in the presence of ryanodine (n = 8 cells from 7 rat kidneys).

Effects of 8-br-cADPR or ryanodine on U46619-induced increase in $[Ca^{2+}]_i$ in renal arterial myocytes

To determine the selectivity of 8-br-cADPR or ryanodine working on cADPR signaling or ryanodine receptor-associated Ca^{2+} release, we determined their effects on U46619-induced Ca^{2+} response. This U46619-induced Ca^{2+} release has been reported to be mediated by IP_3 signaling pathway (Kurata et al., 1993; Yanagisawa et al., 1993). As presented in Fig. 4, U46619 produced a Ca^{2+} release response with an increase of 132 ± 8.7 nM in $[Ca^{2+}]_i$. Pretreatment of the renal arterial myocytes with either 8-br-cADPR (30 μM) or ryanodine (50 μM) for 20 min was without significant effect on the Ca^{2+} release response induced by U46619.

$CaCl_2$-induced contractile response in small renal arteries

Fig. 5A presents representative video microscopic images depicting the effects of $CaCl_2$ on the diameter of isolated and pressurized small rat renal arteries under control condition or after pretreatment with different reagents. Under control condition, addition of $CaCl_2$ (0.01–4 mM) in the Ca^{2+}-free bath solution produced a concentration-dependent vasoconstrictor response, as shown by decrease in arterial diameter. In the presence of 8-br-cADPR (30 μM) or ryanodine (50 μM), the contractile response of these arteries to $CaCl_2$ was significantly attenuated, although not totally abolished. It should be noted that no alterations of the resting diameter of the arteries were detected in the presence of 8-br-cADPR or ryanodine in the Ca^{2+}-free bath solution. Fig. 5B summarized the effects of 8-br-cADPR and ryanodine on $CaCl_2$-induced vasoconstriction of these isolated rat renal arteries. A concentration-dependent contractile response to $CaCl_2$ with a maximal decrease in arterial diameter of 41.0% was observed. The contractile response curve was shifted down and left when the arteries were pretreated by either 8-br-cADPR

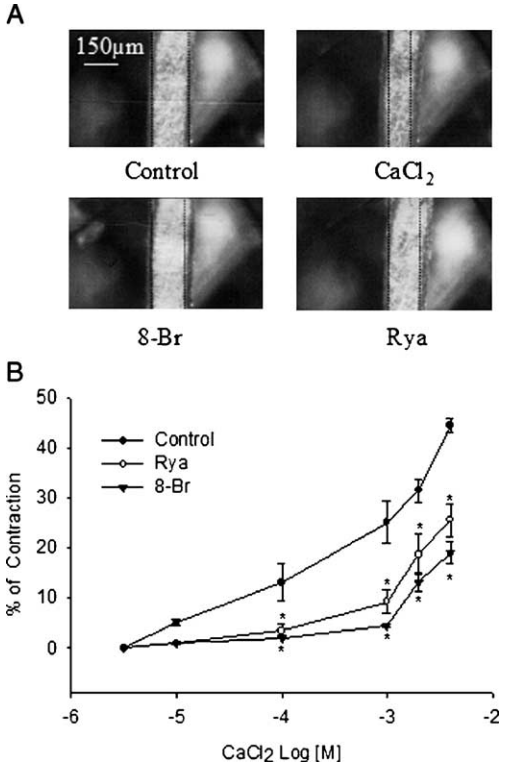

Fig. 5. $CaCl_2$-induced vasoconstriction in isolated and pressurized small renal arteries in the absence or presence of 8-br-cADPR (8-Br) or ryanodine (Rya). (A) Representative photo prints showing the changes in the internal diameters of small renal arteries treated with control, $CaCl_2$ alone, $CaCl_2$ + 8-br-cADPR or $CaCl_2$ + ryanodine. (B) Summarized data showing the effects of $CaCl_2$ before and after pretreatment of the arteries with 8-br-cADPR or ryanodine. *$P < 0.05$ indicates significant difference compared to the values obtained from the arteries only treated with $CaCl_2$ (n = 6 arteries from 6 rats).

