

# cADP-ribose/ryanodine channel/ $\text{Ca}^{2+}$ -release signal transduction pathway in mesangial cells

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**Yusufi, Ahad N. K., Jingfei Cheng, Michael A. Thompson, Thomas P. Dousa, Gina M. Warner, Henry J. Walker, and Joseph P. Grande.** cADP-ribose/ryanodine channel/ $\text{Ca}^{2+}$ -release signal transduction pathway in mesangial cells. *Am J Physiol Renal Physiol* 281: F91–F102, 2001.—Signaling via release of  $\text{Ca}^{2+}$  from intracellular stores is mediated by several systems, including the inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) and cADP-ribose (cADPR) pathway. We recently discovered a high capacity for cADPR synthesis in rat glomeruli and cultured mesangial cells (MC). We sought to determine whether 1) cADPR synthesis in MC is regulated by cytokines and hormones, 2) ryanodine receptors (RyRs) are expressed in MC, and 3)  $\text{Ca}^{2+}$  is released through RyRs in response to cADPR. We found that ADP-ribosyl cyclase, a CD38-like enzyme that catalyzes cADPR synthesis, is upregulated in MC by tumor necrosis factor- $\alpha$ , interleukin-1 $\beta$ , and all-*trans* retinoic acid (atRA). [<sup>3</sup>H]ryanodine binds to microsomal fractions from MC with high affinity in a  $\text{Ca}^{2+}$ -dependent manner; binding is enhanced by specific RyR agonists and blocked by ruthenium red and cADPR. Western blot analysis confirmed the presence of RyR in MC. Release of <sup>45</sup>Ca<sup>2+</sup> from MC microsomes was stimulated by cADPR; release was blocked by ruthenium red and 8-bromo-cADPR. ADPR (non-cyclic) was without effect. In MC, TNF- $\alpha$  and atRA amplified the increment of cytoplasmic  $\text{Ca}^{2+}$  elicited by vasopressin. We conclude that MC possess elements of a novel ADP-ribosyl cyclase→cADPR→RyR→ $\text{Ca}^{2+}$ -release signaling pathway subject to regulation by proinflammatory cytokines and steroid superfamily hormones.

cytokines; retinoids; calcium-induced calcium release; adenosine 5'-diphosphate-ribosyl cyclase; crosstalk

RELEASE OF CALCIUM FROM INTRACELLULAR stores, endoplasmic/sarcoplasmic reticulum (ER/SR), is one of the key signal transduction mechanisms that play a pivotal role in the regulation of numerous cellular functions (5). There are two major systems for  $\text{Ca}^{2+}$  release from intracellular stores (46). Widespread, and probably ubiquitous (45, 26), is a signaling system (6) mediated by the second messenger, inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ). In this system, fast-onset, short-acting hormones such as vasoactive peptides bind receptors that are coupled within the plasma membrane to phos-

phatidylinositol phospholipase C (PI-PLC). This enzyme catalyzes generations of  $\text{IP}_3$ ; de novo generated  $\text{IP}_3$  binds the  $\text{IP}_3$  receptor channel ( $\text{IP}_3\text{Rs}$ ) that opens and releases  $\text{Ca}^{2+}$  from the ER/SR into the cytoplasm (6).

Another route for  $\text{Ca}^{2+}$  release from the ER/SR is through the ryanodine receptor channel (RyRs). This channel is activated by  $\text{Ca}^{2+}$  itself (19, 40), and is so named the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR) pathway (19). cADP-ribose (cADPR), a novel second messenger, is synthesized from  $\beta$ -NAD by the enzyme, ADP-ribosyl cyclase (40). cADPR increases sensitivity of RyR to  $\text{Ca}^{2+}$  and thereby regulates  $\text{Ca}^{2+}$  release through RyRs via the CICR mechanism (19, 40). In our recent studies (11, 17, 3), we discovered that ADP-ribosyl cyclase is upregulated in various tissues and cell types by hormones of the steroid superfamily such as  $\beta$ -estradiol (11), 3,5,3'-triiodothyronine (17), or all-*trans* retinoic acid (atRA) (17, 3), which act with a slow onset and have a prolonged effect. Furthermore, RyRs can be activated or blocked by various specific pharmacological agonists and antagonists (70).

In contrast to the ubiquitous  $\text{IP}_3\text{Rs}$  (46, 45), RyRs have a more limited tissue distribution (4). Furthermore, the expression of RyRs and  $\text{IP}_3\text{Rs}$  is regulated independently, and often in opposing directions. For example, incubation of cells with the cytokine transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) enhances expression of RyRs (25, 2) but suppresses expression of  $\text{IP}_3\text{Rs}$  (59). Stimulation of muscarinic receptors reduces abundance of  $\text{IP}_3\text{Rs}$  without having an effect on expression of RyRs; conversely, incubation in a differentiating medium markedly increases expression of RyRs, but not of  $\text{IP}_3\text{Rs}$  (45). In end-stage heart failure, RyRs are downregulated in left ventricular myocardium, whereas  $\text{IP}_3\text{Rs}$  are upregulated (27).

Mesangial cells (MC), specialized pericytes in glomeruli, are essential for maintenance and regulation of glomerular functions (8, 58, 60). In addition, MC are prime targets of immunoinflammatory injury in various types of glomerulonephritis (1, 58). Contractility and other MC functions are regulated, in major part,

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by  $\text{Ca}^{2+}$  released from ER/SR (1, 8, 58, 60). Numerous vasoactive peptides, bioactive lipids, and biogenic amines modulate MC functions by binding to requisite receptors in the plasma membrane and act via the  $\text{IP}_3\text{-Ca}^{2+}$ -release pathway (1). A recent immunohistochemical study documented the presence of  $\text{IP}_3\text{Rs}$  in MC (52).

Although the  $\text{IP}_3\text{-Ca}^{2+}$ -release signaling system has been well characterized in MC (1, 8, 58, 60), much less is known about the  $\text{cADPR}\rightarrow\text{RyR}\rightarrow\text{Ca}^{2+}$  signaling system. In particular, it is not known whether RyRs are expressed or play a functionally significant role in  $\text{cADPR}$ -elicited  $\text{Ca}^{2+}$  release in MC. In our recent study examining  $\text{cADPR}$  metabolism in the rat kidney (13), we found a strikingly high capacity for  $\text{cADPR}$  synthesis in isolated glomeruli, 50 times higher than in the rest of the examined renal parenchyma (13). We also identified ADP-ribosyl cyclase activity in cultured MC. As mentioned, in studies of nonrenal tissues we determined that ADP-ribosyl cyclase is regulated by long-acting hormones (11, 17, 3).

In view of all these considerations, we now set out to determine whether 1) ADP-ribosyl cyclase in MC is modulated by proinflammatory cytokines and by hormonal agents that act with slow onset and have prolonged action; 2) RyRs are expressed in MC and to what extent; and 3)  $\text{Ca}^{2+}$  is released through RyRs in response to a natural second messenger,  $\text{cADPR}$  (19, 40), and to typical pharmacological agonists of RyRs (70).

## MATERIALS AND METHODS

$[^3\text{H}]$ ryanodine and  $^{45}\text{Ca}^{2+}$  were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).  $\text{cADPR}$ , 8-bromo- $\text{cADPR}$  (8-Br- $\text{cADPR}$ ), ADPR, bastadin (bastadin mixture no. 196710), ruthenium red, and lipopolysaccharide (LPS, *Pseudomonas aeruginosa*; F-D type 1) were purchased from Calbiochem (La Jolla, CA). 4-Chloromethyl-*m*-cresol (4-CmC), 4-chloro-3-ethyl phenol (4-CEP), and caffeine were from Aldrich (Milwaukee, WI). Monoclonal antibodies (MAb) directed against ryanodine receptors, MA3-925 MAb (34C) and MA3-916 MAb (C3-33), were purchased from Affinity Bioreagents (Golden, CO). Recombinant human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and  $\alpha\text{TR}$  were from Biomol Research Laboratories (Plymouth Meeting, PA). Nicotinamide guanine dinucleotide (NGD), cGDP-ribose (cGDPR), 3,5,3'-triiodothyronine ( $\text{T}_3$ ), and all other chemicals and biochemicals, all of highest purity grades, were from Sigma (St. Louis, MO) or other standard suppliers.

