Discovery of a small-molecule inhibitor for kidney ADP-ribosyl cyclase: Implication for intracellular calcium signal mediated by cyclic ADP-ribose

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Abbreviations: ADPR-cyclase, ADP-ribosyl cyclase; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; $[Ca^{2+}]_o$, extracellular calcium ion; cADPR, cyclic ADP-ribose; CaSR, extracellular calcium-sensing receptor; DHAB, 4,4'-dihydroxy-azobenzene; MMC, mouse kidney mesangial cells; NGD⁺, nicotinamide guanine dinucleotide

Abstract

ADP-ribosyl cyclase (ADPR-cyclase) produces a Ca^{2+} -mobilizing second messenger, cyclic ADP-ribose (cADPR), from β -NAD⁺. A prototype of mammalian ADPR-cyclases is a lymphocyte antigen CD38. Accumulating evidence indicates that ADPR-cyclases other than CD38 are expressed in various cells and organs. In this study, we discovered a small molecule inhibitor of kidney ADPR-cyclase. This compound inhibited kidney ADPR-cyclase activity but not CD38, spleen, heart or brain ADPR-cyclase activity *in vitro*. Characterization of the compound in a cell-based system revealed that an extracellular calcium-sensing receptor (CaSR)-mediated cADPR production and a later long-lasting increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse mesangial cells were inhibited by the pre-treatment with this compound. In contrast, the compound did not block CD3/TCR-induced cADPR production and the increase of $[Ca^{2+}]_i$ in Jurkat T cells,

which express CD38 exclusively. The long-lasting Ca^{2+} signal generated by both receptors was inhibited by pre-treatment with an antagonistic cADPR derivative, 8-Br-cADPR, indicating that the Ca^{2+} signal is mediated by the ADPR-cyclase metabolite, cADPR. Moreover, among structurally similar compounds tested, the compound inhibited most potently the cADPR production and Ca^{2+} signal induced by CaSR. These findings provide evidence for existence of a distinct ADPR-cyclase in the kidney and basis for the development of tissue specific inhibitors.

Keywords: ADP-ribosyl cyclase; antagonists and inhibitors; antigens, CD38; calcium signaling; kidney; mesangial cells

Introduction

ADP-ribosyl cyclase (ADPR-cyclase) is widely distributed and plays a critical role in regulation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) via cyclic ADP-ribose (cADPR) production (Guse *et al.*, 1999; Galione *et al.*, 2000; Lee, 2001). The metabolite cADPR is known to increase $[Ca^{2+}]_i$ by releasing from intracellular Ca^{2+} stores or by Ca^{2+} influx through plasma membrane Ca^{2+} channels in a variety of cells (Guse *et al.*, 1999; Galione *et al.*, 2000; Lee, 2001; Partida-Sanchez *et al.*, 2001). Studies have indicated that a Zn^{2+} -sensitive and reducing agent-insensitive ADPR-cyclase(s), which differs from CD38 or *Aplysia californica* ADPR-cyclase, is present in brain, heart, kidney, arterial smooth muscle cells, and bone marrow cells (Hirata *et al.*, 1994; de Toledo *et al.*, 2000; Ceni *et al.*, 2003; Zielinska *et al.*, 2004; Xie *et al.*, 2005). A study with mice disrupted CD38 gene has demonstrated that formation of cADPR is not reduced greatly in mouse kidney, brain, and heart (Partida-Sanchez *et al.*, 2001), suggesting that ADPR-cyclases other than CD38 exist. However, the ADPR-cyclase(s) present in these tissues has not been cloned, and the cADPR antagonistic derivatives such as 8-Br-cADPR and 8-NH₂-cADPR do not distinguish the Ca^{2+} signals induced by the unidentified ADPR-cyclase or CD38 (Walseth and Lee, 1993). Moreover, specific inhibitors of ADPR-

cyclases have neither been found nor synthesized.