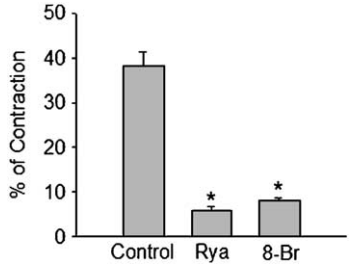

Fig. 6. Caffeine-induced vasoconstriction in isolated and pressurized small renal arteries in the absence or presence of 8-br-cADPR (8-Br) or ryanodine (Rya). *$P < 0.05$ indicates significant difference compared to the values obtained from the arteries only treated with caffeine ($n = 6$ arteries from 6 rats).

or ryanodine, and the maximal reduction of arterial diameter in response to $CaCl_2$ was reduced to 25.5% or 28.9%, respectively ($n = 6$ vessels from 6 rats).

Caffeine-induced contractile response in small renal arteries

In isolated and pressurized small renal arteries, caffeine (1 mM) produced a rapid contractile response when added into the bath solution, and this caffeine-induced vasoconstrictor response only sustained about 1 min. As summarized by Fig. 6, the maximal contractile response to caffeine was 38.2%, as shown by reduction of the internal diameter of the arteries. Pretreatment of the arteries with 8-br-cADPR or ryanodine reduced the maximal contractile response to 5.8% or 8.1%, respectively, corresponding to a decrease of 80% ($n = 6$ vessels from 6 rats).

Endogenous levels of cADPR in renal arterial myocytes before and after exposure to $CaCl_2$ or caffeine

Using cultured renal arterial myocytes, we determined cADPR levels in the absence or presence of $CaCl_2$ or caffeine. As summarized in Fig. 7A, cADPR was detectable in renal arterial myocytes with a concentration range at 1–5 pmol/mg protein. In control renal arterial myocytes, the basal level of cADPR was 2.92 ± 0.52 pmol/mg protein corresponding cADPR concentration of 60 nM by calculation ($n = 8$). Pretreatment of renal arterial myocytes with $CaCl_2$ (1 mM) or caffeine (1 mM) for 5 min was not found to have a significant change in intracellular cADPR levels.

ADP-ribosyl cyclase activity in renal arterial myocytes before and after exposure to $CaCl_2$ or caffeine

Using fluorescence HPLC analysis, cyclic GDP-ribose (cGDPR) production converted from β-NGD^+ by cultured renal arterial myocytes was measured. This conversion of β-NGD^+ into cGDPR was used since cGDPR cannot be metabolized by ADP-ribosyl cyclase, leading to more accurate quantitation of ADP-ribosyl cyclase. This product coeluted with the cGDPR standard at a retention time of 2.2 min. Under control conditions, the conversion rate of β-NGD^+ into cGDPR was 25.67 ± 2.40 pmol/min/mg protein ($n = 8$). In the presence of the ADP-ribosyl cyclase inhibitor, nicotinamide (6 mM), the conversion of β-NGD^+ to cGDPR was substantially inhibited ($n = 8$). However, in renal arterial myocytes incubated with $CaCl_2$ or caffeine, no significant changes in cGDPR production were observed (Fig. 7B).