**MC.** Glomeruli were isolated from 200-g male Sprague-Dawley rats by differential sieving, as previously described (10, 13, 49). Cell outgrowths were characterized as MC by positive immunohistochemical staining for vimentin, smooth muscle-specific actin, and desmin; stains for high- and low-molecular-weight cytokeratins, factor VIII-related antigen, and leukocyte-common antigens were negative. For all experiments described herein, MC were used before *passage 20*. There was no apparent effect of passage number on ADP-ribosyl cyclase activity, RyRs expression, or  $\text{cADPR}$ -mediated microsomal  $\text{Ca}^{2+}$  release activity. To evaluate the effect of hormones and cytokines on ADP-ribosyl cyclase, MC were grown in complete Waymouth's medium with 20% FCS in 100-mm culture dishes to confluence, rendered quiescent for

48 h in Waymouth's medium with 0.5% FCS, and then incubated with cytokines or hormones for 24 h. At the end of this incubation period, MC were rinsed, harvested, and subjected to sonication (3 cycles of 10 s each, 8- $\mu\text{m}$  amplitude) in a "homogenizing buffer" containing (final concentrations) 0.25 M sucrose and 20 mM Tris·HCl (pH = 7.2). The homogenate was centrifuged first at 2,000 *g* for 10 min, and the resulting supernatant was then centrifuged at 40,000 *g* for 30 min. The pellet resuspended in homogenizing medium, designated as "membrane fraction" (11, 70), was assayed for ADP-ribosyl cyclase activity. In pilot experiments, we determined that all ADP-ribosyl cyclase activity in homogenates of MC is associated with membranes, and none was detected in supernatants from 100,000 *g* for 90 min (43). Homogenization and subsequent procedures were conducted at 0–4°C.

**ADP-ribosyl cyclase activity.** Cyclase activity was determined by the fluorometric method of Lee et al. (25, 26), which is based on the conversion of NGD, a structural analog of NAD, to its fluorescent product, cGDPR (28), which we employed in our previous studies (11, 17). Membrane fractions from MC (see above) (~0.1 mg protein/ml) were incubated in the homogenizing buffer (see above) in a thermostated cuvette at 37°C with 0.4 mM NGD substrate, and generation of fluorescent product cGDPR was continually monitored at 300-nm excitation wavelength and 410-nm emission wavelength, using a Hitachi model F-2000 spectrofluorometer (11, 17). The enzymatic activity was calculated from the initial linear slope; change in fluorescence ( $\Delta$ ) was calibrated from standard curves construed with known concentrations of cGDPR (3, 17, 28, 43). All measurements were conducted in duplicate and are from four independent experiments (17). The assay for ADP-ribosyl cyclase with use of NGD substrate accurately determines ADP-ribosyl cyclase activity, because the product cGDPR is, unlike  $\text{cADPR}$ , resistant to degradation by  $\text{cADPR}$  hydrolase (28); in our preceding studies we affirmed concordance of results of ADP-ribosyl cyclase measured with NGD and NAD substrates, respectively (11, 17).

**cADPR hydrolase activity.** Hydrolase activity was determined by thin-layer chromatography (TLC) using 20 × 20-cm ion-exchange, polyethylenimine cellulose TLC plates with ultraviolet (UV) indicator. Membranes isolated from control and cytokine-treated MC (0.5 mg protein/ml) were incubated, in duplicate, for 30 min at 37°C in medium containing (final concentrations) 50  $\mu\text{M}$   $\text{cADPR}$ , 20 mM Tris·HCl (pH 7.4), and 0.2  $\mu\text{Ci/ml}$   $[^3\text{H}]\text{cADPR}$ . The reaction was terminated by mixing a 20- $\mu\text{l}$  aliquot of the reaction mixture with a 20- $\mu\text{l}$  stop solution containing (final concentration) 1 mM  $\text{cADPR}$ , 1 mM ADPR, and 10% glacial acetic acid, and storing on ice. The 40- $\mu\text{l}$  aliquot was placed on the TLC plates, and the mixture was separated in a TLC chamber containing 0.2 M NaCl and 30% ethanol at room temperature for a period of 2.5 h. The plates were dried, and the fractions were identified by a 254-nm UV light. The  $\text{cADPR}$  fraction was removed from the plate, placed in liquid scintillation vials, radioactivity was determined by a Beckman LS6000 liquid scintillation counter, and  $\text{cADPR}$  hydrolase activity was calculated by comparing initial  $[^3\text{H}]\text{cADPR}$  levels with those measured after 30 min of incubation.

**Isolation of microsomes.** Microsomal fractions were isolated (at 0–4°C) from cultured rat MC or from rat myocardium. MC or myocardium, finely minced with a razor blade, were suspended in a buffer containing 300 mM sucrose, 10 mM HEPES, 0.1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF) (pH = 7.4), and homogenized by Polytron (3 × 30 s at *setting 10*). The homogenate was centrifuged for 10 min at 1,600 *g*, and the pellet was discarded. The supernatant was further centrifuged at 11,000 *g* for 20 min,

and the supernatant thus obtained was ultracentrifuged at 100,000 *g* for 1 h. The pellet was resuspended in a small volume of homogenizing buffer by Dounce homogenizer. The microsomes were either used fresh for measurement of  $^{45}\text{Ca}^{2+}$  release or were divided into aliquots, quickly frozen, and stored at  $-80^{\circ}\text{C}$  for measurement of [ $^3\text{H}$ ]ryanodine binding. Storage at  $-80^{\circ}\text{C}$  preserved the [ $^3\text{H}$ ]ryanodine binding capacity of microsomes. The protein content of fractions was determined by the method of Lowry et al. (44).

**[ $^3\text{H}$ ]ryanodine binding.** Microsomes (100–200  $\mu\text{g}$  protein) were incubated for 2 h at  $37^{\circ}\text{C}$  in a medium containing (final concentration) 600 mM KCl, 100  $\mu\text{M}$  EGTA, 150  $\mu\text{M}$   $\text{Ca}^{2+}$ , 0.2 mM PMSF, 25 mM HEPES (pH 7.2), and 30 nM [ $^3\text{H}$ ]ryanodine (54.7 Ci/mmol). Free [ $^3\text{H}$ ]ryanodine was separated from [ $^3\text{H}$ ]ryanodine, bound to microsomes by a rapid filtration technique using Whatman GF/B filters, followed by three subsequent washes with 3 ml of ice-cold water. The [ $^3\text{H}$ ]ryanodine radioactivity that remained on the filters was measured by liquid scintillation counting (18, 57). The high-affinity specific [ $^3\text{H}$ ]ryanodine binding was calculated as the difference of total binding and nonspecific binding, determined in the presence of  $>3,000$ -fold higher concentration of unlabeled ryanodine (0.1 mM).