As bioactive small-molecules capable of inducing a specific phenotype serve as tools to probe the functions of protein and signaling pathways (Schreiber, 1998; Kwon, 2003), we expect that specific ADPR-cyclase inhibitors would be useful for demonstrating the existence of kidney ADPR-cyclase, for providing specific tools to distinguish the ADPR-cyclase from other ones originated from different tissues, and as potential lead molecules targeting the ADPR-cyclase for therapeutic benefit. To this end, we partially purified ADPR-cyclase from rat kidney, characterized, and screened a chemical library. Here, we report the discovery of a bioactive small-molecule inhibitor of the kidney ADPR-cyclase.

Materials and Methods

Materials

Anti-human CD3 monoclonal antibody (clone OKT3) was obtained from eBioscience (San Diego, CA). Nicotinamide 1,N⁶-ethenoadenine dinucleotide (ϵ -NAD⁺), nicotinamide guanine dinucleotide (NGD⁺), protease inhibitors, heparin-agarose and other chemicals were from Sigma-Aldrich (St. Louis, Mo). Q-Sepharose, Chelating-Sepharose, and CM-Sepharose were purchased from Amersham Biosciences (Piscataway, NJ). 4,4'-Dihydroxyazobenzene (DHAB) and chemical library were purchased from ComGenex (Budapest, Hungary) and Chembridge (San Diego, CA).

Partial purification of ADPR-cyclase from rat kidney

Kidneys from Sprague-Dawley rats weighing 250–300 g were washed with ice-cold PBS, minced in a lysis buffer (50 mM HEPES buffer, pH 7.2, 150 mM NaCl, 10% Glycerol, and 1 mM PMSF) and homogenized with a Teflon glass homogenizer in an ice bath. After centrifugation at $500 \times g$ for 10 min, the supernatant was centrifuged at $100,000 \times g$ for 1 h. Pellets obtained were solubilized with 1% Triton X-100 in the lysis buffer for 1 h. After centrifugation at $100,000 \times g$ for 1 h, the extract was used to purify ADPR-cyclase. We found that ADPR-cyclase did not bind to CM-Sepharose and heparin-agarose and therefore, they were used for the negative purification. The flow-through fractions from these columns were loaded onto Cu²⁺-chelating Sepharose, washed with five column volume of the lysis buffer containing 0.1% Triton X-100, and eluted with 200 mM imidazole in the same buffer. Overall yield was approximately 15% with 64-fold purification.

Assays for ADPR-cyclase and cADPR-hydrolase assays

Specific ADPR-cyclase activity was determined by measuring cyclic GDP-ribose (cGDPR) or etheno-ADP-ribose (ϵ -ADPR) fluorometrically using NGD⁺ or ϵ -NAD⁺ as a substrate (Greaff *et al.*, 1994). Samples were incubated in the presence of 200 μ M NGD⁺ or ϵ -NAD⁺ with and without an appropriate agent in an assay buffer (25 mM HEPES, pH 7.4, 100 mM NaCl, and 0.1% Triton X-100) in a 50 μ l-final volume. The reaction mixture was incubated at 37°C for 10 min. The reaction was stopped by adding 50 μ l trichloroacetic acid (10%). The samples were centrifuged at $21,000 \times g$ for 10 min and the supernatant (80 μ l) was diluted with 920 μ l of 100 mM sodium phosphate buffer (pH 7.2). Fluorescence of cGDPR or ϵ -ADPR in the solution was determined at excitation/emission wavelengths of 297/410 nm (HITACHI F-2000 fluorescence spectrophotometer). cADPR-hydrolase activity was determined by incubating cADPR with ADPR-cyclase or CD38 at 37°C for 20 min. Hydrolysis of cADPR was analyzed by high performance liquid chromatography as described (White *et al.*, 2000).

Chemical library screening

Using a chemical library of approximately 10,000 compounds, the screening of ADPR-cyclase inhibitor was conducted in the reaction mixture with partially purified rat kidney ADPR-cyclase, chemical compound, and 200 μ M ϵ -NAD⁺ in the assay buffer in a 50 μ l-final volume. Formation of cADPR was determined as described above after incubation of the reaction mixtures at room temperature for 60 min. Concentration of compounds used in all enzyme inhibitory assays was 200 μ M unless otherwise indicated.