Colocalization of FKBP12.6 and ryanodine receptors in renal arterial myocytes before and after exposure to $CaCl_2$

Fig. 8 presents typical confocal microscopic images showing the effects of $CaCl_2$ on the colocalization of FKBP12.6 and ryanodine receptors in renal arterial myocytes under control condition or after pretreatment with 8-Br-cADPR. Under control condition, FKBP12.6, visualized as several intense spots of fluorescence within the cells, was found to colocalize with ryanodine receptors (yellow in overlay images). Addition of $CaCl_2$ (1 mM) produced a significant decrease in FKBP12.6 fluorescence intensity (colocalized with ryanodine receptors). In the presence of 8-Br-cADPR (30 μM), this decrease in FKBP12.6 colocalization with ryanodine

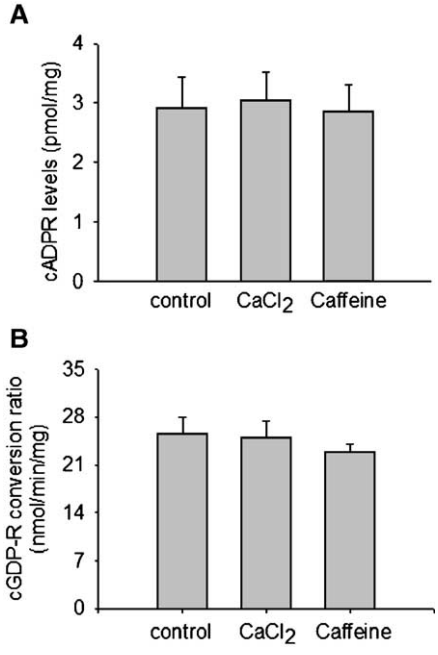

Fig. 7. Effects of $CaCl_2$ or caffeine on cADPR levels and ADP-ribosylcyclase activity in renal arterial myocytes. (A) Endogenous levels of cADPR in renal arterial myocytes before and after exposure to $CaCl_2$ ($P > 0.05$) ($n = 8$ cells from 7 rat kidneys). (B) ADP-ribosyl cyclase activity in renal arterial myocytes before and after exposure to caffeine ($P > 0.05$) ($n = 8$ cells from 7 rat kidneys).

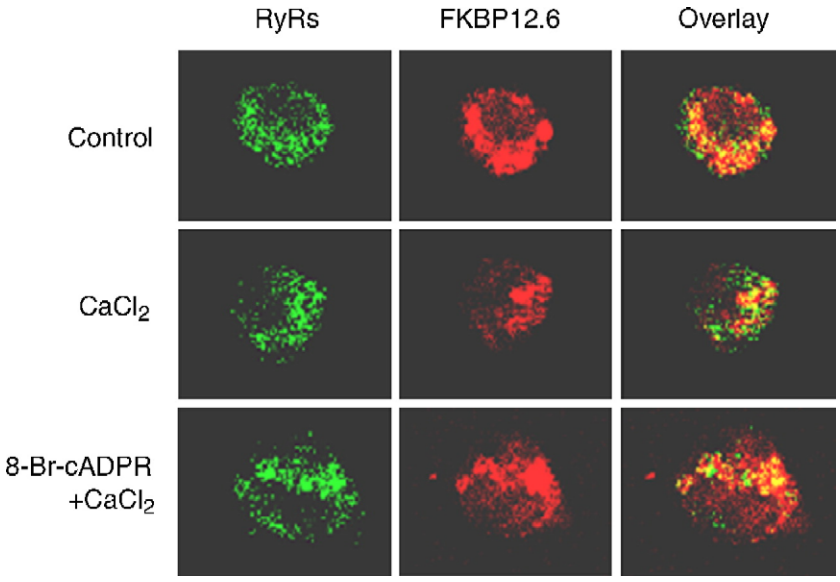

Fig. 8. Colocalization of FKBP12.6 and ryanodine receptors (RyRs) in renal arterial myocytes before and after exposure to $CaCl_2$. Representative confocal microscopic images showing colocalization of BODIPY-tagged RyR2 TM domains (green), Texas Red (TR)-conjugated F(ab)2-fragments of FKBP12.6 antibody domain (red), and overlay images (yellow). FKBP12.6: FK506 binding protein 12.6.

receptors in renal arterial myocytes-induced by $CaCl_2$ was substantially blocked.