**$^{45}\text{Ca}^{2+}$  release from microsomes (32).** Freshly prepared microsomes ( $\sim 100$   $\mu\text{g}$  protein) were passively loaded by incubating for 3 h at room temperature ( $21^{\circ}\text{C}$ ) in a medium containing 100 mM NaCl, 25 mM HEPES (pH 7.2), 1 mM  $\text{CaCl}_2$ , and 1  $\mu\text{Ci}$  of  $^{45}\text{Ca}^{2+}$ . Release of  $^{45}\text{Ca}^{2+}$  from loaded microsomes was initiated by 10-fold dilution of microsomal suspension with a buffer containing 100 mM NaCl, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , and 25 mM HEPES, pH 7.2 (32). After 10 s, the suspension was further diluted in a medium of identical composition that contained tested agonists with or without 10  $\mu\text{M}$  ruthenium red (final dilution 50-fold).  $^{45}\text{Ca}^{2+}$  efflux was stopped at 90 s after the second dilution with added test agents, and the  $^{45}\text{Ca}^{2+}$  retained in microsomes was separated from free  $^{45}\text{Ca}^{2+}$  by a rapid filtration technique using Whatman GF/B filters. The filters were rinsed three times with a solution containing 100 mM NaCl, 1 mM EGTA, 4 mM  $\text{MgCl}_2$ , 10  $\mu\text{M}$  ruthenium red, and 25 mM HEPES, pH 7.2. The  $^{45}\text{Ca}^{2+}$  retained in microsomes was determined by liquid scintillation counting.

**Measurement of cytoplasmic  $\text{Ca}^{2+}$  concentration.** Cytoplasmic  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in intact MC was measured in monolayers grown to confluence on  $9 \times 22$ -mm coverslips (69). MC were treated with cytokines for 24 h, as described above. Coverslips with MC were rinsed two times in PBS and then incubated with 1  $\mu\text{M}$  of the acetoxymethyl ester of fura 2 (fura 2-AM) for 1 h at room temperature in a solution of (final concentrations) 135 mM NaCl, 5 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 5.5 mM D-glucose, and 10 mM HEPES, pH 7.4. After fura 2-AM loading, coverslips were washed and placed into thermostated ( $37^{\circ}\text{C}$ ) cuvettes of the Hitachi model F-2000 fluorescence spectrophotometer. Vasopressin was added to cells, and emitted fluorescence was measured using dual wavelength ratios of 340/380 nm for excitation and 510 nm for emissions, and  $[\text{Ca}^{2+}]_i$  values were determined from the standard curve using Cation Measurement software.

**PCR analysis.** Total RNA was isolated from rat MC using the RNeasy Total RNA Isolation Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Reverse transcription and PCR amplification were performed using the GeneAmp System (Perkin-Elmer, Branchburg, NJ).

**CD38.** PCR analysis of CD38 was performed using primers homologous to the rat ADP-ribosyl cyclase/cADPR hydrolase (CD38; GenBank accession no. D29646) (37). The sense

primer, 5'-CCTTGGTAGTGGTCTGGTCATAG-3', and antisense primer, 5'-TCACATTCCAGAACACAGCAACAG-3', were designed to produce a 480-bp product. The PCR conditions were  $95^{\circ}\text{C}$  for 5 min, and then 40 cycles at  $95^{\circ}\text{C}$  for 1 min,  $42^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min, followed by a final extension at  $72^{\circ}\text{C}$  for 7 min, and storage at  $4^{\circ}\text{C}$ . The PCR product was subcloned into the pCRII vector (Invitrogen, Carlsbad, CA) and sequenced in both orientations. The cDNA product was used in subsequent Northern blot analyses (see below).

**RyRs.** PCR analysis of RyR-1, RyR-2, and RyR-3 was performed essentially as previously described (16), with the addition of a DNase I treatment (RNase-free DNase I; Roche Molecular Chemicals, Indianapolis, IN) to eliminate residual genomic DNA. Design of PCR primers was based on GenBank accession nos. X83932 (RyR-1), X83933 (RyR-2), and X83934 (RyR-3) (16). The nucleotide sequence and length of expected PCR products for each primer pair are RyR-1 (sense), GAAGTTCTGGACAAACACGGG; RyR-1 (antisense), TCGCTCTTGTGTAGAAATTTGCGG (435 bp); RyR-2 (sense), GAATCAGTGAGTTACTGGGCATGG; RyR-2 (antisense), CTGGTCTCTGAGTTCTCCAAAAGC (635 bp); RyR-3 (sense), AGAAGAGGCCAAAGCAGAGG; and RyR-3 (antisense), GGAGGCCAACGGTCAGA (269 bp). All PCR products were sequenced in both orientations.

**Northern blot analysis.** Quiescent rat MC were treated with atRA (1  $\mu\text{M}$ ), TNF- $\alpha$  (20 ng/ml), or both atRA (1  $\mu\text{M}$ ) and TNF- $\alpha$  (10 ng/ml) for 18 h before isolation of total cellular RNA. RNA (10  $\mu\text{g}/\text{lane}$ ) was electrophoresed through a 1% agarose, 2.2 M formaldehyde denaturing gel, then transferred to nylon membranes (Schleicher and Schuell, Keene NH), as previously described (30, 29). Ethidium bromide was added to each lane to allow visualization of the RNA with UV light. The complete transfer of the RNA from the gel to the membrane was documented by examining the membrane under UV light. The CD38 PCR product was excised from the pCRII vector and labeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP by the random primer method (20). Membranes were hybridized at  $65^{\circ}\text{C}$  in a 0.5 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 7% SDS, and 1% BSA, as described by Church and Gilbert (14). Gels were reprobated with a cDNA-encoding housekeeping gene, GAPDH. Autoradiograms were quantitated by computer-assisted video densitometry. Data for 18S ribosomal RNA were obtained from a negative image of the ethidium bromide-stained nylon membrane, according to the method of Correa-Rotter et al. (15). The CD38 signal in each lane was normalized to the corresponding GAPDH band, as previously described (29).

**Western blot analysis.** SDS-PAGE was performed using 7.5% gels cast in the PROTEAN II minigel system (Bio-Rad). Microsomes ( $\sim 100$   $\mu\text{g}$  protein) were denatured for 3 min at  $95^{\circ}\text{C}$  in loading buffer according to Laemmli (38). Electrophoresis was performed at a constant current (20 mA/gel) and transferred to nitrocellulose membranes, and the blots were incubated with mouse anti-RyR monoclonal antibodies using either MAb MA3-925 (34C) that reacts with both RyR-1 and RyR-2 or MAb MA3-916 (C3-33) that reacts strongly with RyR-2 type, and only faintly with RyR-1. Blots were incubated with anti-mouse secondary antibody for 1 h and visualized by exposure to X-ray film using an enhanced chemiluminescence technique (Amersham Pharmacia Biotech). Western blots prepared from membranes isolated from control, TNF- $\alpha$ -treated, and TNF- $\alpha$ +atRA-treated renal MC were incubated with an anti-CD38 antibody [goat polyclonal anti-CD38 (M-19), Santa Cruz] and visualized, as described above.

All results were evaluated statistically with use of the Student's *t*-test for group or paired comparison; values  $P < 0.05$  were considered statistically significant.

## RESULTS

ADP-ribosyl cyclase activity in MC membranes was upregulated by incubation with cytokines and with steroid superfamily hormones (Fig. 1). The proinflammatory cytokines TNF- $\alpha$  and IL-1 significantly induced ADP-ribosyl cyclase activity in membranes isolated from MC (TNF- $\alpha$   $\Delta+46\%$ ,  $P < 0.05$ ; and IL-1  $\Delta+38\%$ ,  $P < 0.05$ ). Similarly, the steroid superfamily hormone atRA (1  $\mu\text{M}$ ) increased ADP-ribosyl cyclase activity ( $\Delta+76\%$ ,  $P < 0.05$ ) (Fig. 1). Combined treatment of MC with TNF- $\alpha$  and atRA, which signal through different pathways, produced at least an additive upregulation of ADP-ribosyl cyclase activity ( $\Delta+145\%$ ,  $P < 0.05$ ) compared with the effects of atRA or TNF- $\alpha$  alone. T<sub>3</sub>, another steroid superfamily hormone, did not significantly augment ADP-ribosyl cyclase activity ( $\Delta+19\%$ ,  $P > 0.05$ , Fig. 1). Under these experimental conditions, bacterial LPS did not significantly increase ADP-ribosyl cyclase activity in MC ( $\Delta+12\%$ ,  $P > .05$ ).