Cell culture

Mouse kidney mesangial cells (MMC) were kindly provided by S. H. Cha (Kwak *et al.*, 2005) and cultured in DMEM supplemented 5% heat inactivated FBS, 0.25 μ g/ml amphotericin B, penicillin (100 U/ml), and streptomycin (100 U/ml). Jurkat T cells were obtained from American Tissue Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 supplemented with 10% FBS and antibiotics described above. The cells were maintained in a humidified incubator at 37°C in the presence of 5% CO₂ and 95% air.

Measurement of intracellular cADPR concentration [cADPR]_i

[cADPR]_i was measured using a cyclic enzymatic

Figure 1. Effects of Zn²⁺ and DTT on the activity of rat kidney ADPR-cyclase and CD38. Partially purified rat kidney ADPR-cyclase (10 µg/assay) or recombinant human CD38 (10 µg/assay) was incubated with various concentrations of ZnCl₂ (A), and DTT (B) at 37°C for 10 min and the ADPR-cyclase activity was measured by using NGD⁺ as the substrate.

assay as described previously (Graeff and Lee, 2002). *Aplysia californica* ADPR-cyclase was purified from sea urchin egg according to the method described (Lee and Aarhus, 1991).

Measurement of [Ca²⁺]_i

Changes in [Ca²⁺]_i in MMC and Jurkat T cells were determined as described previously (Kim *et al.*, 2006). Briefly, MMC cultured on confocal dishes for 48 h or Jurkat T cells seeded on 100 µg/ml poly L-lysine-coated confocal dishes at a concentration of 10⁴ cells/dish were washed with Hank's balanced salt solution (HBSS) (2 mM CaCl₂, 145 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 5 mM D-glucose, and 20 mM HEPES, pH 7.3) and then loaded with 5 µM Fluo3 AM (Molecular Probe, Eugene, OR). Changes in Ca²⁺ fluorescence were determined at 488 nm/530 nm (excitation/emission) by air-cooled argon laser system. [Ca²⁺]_i was calculated using a K_d of 325 nM for Fluo3 AM by the method of Tsien *et al.* (1982).

Cell proliferation assay

MMC was seeded at a concentration of 5 × 10³ cells/well on a 12-well plate. After incubating for 1 day, 10 mM [Ca²⁺]_o was treated for 2 days (Kwak *et al.*, 2005). The cells were then detached using 0.25% trypsin-EDTA, and the number of cells was counted after trypan blue staining to exclude dead cells.

Statistical analysis

Data represent means ± standard error of the mean (SEM) of at least three separate experiments.

Statistical analysis was performed using Student's *t*-test. A value of *P* < 0.05 was considered significant.

Results and Discussion

Characterization of ADPR-cyclase from rat kidney

As a confirmation for the study, we firstly examined whether kidney ADPR-cyclase exhibits different biochemical properties from those of CD38. Zinc ion is known to stimulate the cADPR forming cyclase activity of CD38 but inhibits its cADPR-hydrolase activity and a reducing agent, 1,4-dithiothreitol (DTT) has been shown to inhibit both ADPR-cyclase and cADPR-hydrolase activities (Kukimoto *et al.*, 1996). The effects of these molecules on the kidney ADPR-cyclase and CD38 were evaluated. Zinc stimulated the cyclase activity of CD38 but inhibited kidney ADPR-cyclase activity (Figure 1A). On the other hand, DTT inhibited the cyclase activity of CD38 but exhibited no effect on the kidney ADPR-cyclase (Figure 1B). When cADPR-hydrolase activity of the kidney ADPR-cyclase was determined, we found that cADPR-hydrolase activity of the kidney enzyme was not detected whereas CD38 exhibited cADPR-hydrolase activity that was inhibited by Zn²⁺ (data not shown). These results demonstrate that the kidney ADPR-cyclase is distinct from CD38 in biochemical properties.

Identification of 4,4'-dihydroxy azobenzene (DHAB) as a kidney ADPR-cyclase inhibitor

Having known that kidney ADPR-cyclase differs

from CD38, we screened a chemical library of approximately 10,000 compounds for small-molecule inhibitors of the kidney ADPR-cyclase. This screen resulted in the selection of DHAB as a small-molecule inhibitor (Figure 2A). The compound was able to inhibit generation of cGDPR and ϵ -ADPR from NGD^+ and $\epsilon\text{-NAD}^+$, respectively, by the kidney

ADPR-cyclase in a concentration-dependent manner. These data suggest that the compound may bind to the active site of the enzyme. Half maximal inhibition (IC_{50}) of the enzyme activity was approximately 100 μM (Figure 2B). CD38 and ADPR-cyclases purified partially from rat brain, heart, and spleen tissues were insensitive to DHAB at 200 μM (Figure 2C).