Discussion

Recent studies in our laboratory have shown that an enzymatic pathway for cADPR production and metabolism is present along the renal vasculature (Li et al., 2000), but the physiological role of endogenous cADPR in these blood vessels remains unclear. To further determine the contribution of cADPR to the regulation of intracellular Ca^{2+} concentrations in renal arterial myocytes, the present study was designed to first examine the effects of a cADPR antagonist, 8-br-cADPR on $CaCl_2$- and caffeine-induced Ca^{2+} release and vasoconstriction, and then elucidate the mechanism by which cADPR exerts its action during this elevation of intracellular Ca^{2+} in renal arterial myocytes. We demonstrated that extracellular Ca^{2+} or caffeine stimulated ryanodine receptor-mediated Ca^{2+} release in freshly dissociated renal arterial myocytes. Blockade of cADPR actions by 8-br-cADPR significantly attenuated Ca^{2+} release response of the renal arterial myocytes to Ca^{2+} influx or caffeine, and also reduced $CaCl_2$ or caffeine-induced vasoconstriction in small renal arteries. By confocal microscopy, extracellular Ca^{2+} was found to result in FKBP12.6 dissociation from ryanodine receptors on the SR of renal arterial myocytes. These results provide evidence that cADPR-mediated Ca^{2+} signaling importantly participates in Ca^{2+}-induced Ca^{2+} release (CICR) in renal arterial myocytes and consequent renal vasoconstriction.

In the first series of experiments, we determined the effects of cADPR antagonism on $CaCl_2$-induced Ca^{2+} release in freshly isolated renal arterial myocytes. These cells were incubated with a Ca^{2+}-free bath solution, and then different concentrations of $CaCl_2$ were added into the bath solution to activate Ca^{2+} release response from their intracellular stores. This Ca^{2+} release is dependent on the initial Ca^{2+} influx and therefore is known as a CICR testing model. By using fluorescent microscopic spectrometry, an intracellular Ca^{2+} transient increase that consisted of a rapid peak increase in $[Ca^{2+}]_i$ followed by a sustained rise in $[Ca^{2+}]_i$ was observed when $CaCl_2$ was introduced into the bath. In the previous studies, the rapid peak increase in $[Ca^{2+}]_i$ in single cell recordings was characterized, and it was primarily determined by intracellular Ca^{2+} release. The sustained increase in $[Ca^{2+}]_i$ in this transient Ca^{2+} response was mainly due to Ca^{2+} influx (Salomonsson and Arendshorst, 1999; Inscho et al., 1999a,b). As shown in the representative microscopic spectrometric recordings (Fig. 1A), both Ca^{2+} influx (sustained increase) and Ca^{2+} release (peak increase) in rat renal arterial myocytes can be observed when $CaCl_2$ was added into the Ca^{2+}-free bath solution. Pretreatment of these renal arterial myocytes with 8-br-cADPR, a cell permeant antagonist of cADPR, substantially attenuated $CaCl_2$-induced rapid peak increase in $[Ca^{2+}]_i$, suggesting the contributing role of cADPR-mediated signaling pathway in the CICR in these cell. However, 8-br-cADPR had no effect on the sustained increase in $[Ca^{2+}]_i$ induced by $CaCl_2$ (Fig. 1B). This cADPR antagonist also had no effect on Ca^{2+} release response to U46619, a well-known IP_3 signaling activator (Yamagishi et al., 1992; Kurata et al., 1993; Yanagisawa et al., 1993; Tosun et al., 1998) (Fig. 4). These results further support the view that endogenous cADPR could activate or enhance Ca^{2+} influx-induced Ca^{2+} release in renal arterial myocytes.