In other cell systems, CD38 and related enzymes are recognized as multifunctional enzymes (39). Whereas CD38 promotes cADPR formation from  $\beta$ -NAD through cyclase activity, CD38 also has hydrolase activity, which promotes the catabolism of cADPR to ADPR (39). To determine whether TNF- $\alpha$ , atRA, or LPS induces ADP-ribosyl cyclase activity to a greater extent than cADPR hydrolase activity, we assayed ADP-ribosyl cyclase activity and cADPR hydrolase activity in

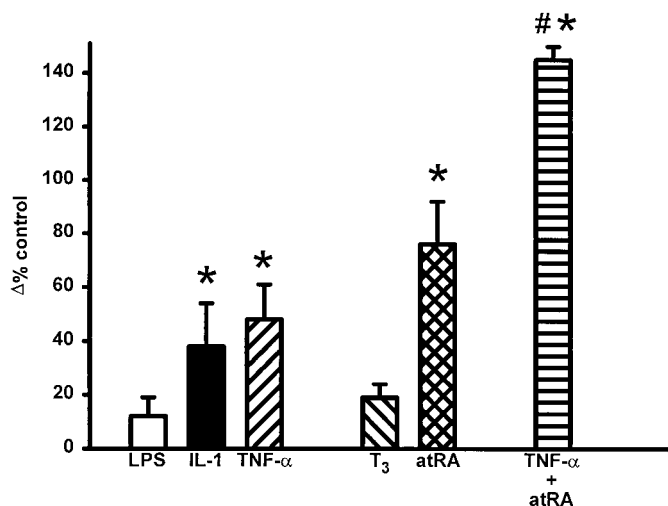


Fig. 1. Upregulation of ADP-ribosyl cyclase activity in mesangial cells (MC) by proinflammatory cytokines and hormones. The basal specific activity of ADP-ribosyl cyclase [ $22.8 \pm 4.1$  nmol cGDP-ribose (cGDPR)  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>; means  $\pm$  SE,  $n = 4$  experiments, each performed in duplicate] is taken as 100%, and the effects of tested agents are expressed as  $\Delta + \%$ . Each bar denotes means  $\pm$  SE. MC were treated with 20 ng/ml tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), 30 ng/ml lipopolysaccharide (LPS), 10 ng/ml interleukin-1 (IL-1); 1  $\mu\text{M}$  all-*trans* retinoic acid (atRA), 0.1  $\mu\text{M}$  3,5,3'-triiodothyronine (T<sub>3</sub>), or 10 ng/ml TNF- $\alpha$  + 1  $\mu\text{M}$  atRA. #Value significantly higher ( $P < 0.05$ , *t*-test) than TNF- $\alpha$  alone or atRA alone. \*Significantly ( $P < 0.05$ , *t*-test,  $n = 4$  experiments) higher than untreated controls.

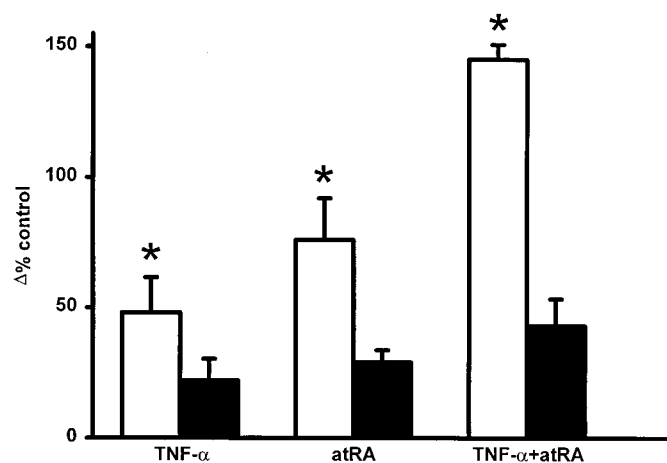


Fig. 2. Comparison of ADP-ribosyl cyclase activity and cADPR hydrolase activity. The basal specific activity of ADP ribosyl cyclase ( $36.3 \pm 4.3$  nM cGDPR  $\cdot$  min<sup>-1</sup>  $\cdot$  mg protein<sup>-1</sup>; means  $\pm$  SE,  $n = 3$ ) is taken as 100%, and the effect of TNF- $\alpha$  (20 ng/ml), atRA (1  $\mu\text{M}$ ), or the combination of TNF- $\alpha$  + atRA (10 ng/ml and 1  $\mu\text{M}$ , respectively) is expressed as  $\Delta + \%$ . cADPR hydrolase activity was quantitated from the amount of [<sup>3</sup>H]cADPR hydrolyzed after 30 min of incubation. Open bars, ADP-ribosyl cyclase activity; solid bars, cADPR hydrolase activity. Each bar denotes means  $\pm$  SE. \*Significantly ( $P < 0.05$ , *t*-test) higher than untreated controls.

membranes isolated from parallel cultures of MC treated with TNF- $\alpha$ , atRA, and atRA plus TNF- $\alpha$ . TNF- $\alpha$  and atRA increased ADP-ribosyl cyclase activity to a much greater extent than cADPR hydrolase activity (Fig. 2). Although the combined treatment of MC with TNF- $\alpha$  plus atRA significantly augmented ADP-ribosyl cyclase activity, these combined treatments did not significantly alter cADPR hydrolase activity (Fig. 2). Similarly, combined treatment of MC with LPS and TNF- $\alpha$  did not significantly alter cADPR hydrolase activity, compared with treatment with LPS or TNF- $\alpha$  alone (data not shown).

Several approaches were employed to define the nature of MC ADP-ribosyl cyclase activity. By using appropriate primers, a 480-bp cDNA was amplified from reverse-transcribed MC RNA with 100% sequence identity to the rat CD38-like ADP-ribosyl cyclase/cADPR hydrolase isolated from rat pancreatic islets (GenBank accession no. D29646, data not shown) (37). By Northern blot analysis, a distinct 2.2-kb band was identified in MC, corresponding to CD38 mRNA. Relatively low levels of CD38 expression were detected in untreated control rat MC (Fig. 3). atRA (1  $\mu\text{M}$ ) and TNF- $\alpha$  induced steady-state CD38 mRNA levels by 2.2-fold ( $P < 0.001$ ) and 1.4-fold ( $P < 0.05$ ), respectively (Fig. 3). Increased CD38 mRNA levels were observed after 2-h treatment with atRA and reached maximal levels after 12–18 h (data not shown). The combined treatment of rat MC for 18 h with atRA (1  $\mu\text{M}$ ) and TNF- $\alpha$  induced CD38 mRNA levels by 2.8-fold ( $P < 0.001$ ) (Fig. 3). These studies indicate that TNF- $\alpha$ - and atRA-mediated increases in ADP-ribosyl cyclase activity are associated with a concomitant induction of steady-state CD38 mRNA levels.

By Western blot analysis, a 42-kDa CD38-like protein was identified in membranes isolated from rat MC

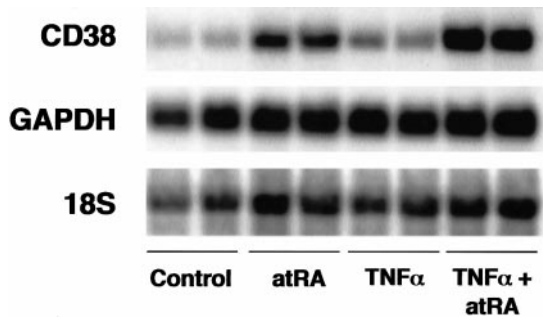


Fig. 3. Induction of CD38 mRNA by TNF- $\alpha$  and atRA. Northern blots prepared from rat MC treated with atRA (1  $\mu$ M), TNF- $\alpha$  (20 ng/ml), or both TNF- $\alpha$  (10 ng/ml) and atRA (1  $\mu$ M) were probed with a rat CD38 cDNA, as described in MATERIALS AND METHODS. Data are representative of 3 independent experiments.