Figure 2. DHAB competes with the substrate of ADPR-cyclase. (A) Structure of DHAB. (B) Inhibition of rat kidney ADPR-cyclase by DHAB. The enzyme (10 $\mu\text{g}/\text{tube}$) was incubated with 200 μM NGD^+ or $\epsilon\text{-NAD}^+$ in the presence of various concentrations of DHAB at 37°C for 10 min. DHAB was preincubated with the enzyme at 4°C for 1 h. (C) Effects of DHAB on human CD38, rat brain, heart, spleen, and kidney ADPR-cyclase. The mean $\pm$ SEM of three independent experiments is shown. *Control versus DHAB, $P < 0.001$.

Stimulation of extracellular calcium ion ($[\text{Ca}^{2+}]_o$)-sensing receptor (CaSR) in mouse mesangial cells (MMC) produces cADPR and a long-lasting Ca^{2+} signal

Inhibitory potency of DHAB on the ADPR-cyclase activity is critical for studying the signaling or inducing phenotype in a cell-based system. In MMC, an ADPR-cyclase activating receptor has not been identified. However, a number of G protein-coupled receptors (GPCRs) have been shown to utilize ADPR-cyclase in regulation of $[\text{Ca}^{2+}]_i$ (Higashida *et al.*, 1999; 2000; Boittin *et al.*, 2003; Ge *et al.*, 2003; Sternfeld *et al.*, 2003; Fellner and Arendshorst, 2005). We examined several ligands of GPCRs, which are known to stimulate cADPR production in various cells upon binding to the cognate receptor. Among them, stimulation of CaSR with $[\text{Ca}^{2+}]_o$ resulted in a significant increase of $[\text{cADPR}]_i$ (Figure 3A) and a generation of long-lasting increase of $[\text{Ca}^{2+}]_i$, a burst Ca^{2+} rise followed by a sustained Ca^{2+} rise that was gradually decreased (Figure 3B). The sustained Ca^{2+} signal, but not the initial burst Ca^{2+} rise, was blocked by pre-treatment with an antagonistic cADPR, 8-Br-cADPR (Figure 3C). On the basis of these results that stimulation of CaSR activates ADPR-cyclase in MMC, we evaluated DHAB as a possible candidate inhibitor of ADPR-cyclase. This compound was able to inhibit $[\text{Ca}^{2+}]_o$ -mediated later sustained elevation of $[\text{Ca}^{2+}]_i$ but not the initial rise of $[\text{Ca}^{2+}]_i$ in a dose-dependent manner (Figure 4A-G). Further, $[\text{Ca}^{2+}]_o$ -induced production of cADPR was also blocked by pre-treatment of DHAB in a concentration-dependent manner (Figure 4H). IC_{50} was approximately 2.5 nM. In addition, since it has been reported that CaSR-mediated Ca^{2+} signal is involved in MMC proliferation (Kwak *et al.*, 2005), we examined whether DHAB inhibits the $[\text{Ca}^{2+}]_o$ -induced MMC proliferation. As presented in Figure 4I, the $[\text{Ca}^{2+}]_o$ -induced increment of proliferation was also inhibited by DHAB in a similar range of concentrations observed in the inhibition of the sustained Ca^{2+} signal, suggesting that cADPR-induced sustained Ca^{2+} signal regulates MMC proliferation.