The present study also addressed the direct relationship of $CaCl_2$-induced Ca^{2+} release to ryanodine-sensitive intracellular Ca^{2+} stores in the sarcoplasmic reticulum (SR) of renal arterial myocytes. Recent studies have indicated that cADPR activates ryanodine receptor-mediated Ca^{2+} release from the SR in a variety of tissues or cells, which is one of the most important mechanisms of CICR (Galione et al., 1991; Lee, 1993; Lee et al., 1995). However, little is known whether this ryanodine receptor-mediated Ca^{2+} release contributes to CICR and cADPR signaling in renal vascular smooth muscle. In a recent study, we reported that a CICR blocker, tetracaine markedly decreased exogenous cADPR-induced Ca^{2+} release response in renal arterial myocytes (Li et al., 2000), which suggested the functional presence of CICR and the potential involvement of cADPR in CICR of these cells. Demonstration in the present study that a relatively high concentration of ryanodine (50 μM) blocked $CaCl_2$-induced Ca^{2+} release further confirms that $CaCl_2$-stimulated, cADPR-mediated Ca^{2+} release is attributed to activation of ryanodine receptors on the SR of renal arterial myocytes, which is also consistent with previous evidence that the entry of Ca^{2+} through voltage-operating channels triggers CICR, resulting in a global Ca^{2+} increase throughout the cytoplasma and nucleus (Berridge, 1997).

To further determine the role of cADPR in CICR, we examined the effects of 8-br-cADPR on caffeine-stimulated Ca^{2+} release in renal arterial myocytes. Caffeine is a well-known CICR activator, and it can produce Ca^{2+} release from the SR through ryanodine receptors in a variety of cell types including vascular myocytes. It was found that caffeine at a concentration of 1 mM produced a rapid Ca^{2+} release response in renal arterial myocytes that were exposed to a Ca^{2+}-free bath solution. This caffeine-induced Ca^{2+} release can be repeatedly activated after the cells were reloaded with Hanks' buffer containing 2.5 mM $CaCl_2$. Since caffeine-activated CICR is independent of Ca^{2+} influx, these experiments could directly determine Ca^{2+} release from the SR without interference of Ca^{2+} influx. We found that the cADPR antagonist, 8-br-cADPR completely blocked caffeine-induced Ca^{2+} release response, which was similar to the inhibiting action of blockade of ryanodine receptors by ryanodine. This suggests that the action of cADPR in CICR does couple with ryanodine receptor-mediated mechanisms in the SR of renal arterial myocytes.

We next determined whether this cADPR-activated CICR via ryanodine receptors is of physiological significance with respect to the vasomotor response. In previous studies, it has been shown that it is CICR that amplifies intracellular Ca^{2+} signaling activated by Ca^{2+} influx in vascular smooth muscle cells and thereby produces global Ca^{2+} increase, resulting in vasoconstriction in the small arteries or arterioles. By simultaneous recording of the voltage-gated Ca^{2+} channel currents and Ca^{2+} fluorescence imaging, those studies demonstrated that opening of Ca^{2+} channels by a pulse increase in membrane potential produced a large Ca^{2+} release response and consequent vasoconstriction, which provides direct evidence that Ca^{2+} influx-induced Ca^{2+} release is present in vascular myocytes and it contributes to the vasomotor response (Ganitkevich and Isenberg, 1995; Asano et al., 1996; Garcha and Hughes, 1997). The findings from our renal arterial preparations in the present study further confirm the role of CICR via ryanodine receptors in mediating renal vasoconstriction. We found that $CaCl_2$ produced a concentration-dependent contractile response in isolated and pressurized small renal arteries bathed with a Ca^{2+}-free solution, and that this response was significantly attenuated by pretreatment of the arteries with cADPR antagonist, 8-br-cADPR. However, 8-br-cADPR did not completely block the vasoconstrictor response to $CaCl_2$. This reflects that cADPR may partially contribute to this CICR-associated renal vasoconstriction. Similarly, the present study demonstrated that ryanodine also partially diminished the vasoconstrictor response of the renal arteries to $CaCl_2$. Taken together, these results indicate that the cADPR/ryanodine receptor-mediated Ca^{2+} signaling pathway is one of the mechanisms mediating CICR in renal arterial myocytes and related renal vasoconstriction.