(Fig. 4). In parallel with ADP-ribosyl cyclase activity (Fig. 1), atRA and TNF- $\alpha$  + atRA increased expression of the CD38-like protein [ $\Delta$ +%  $24 \pm 5$  vs. untreated control ( $P < 0.05$ ) and  $\Delta$ +%  $42 \pm 6$  vs. untreated control ( $P < 0.05$ ), respectively;  $n = 6$  experiments, each performed in duplicate] (Fig. 4). These studies indicate that cytokine-mediated ADP-ribosyl cyclase activity may at least in part be mediated through increased production of a CD38-like protein. Furthermore, cytokine-mediated induction of CD38 mRNA and protein leads to preferential activation of ADP-ribosyl cyclase activity relative to cADPR hydrolase activity.

In other cell systems, intracellular  $\text{Ca}^{2+}$  release by cADPR is mediated by the RyR channel. However, the presence of such an RyR channel in MC has not been previously documented. We found that [ $^3\text{H}$ ]ryanodine specifically binds to microsomal fractions isolated from MC in a  $\text{Ca}^{2+}$ -dependent fashion (Fig. 5A). The [ $^3\text{H}$ ]ryanodine binding was suppressed by the addition of molar excess (greater than  $\times 3,000$ ) of unlabeled ryanodine and was also blocked by 10  $\mu\text{M}$  ruthenium red (Fig. 5A), an established inhibitor of RyRs (19, 40). Specific binding of [ $^3\text{H}$ ]ryanodine to microsomes prepared from MC ( $91 \pm 22 \text{ fmol} \cdot \text{mg protein}^{-1} \cdot 120 \text{ min}^{-1}$ ) was about threefold less ( $P < 0.01$ ) than [ $^3\text{H}$ ]ryanodine binding to microsomes prepared from rat myocardium ( $324 \pm 20 \text{ fmol} \cdot \text{mg protein}^{-1} \cdot 120 \text{ min}^{-1}$ ) (data not shown).

To further document the specificity of [ $^3\text{H}$ ]ryanodine binding to MC microsomes, we measured [ $^3\text{H}$ ]ryanodine binding in the presence of caffeine, a well-known and established RyR agonist (19, 36, 40), and in the presence of several recently described pharmacological agonists that interact specifically with RyRs (18, 32, 36, 55, 57). We found that 4-CmC, 4-CEP, and the

alkaloids bastadin and caffeine all significantly enhanced [ $^3\text{H}$ ]ryanodine binding to microsomes of MC (Fig. 5B). Moreover, [ $^3\text{H}$ ]ryanodine binding to MC microsomes was inhibited by 10  $\mu\text{M}$  cADPR. This effect was blocked by 8-Br-cADPR, a selective antagonist of cADPR. ADPR (non-cyclic) had no effect on [ $^3\text{H}$ ]ryanodine binding to MC microsomes (Fig. 5C). The presence of RyR-1, -2, and -3 was identified in MC by RT-PCR (Fig. 6A).

Immunoblot analysis, using RyR isoform-specific antibodies, confirmed the presence of RyR-1 in MC microsomes (Fig. 6B). When a Western blot was probed with MAb MA3-925 (that recognizes RyR-1 and RyR-2 isoforms equally), two major bands were detected at  $M_r \approx 560 \text{ kDa}$  (Fig. 6B, lanes 1 and 2). When probed with MAb MA3-916, which strongly reacts with RyR-2 but weakly with RyR-1, strong bands were detected in microsomal preparations from rat myocardium, but no bands were detected in microsomes from MC even after prolonged exposure (Fig. 6B, lanes 3 and 4), indicating that MC expression of RyR-2 is extremely low. Taken together, these data indicate that MC possess RyR-1 and possibly RyR-3, with low levels of RyR-2 expression.

To determine the functional significance of RyRs in MC, we measured  $\text{Ca}^{2+}$  release from MC microsomes that were preloaded with  $^{45}\text{Ca}^{2+}$ . cADPR elicited  $^{45}\text{Ca}^{2+}$  release from rat MC microsomes in a dose-dependent fashion (Fig. 7). The release of  $^{45}\text{Ca}^{2+}$  was significantly ( $P < 0.01$ ) increased by cADPR, a natural RyR agonist (19, 40), by caffeine (19, 36, 40), and by 4-CmC (57, 69) (Fig. 8A). The stimulatory effects of cADPR and pharmacological agonists on  $^{45}\text{Ca}^{2+}$  release from MC microsomes were all blocked by ruthenium red (Fig. 8, A and B). The cADPR-stimulated  $^{45}\text{Ca}^{2+}$  release ( $\Delta\% + 62 \pm 9$ ; means  $\pm$  SE,  $P < 0.025$ ,  $t$ -test;  $n = 3$ ) was blocked by the selective cADPR antagonist 8-Br-cADPR (64); furthermore, ADPR (non-cyclic), a hydrolytic metabolite of cADPR (19, 40), showed no effect on  $^{45}\text{Ca}^{2+}$  release (Fig. 8B). Release of  $^{45}\text{Ca}^{2+}$  mediated by cADPR was 60–70% of  $^{45}\text{Ca}^{2+}$  release induced by ionomycin or  $\text{IP}_3$  (Fig. 8, B and C).  $\text{IP}_3$ -mediated  $^{45}\text{Ca}^{2+}$  release was blocked by heparin and not by ruthenium red, as expected (Fig. 8C).

Finally, we determined whether the RyR agonist caffeine can enhance the increment of cytoplasmic  $\text{Ca}^{2+}$  level evoked by vasopressin in intact MC (Fig. 9A). The extent of  $\text{Ca}^{2+}$  increase in intact MC elicited by vasopressin ( $\Delta\% + 137 \pm 17$ ;  $n = 5$ ) was enhanced twofold ( $P < 0.01$ ) in the presence of 10 mM caffeine ( $\Delta\% + 281 \pm 33$ , means  $\pm$  SE;  $n = 5$ ). The vasopressin-

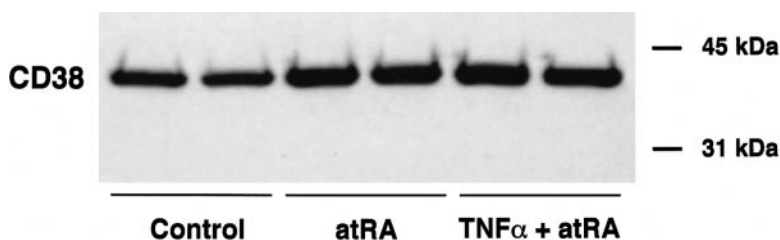


Fig. 4. Upregulation of CD38-like ADP-ribosyl cyclase by TNF- $\alpha$  and atRA. Western blots were prepared from membranes of rat MC treated with atRA alone (1  $\mu\text{M}$ ) or both TNF- $\alpha$  (10 ng/ml) and atRA (1  $\mu\text{M}$ ), as described in MATERIALS AND METHODS. Data are representative of 6 experiments.

elicited  $\text{Ca}^{2+}$  release was also significantly increased by TNF- $\alpha$  and atRA combined (Fig. 9B).

## DISCUSSION

Release of  $\text{Ca}^{2+}$  from intracellular stores governs major functions of MC, such as contractility (58, 60), and plays a key role in signal transduction pathways by which many hormonal agents regulate MC functions in physiological and pathobiological states (1, 8, 58, 60).