An efficacy of DHAB for inhibition of kidney ADPR-cyclase was further evaluated utilizing DAHB

Figure 3. Stimulation of CaSR with $[Ca^{2+}]_o$ resulted in a significant increase of $[cADPR]_i$ and a long-lasting $[Ca^{2+}]_i$. (A) $[cADPR]_i$ is increased in response to 10 mM $CaCl_2$ treatment. MMC was incubated with 10 mM $CaCl_2$ at 37°C for 2 min. Formation of cADPR was determined as described under Experimental Procedures. The mean $\pm$ SEM of three independent experiments is shown. *Control versus 10 mM $CaCl_2$, $P < 0.001$. (B) Treatment of MMC with 10 mM $CaCl_2$ generates a long-lasting intracellular Ca^{2+} signal. (C) Pre-treatment of MMC with 8-Br-cADPR (100 μ M) for 30 min abolishes $[Ca^{2+}]_o$ -induced sustained Ca^{2+} increase, whereas the initial Ca^{2+} increase remains. n indicates the cell numbers.

Figure 4. DAHB inhibits $[Ca^{2+}]_o$ -induced Ca^{2+} signal in MMC in a concentration-dependent manner. (A) Treatment of MMC with 10 mM $CaCl_2$ generates a long-lasting intracellular Ca^{2+} signal. (B-E) $[Ca^{2+}]_o$ -induced sustained Ca^{2+} signal in MMC is inhibited by pre-treatment of various concentrations of DAHB (0.5, 1, 5, and 10 nM) for 1 h. (F) Effects of various concentrations of DAHB on $[Ca^{2+}]_o$ -induced initial Ca^{2+} increase in MMC at 54 sec. *Control versus 10 mM $CaCl_2$ or 10 mM $CaCl_2$ versus DAHB, $P < 0.001$. (G) Effects of various concentrations of DAHB on $[Ca^{2+}]_o$ -induced Ca^{2+} increase in MMC at 300 s. *Control versus 10 mM $CaCl_2$, $P < 0.001$; **10 mM $CaCl_2$ versus 10 mM $CaCl_2$ plus DAHB, $P < 0.001$. (H) $[Ca^{2+}]_o$ -induced production of cADPR was blocked by pre-treatment of DAHB in a concentration-dependent manner. (I) DAHB inhibits $[Ca^{2+}]_o$ -induced increment of proliferation of MMC in a dose-dependent manner. The mean $\pm$ SEM of three independent experiments is shown. *Control versus 10 mM $CaCl_2$, $P < 0.001$; **10 mM $CaCl_2$ versus 10 mM $CaCl_2$ plus DAHB, $P < 0.001$.

analogs, 2,2'-dihydroxybenzene and azobenzene, and structurally similar compounds, resveratrol and piceatannol that are known to activate SIRT1 activity, which uses NAD as a cofactor (Howitz *et al.*, 2003). Production of cADPR and the later sustained Ca^{2+} signal in response to $[Ca^{2+}]_o$ were inhibited with

different efficacy by these small molecules (Table 1). As expected, among these small molecules, DAHB showed the strongest inhibitory potency. The order of IC_{50} was DAHB > resveratrol > azobenzene = piceatannol > 2,2'-dihydroxy azobenzene. These observations suggest that the biphenyl moiety but

Table 1. Inhibitory potency of DAHB, DAHB analogs, and structurally similar compounds on Ca^{2+} signal and cADPR production in response to extracellular calcium.

Structure	Compound	IC_{50} ($[\text{Ca}^{2+}]_i$, μM) (mean $\pm$ SEM)	IC_{50} ($[\text{cADPR}]_i$, μM) (mean $\pm$ SEM)
	4,4'-Dihydroxy-azobenzene	0.0025 ± 0.0002	0.0025 ± 0.0003
	2,2'-Dihydroxy-azobenzene	15 ± 1.5	16.5 ± 2.5
	Azobenzene	10 ± 1.1	13 ± 2.1
	Resveratrol (3,5,4'-trihydroxy-trans-stilbene)	5 ± 0.8	5.5 ± 0.5
	Piceatannol (3,5,3',4'-tetrahydroxy-trans-stilbene)	10 ± 1.7	8.9 ± 1.5

IC_{50} of the compounds for cADPR production and the later sustained Ca^{2+} signal induced by 10 mM CaCl_2 was determined after incubation for 90 s ($[\text{cADPR}]_i$) and 300 s ($[\text{Ca}^{2+}]_i$) as described in the legend of Figure 4.

not the azo bond and position of hydroxyl group affect the binding of the inhibitor to the enzyme.