In additional experiments, we examined the role of 8-br-cADPR in the vasoconstrictor response of small renal arteries to a direct activation of ryanodine receptors by caffeine. It was found that caffeine at a concentration of 1 mM produced a rapid vasoconstriction in small renal arteries exposed to a Ca^{2+}-free solution, which could be repeated after "recharging" the SR by a short-term pretreatment of the arteries with KCl. However, either 8-br-cADPR or ryanodine significantly blocked this caffeine-induced contractile response. It is concluded that cADPR is capable of altering the activity of ryanodine receptors and thereby enhances CICR, producing the contractile response in renal arterial muscle.

Previous studies proposed two mechanistic models to elucidate the role of endogenous cADPR in regulating Ca^{2+} release response in vascular myocytes. In the first model, cADPR is proposed to serve as a second messenger to mediate the Ca^{2+} release response to different stimuli. Along this line, some agonists or stimuli stimulate ADP-ribosylcyclase to produce cADPR, leading to activation of Ca^{2+} release from the SR through the ryanodine receptors. However, our data do not support this view, because activation of ADP-ribosylcyclase as well as an increase of intracellular cADPR levels in renal arterial myocytes were not observed when these cells were challenged by $CaCl_2$ or caffeine. Another model considers cADPR as a modulator of CICR or ryanodine receptors. In this way, cytosolic cADPR sensitizes the ryanodine receptors, enhancing CICR activated by agonists or Ca^{2+} influx. This sensitizing action is associated with its action to enhance dissociation of ryanodine receptor from accessory protein, FKBP 12.6 (Brillantes et al., 1994; Li et al., 2002; Valdivia, 1998). In the present study, confocal fluorescence imaging analysis was performed and confirmed the presence of FKBP12.6, an accessory protein of the ryanodine receptors and colocaliza-

tion of this protein with ryanodine in renal arterial myocytes (Fleischer and Inui, 1989; Tang et al., 2002). $CaCl_2$ significantly decreased the FKBP12.6 fluorescence colocalized with ryanodine receptors. However, pretreatment of cells with 8-Br-cADPR markedly attenuated $CaCl_2$-induced decrease in colocalization of these proteins, suggesting that cADPR may mediate the dissociation of FKBP 12.6 protein from ryanodine receptors under this condition. To our knowledge, these results provide first evidence that FKBP12.6 serves as a regulatory protein to control the activity of the ryanodine receptors on the SR in renal arterial myocytes and that Ca^{2+} influx by $CaCl_2$ dissociates this accessory protein from ryanodine receptor through cADPR pathway. Therefore, our results suggest that cADPR exerts a modulator action to enhance the sensitivity of ryanodine receptors by dissociating FKBP 12.6 in renal arterial myocytes, which increases Ca^{2+} release responses to different stimuli.

In summary, the present study demonstrated that extracellular Ca^{2+} and caffeine stimulated ryanodine receptor-mediated Ca^{2+} release in freshly dissociated renal arterial myocytes and thereby produced vasoconstriction in small renal arteries. The antagonism of the cADPR action by 8-br-cADPR attenuated both the Ca^{2+} release response of the renal arterial myocytes and vasoconstriction of the arteries. This inhibitory action of 8-br-cADPR in renal arterial smooth muscle was similar to that caused by blockade of ryanodine receptors using ryanodine. It was also found that extracellular increase in Ca^{2+} levels and caffeine did not increase cADPR production, but altered the sensitivity of ryanodine receptors to Ca^{2+} by dissociating their accessory proteins. Taken together, we conclude that cADPR importantly participates in CICR through enhancing ryanodine receptor activation in renal arterial myocytes and thus contributes to the control of $[Ca^{2+}]_i$ in these cells and renal vasoconstrictor response to Ca^{2+} influx.

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