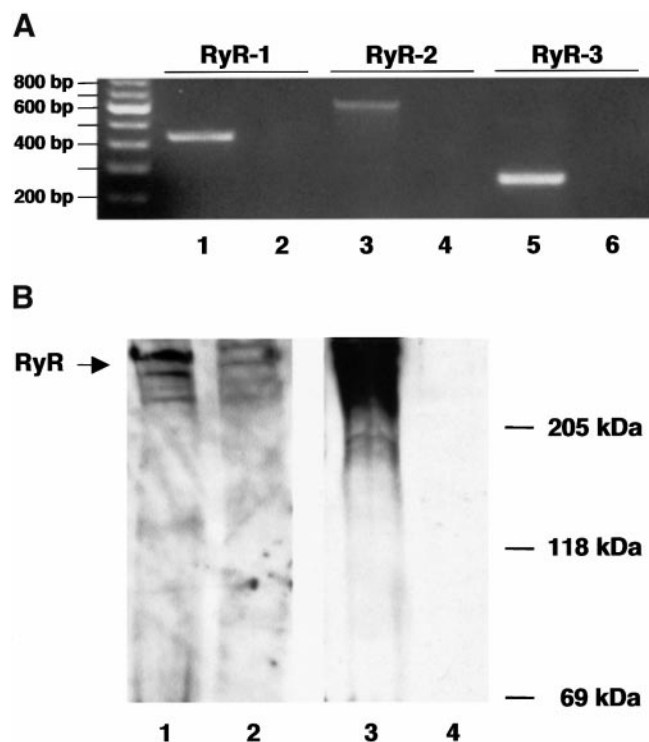
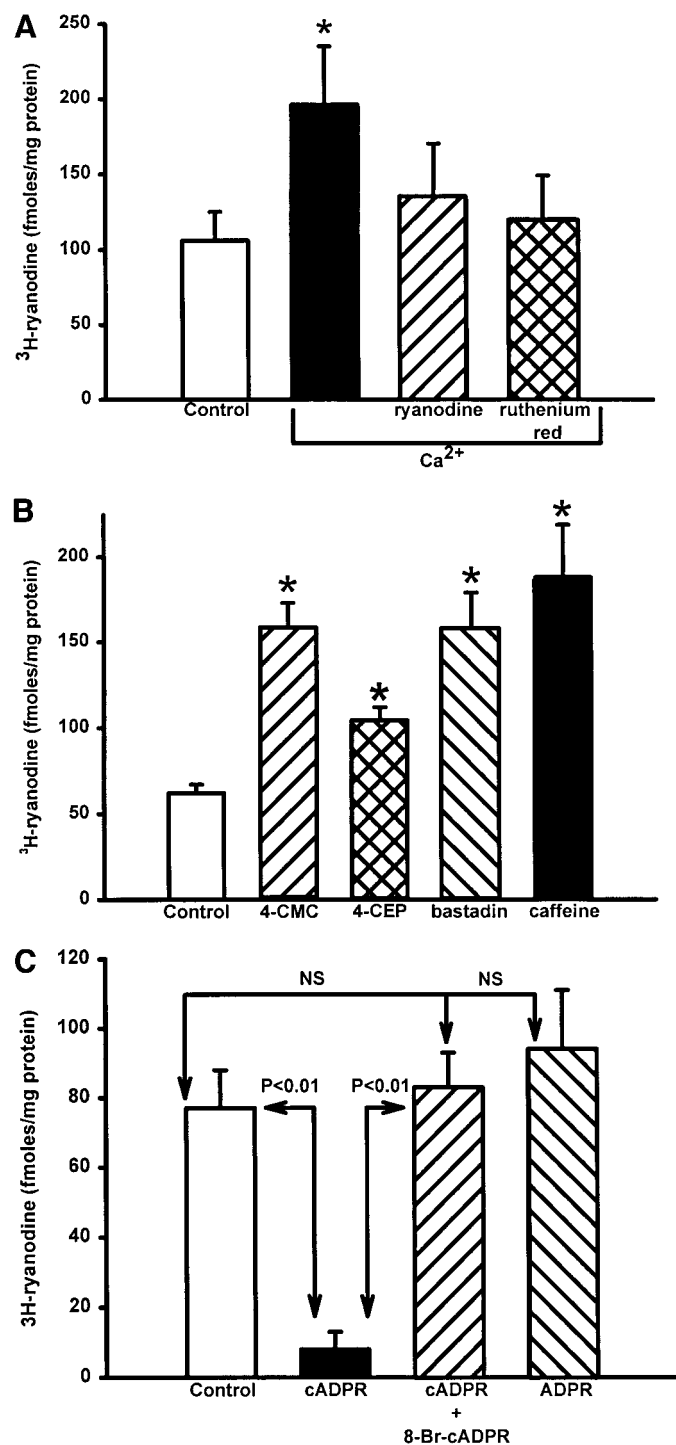


Fig. 6. RyR expression by MC. **A**: PCR amplification of RyR-1, RyR-2, and RyR-3 in MC. cDNA fragments of RyR-1 (lanes 1 and 2), RyR-2 (lanes 3 and 4), and RyR-3 (lanes 5 and 6) were amplified from MC cDNA, as described in MATERIALS AND METHODS. MC express RyR-1 (480 bp, lane 1), RyR-2 (635 bp, lane 3), and RyR-3 (269 bp, lane 5). Lanes 2, 4, and 6: negative controls, in which RT is omitted. **B**: analysis of MC microsomal fractions by Western blot. MC preparations were probed with 2 different RyR mAb (see MATERIALS AND METHODS) and compared with microsome preparations from rat myocardium. Lanes 1 and 3: rat myocardial microsomes. Lanes 2 and 4: MC microsomes. Lanes 1 and 2 were probed with MA3-925 MAb, which reacts strongly with RyR-1 and RyR-2. Lanes 3 and 4 were probed with MA3-916, which reacts strongly with RyR-2 and weakly with RyR-1. Note the strong signal in rat myocardium (lane 3) and the absence of signal in MC (lane 4), despite prolonged exposure time. Two major bands of  $M_r \sim 560$  kDa are identified in both rat myocardium and MC.

In general, signaling pathways that transduce effects of fast-onset, short-acting hormonal agents in MC, such as vasoactive peptides (e.g., endothelin, vasopressin), often employ the well-established  $\text{IP}_3$ - $\text{IP}_3\text{R}$

Fig. 5.  $^3\text{H}$ ryanodine binding to microsomal fractions of MC. **A**:  $^3\text{H}$ ryanodine binding is shown. The microsomes were incubated with 30 nM  $^3\text{H}$ ryanodine (see MATERIALS AND METHODS) without added  $\text{Ca}^{2+}$  (control), with 50  $\mu\text{M}$   $\text{Ca}^{2+}$  alone, with 50  $\mu\text{M}$   $\text{Ca}^{2+}$  + 100  $\mu\text{M}$  ryanodine, or with 50  $\mu\text{M}$   $\text{Ca}^{2+}$  + 10  $\mu\text{M}$  ruthenium red. Each bar denotes means  $\pm$  SE;  $n = 4$ . \*Statistically different from controls ( $P < 0.05$  by  $t$ -test). **B**: the effect of ryanodine receptor (RyR) agonists on specific  $\text{Ca}^{2+}$ -dependent  $^3\text{H}$ ryanodine binding to MC microsomal fraction. Microsomal fractions were treated with no addition (control), 0.5 mM 4-chloromethyl- $m$ -cresol (4-CmC), 0.5 mM 4-chloro-3-ethyl phenol (4-CEP), 10  $\mu\text{M}$  bastadin, or 20 mM caffeine. Each bar denotes means  $\pm$  SE;  $n = 3$ . \*Statistically different ( $P < 0.05$ ,  $t$ -test) from controls. **C**: effect of 10  $\mu\text{M}$  cADPR on  $^3\text{H}$ ryanodine binding to microsomes from MC. Microsomes were treated with no addition (control), 10  $\mu\text{M}$  cADPR, 10  $\mu\text{M}$  cADPR + 40  $\mu\text{M}$  8-bromo-cADPR (8-Br-cADPR), or 10  $\mu\text{M}$  ADPR. Each bar denotes means  $\pm$  SE;  $n = 3$ .