Taken together, these data show that DHAB inhibits cADPR production stimulated by CaSR in MMC at ≤ 5 nM and is far more potent in cell-based system than *in vitro*. Although the reasons for the different efficacy of the DHAB are not clearly understood, it might be due to the environment of the enzyme location such as membranes versus detergent or due to nonspecific hindrance of other proteins in the enzyme preparation. It is also possible that the kidney enzyme is a transmembrane protein as CD38 is, so that accessibility of DAHB to the enzyme in the cell system is far greater than that in detergent. A prototype of ADPR-cyclases, CD38 is a type II transmembrane protein in which the active site is located at the extracellular domain (Han *et al.*, 2000; An *et al.*, 2001). In fact, the kidney enzyme was found in plasma membrane

rich fraction but not cytosol (data not shown).

Specificity of DHAB

Above results obtained from *in vitro* study indicated that DHAB is specific for the kidney ADPR-cyclase. To evaluate this finding further, we utilized a human T cell-derived cell line, Jurkat T cell. Jurkat T cells express exclusively CD38 that is regulated by CD3/TCR (Zubiaur *et al.*, 1997). Treatment of Jurkat T cells with OKT3, which is a ligand for CD3/TCR, showed a typical biphasic increase of $[\text{Ca}^{2+}]_i$, an initial peak rise followed by a sustained rise (Figure 5A). Pre-treatment with 8-Br-cADPR inhibited only the sustained Ca^{2+} rise (Figure 5B). In contrast, DHAB did not show any effects on OKT3-mediated Ca^{2+} rise even at 10 μM (Figure 5C). Effects of these agents on OKT3-induced Ca^{2+} signal was summarized in Figure 5D and 5E. Supporting the

Figure 5. DHAB does not inhibit CD38-involved cADPR production and Ca^{2+} signal in Jurkat T cells. (A) Treatment of Jurkat T cells with 10 $\mu\text{g}/\text{ml}$ OKT3 generates a long-lasting intracellular Ca^{2+} signal. (B) Pre-treatment of Jurkat T cells with 8-Br-cADPR (100 μM) for 30 min abolishes OKT3-induced sustained Ca^{2+} increase, whereas the initial Ca^{2+} increase remains. (C) Pre-treatment of Jurkat T cells with DHAB (10 μM) for 1 h has no effect on the long-lasting intracellular Ca^{2+} signal induced by OKT3. (D) Effects of various agents on OKT3-induced initial Ca^{2+} increase at 54 s. *Control versus OKT3 or OKT3 plus 8-Br-cADPR or OKT3 plus DHAB, $P < 0.001$. (E) Effects of various agents on OKT3-induced Ca^{2+} increase at 300 s. *Control versus OKT3 or OKT3 plus DHAB, $P < 0.001$; **OKT3 versus OKT3 plus 8-Br-cADPR, $P < 0.001$. (F) Effect of various concentrations of DHAB on OKT3-mediated increase of [cADPR]. Jurkat T cells were incubated with 10 $\mu\text{g}/\text{ml}$ OKT3 for 2 min. Formation of cADPR was determined as described under Experimental Procedures. *Control versus OKT3 or OKT3 plus DHAB, $P < 0.001$.

observations, OKT3-induced cADPR production was not blocked by pre-treatment with DHAB (Figure 5F).

In the present study, we have discovered a small and potent molecule, DHAB, that inhibits specifically the kidney ADPR-cyclase at ≤ 5 nM. The discovery of the specific inhibitor for the enzyme enables us to provide further evidence that there are ADPR-cyclases different from CD38. More importantly, our data provide the basis for the discovery of bioactive small-molecule ADPR-cyclase inhibitors, utilizing partially purified enzyme and cell-based system. Benefits of the kidney ADPR-cyclase specific inhibitor are several folds: use of DHAB may facilitate understanding of kidney functions involving regulation of Ca^{2+} homeostasis; the inhibitor may help to understand pathogenesis of the kidney; this compound can be the basis for development of

tissue specific inhibitors of ADPR-cyclases; finally, the compound may be applied to therapeutic purposes for prevention and management of human kidney diseases.

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