$\text{Ca}^{2+}$ -release system (1, 5, 6, 58). This signaling pathway includes binding of a hormone to its receptor, coupled in the plasma membrane via  $G_q$  to PI-PLC, that increases generation of  $\text{IP}_3$ ; the de novo generated  $\text{IP}_3$  binds onto  $\text{IP}_3\text{Rs}$  within SR/ER membranes and triggers release of  $\text{Ca}^{2+}$  as a signal into the cytoplasm (Fig. 10).

Herein we report that MC possess elements of a "cADPR $\rightarrow$ RyR $\rightarrow$  $\text{Ca}^{2+}$ -release" signaling pathway and that this pathway differs substantially in design and function from the classic  $\text{IP}_3$ - $\text{Ca}^{2+}$ -release pathway (6, 14, 19, 40). First,  $\text{Ca}^{2+}$  release from MC is elicited by a novel second messenger, cADPR, that sensitizes RyRs to  $\text{Ca}^{2+}$  and activates and/or facilitates CICR (14, 19, 40). Second, MC are capable of synthesizing cADPR from  $\beta$ -NAD, a reaction catalyzed by ADP-ribosyl cyclase. Finally,  $\text{Ca}^{2+}$  is released from MC through a distinct RyR channel that differs in properties, namely regulation, from  $\text{IP}_3\text{Rs}$  (19, 40, 46).

Several distinct molecules with ADP-ribosyl cyclase activity have been identified, including a soluble ADP-ribosyl cyclase isolated from *Aplysia californica* ovotestis, a cytosolic enzyme capable of metabolizing cADPR isolated from sea urchin egg extracts (15, 19, 37, 39, 41), and the membrane-bound ADP-ribosyl cyclases CD38 and BST-1/CD157 (33). We found that virtually all of the MC  $\text{Ca}^{2+}$  release activity attributable to cADPR was membrane bound; no significant cytosolic  $\text{Ca}^{2+}$  release activity was detected. In several other mammalian cells and tissues, including rat liver homogenates, uterine smooth muscle cells, and vascular smooth muscle cells, we found that almost all of the cADPR-mediated  $\text{Ca}^{2+}$  release activity is membrane associated (11, 17, 43). Furthermore, we failed to demonstrate BST-1 expression in membrane extracts from MC (Walker HJ, Yusufi ANK, and Dousa TP, unpublished observations). Based on these considerations, we sought to determine whether  $\text{Ca}^{2+}$  release activity

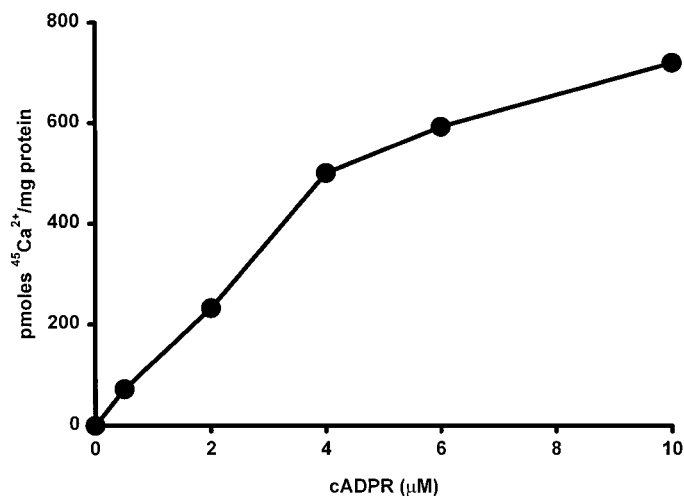


Fig. 7. cADPR elicits  $^{45}\text{Ca}^{2+}$  release from rat MC microsomes in a dose-dependent fashion. Microsomes isolated from rat MC were loaded with  $^{45}\text{Ca}^{2+}$  in the presence of doses of cADPR ranging from 0 to 10  $\mu\text{M}$ .  $^{45}\text{Ca}^{2+}$  release was assessed as described in MATERIALS AND METHODS.

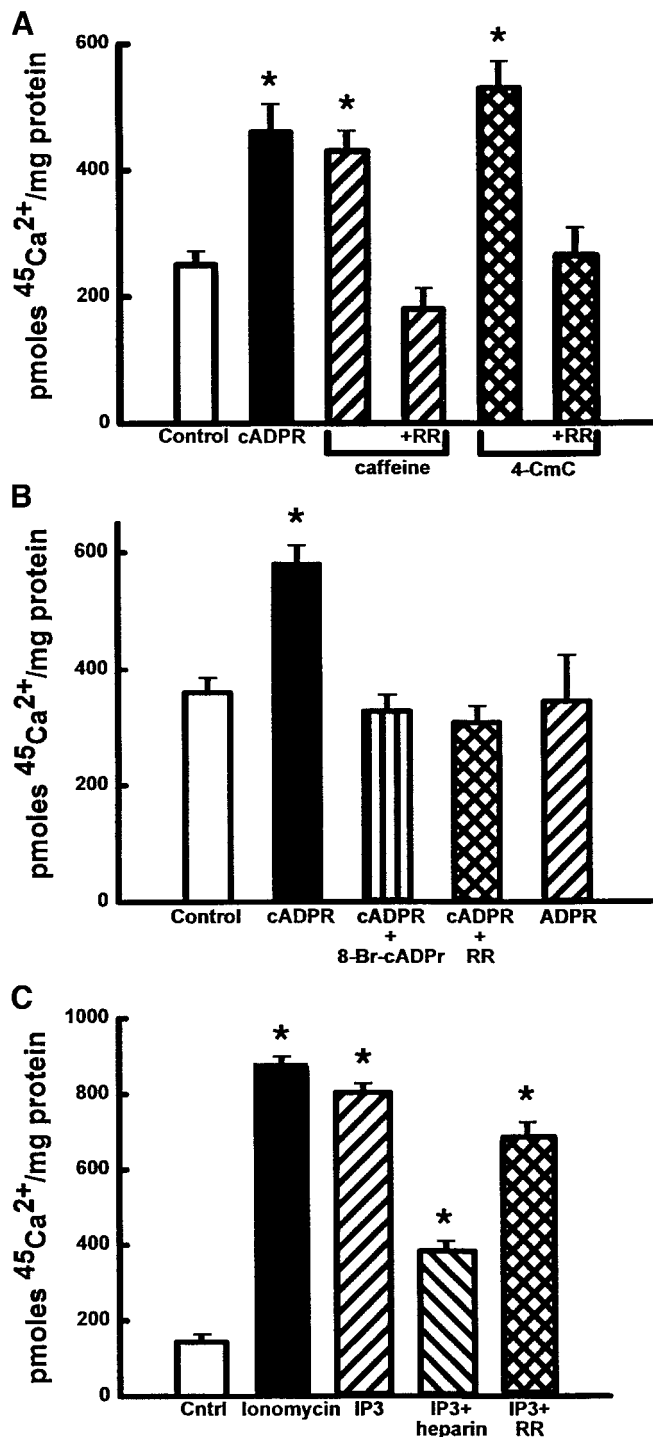


Fig. 8.  $^{45}\text{Ca}^{2+}$  release from MC microsomes. A: effect of cADPR and pharmacological RyR agonists on  $^{45}\text{Ca}^{2+}$  release from preloaded microsomal fraction of MC. Microsomal fractions were treated with no additions (control), 10  $\mu\text{M}$  cADPR, 20 mM caffeine alone or with 10  $\mu\text{M}$  ruthenium red (+RR), or 500  $\mu\text{M}$  4-CmC alone or with 10  $\mu\text{M}$  RR. B: microsomal fractions were treated with no additions (control), 10  $\mu\text{M}$  cADPR, 10  $\mu\text{M}$  cADPR + 40  $\mu\text{M}$  8-Br-cADPR, 10  $\mu\text{M}$  cADPR + 10  $\mu\text{M}$  RR, or 10  $\mu\text{M}$  ADPR. C: effect of ionomycin and  $\text{IP}_3$  on calcium release by microsomes isolated from MC. Microsomes were treated with no addition (control), 10  $\mu\text{M}$  ionomycin, 8  $\mu\text{M}$   $\text{IP}_3$ , 8  $\mu\text{M}$   $\text{IP}_3$  + 1 mg/ml heparin, or 8  $\mu\text{M}$   $\text{IP}_3$  + 10  $\mu\text{M}$  RR. \*Significantly higher ( $P < 0.01$ ;  $t$ -test) than controls; each bar denotes means  $\pm$  SE;  $n = 3-4$ .

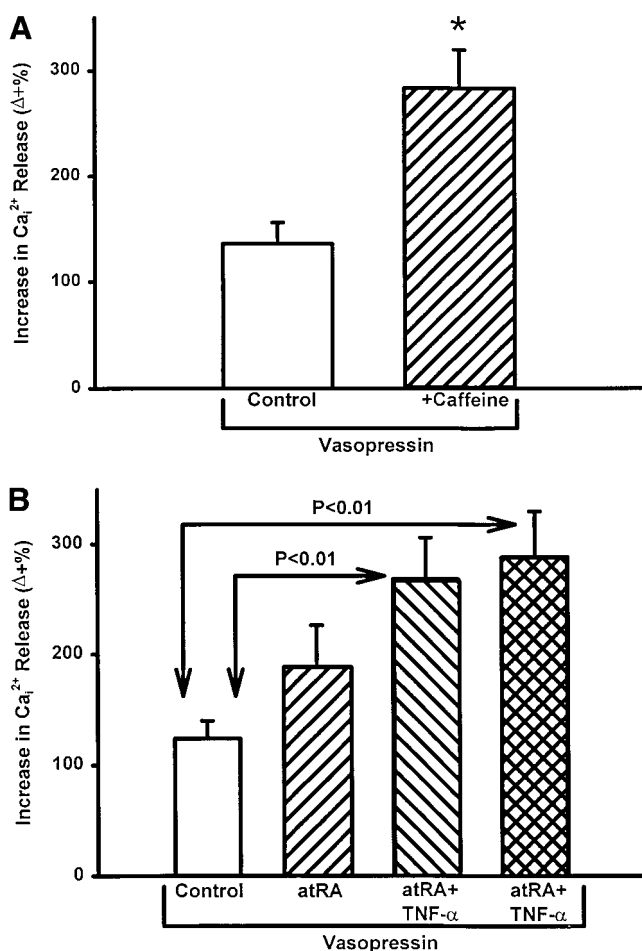


Fig. 9. Intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) release triggered by  $1 \mu M$  vasopressin in MC. A: effect of caffeine. Basal  $[Ca^{2+}]_i$  level in MC was  $140 \pm 11$  nM. Bars (means  $\pm$  SE,  $n = 5$ ) denote increment ( $\Delta$ %) of  $[Ca^{2+}]_i$  in response to  $1 \mu M$  vasopressin without (control) or in the presence of  $10$  mM caffeine. \*Significantly higher than controls ( $P < 0.01$ ,  $t$ -test,  $n = 5$  experiments). B: effects of retinoids and TNF- $\alpha$ . Bars (means  $\pm$  SE,  $n = 3$  experiments) denote increment ( $\Delta$ %) of  $[Ca^{2+}]_i$  in response to  $1 \mu M$  vasopressin alone (control), with  $1 \mu M$  atRA, with  $1 \mu M$  atRA +  $10$  ng/ml TNF- $\alpha$ , and with  $10 \mu M$  atRA +  $10$  ng/ml TNF- $\alpha$ .

elicited by cADPR may be mediated by a CD38-like ADP-ribosyl cyclase.

By RT-PCR, we found that MC possess a CD38-like molecule with 100% sequence homology to the rat CD38-like ADP-ribosyl cyclase/cADPR hydrolase isolated from rat pancreatic islets. The presence of a CD38-like protein in rat MC was confirmed by Western blot analysis (Fig. 3). CD38 has recently been identified in a variety of mammalian cells including liver (43), brain (50), vascular and uterine smooth muscle (11, 17), T cells (31), and pancreatic islets (54, 62). In rat pancreas, the CD38  $\rightarrow$  cADPR  $\rightarrow$  RyR  $\rightarrow$   $Ca^{2+}$  signaling pathway is regulated by glucose and is involved in regulation of insulin secretion (54, 62). In diabetic patients, autoantibodies directed against CD38 (35) and missense mutations in the CD38 gene (67) have been identified.

In concert with increased ADP-ribosyl cyclase activity, we found that atRA and TNF- $\alpha$  induce CD38

mRNA expression and production of a CD38-like protein, as assessed by Western blot analysis. Because we did not identify soluble ADP-ribosyl cyclase activity or BST-1 in MC, we postulate that basal and cytokine-induced ADP-ribosyl cyclase activity in MC is directed by a CD38-like protein. However, further studies such as CD38 antibody neutralization studies, CD38 antisense studies, or  $Ca^{2+}$  release experiments using cells derived from CD38 knockout animals are needed to determine whether atRA or TNF- $\alpha$ -mediated induction of CD38 is causally related to increases in ADP-ribosyl cyclase activity in MC.

CD38 and other ADP-ribosyl cyclases are multifunctional enzymes that are capable of catalyzing the hydrolysis of cADPR to ADPR as well as the production of cADPR from  $\beta$ -NAD (34). Under our experimental conditions, ADP-ribosyl cyclase activity in MC is preferentially induced by atRA and TNF- $\alpha$ , without significant effects on cADPR hydrolase activity. We observed that the extent of  $Ca^{2+}$  increase in intact MC elicited by vasopressin was augmented by pretreatment with TNF- $\alpha$  and atRA or pretreatment with the RyR agonist caffeine. These studies provide indirect evidence that atRA- and TNF- $\alpha$ -mediated induction of ADP-ribosyl cyclase produces functionally significant increases in intracellular  $Ca^{2+}$  fluxes.

Several lines of evidence document that functionally competent RyRs are expressed in MC. The high-affinity, [ $^3$ H]ryanodine  $Ca^{2+}$ -dependent binding is now a well-established method for detection and quantitation of RyRs (18, 45, 57). [ $^3$ H]ryanodine binding to MC microsomes requires relatively high KCl concentrations. This requirement for high KCl concentrations to demonstrate specific ryanodine binding in a variety of mammalian cells and tissues, including smooth muscle cells, LLC-PK $_1$  cells, and extracts from renal cortex, has been reported by other investigators (4, 18, 63). The observation that [ $^3$ H]ryanodine binding to microsomes from MC is significantly enhanced by specific pharmacological agonists of RyRs is diagnostic (Fig. 5B): caffeine (32, 36, 57), 4-CmC (18, 32, 36, 57), 4-CEP (18, 36), and the alkaloid bastadin (18, 55) all acted in a similar manner. All of these agonists enhance binding of [ $^3$ H]ryanodine to microsomes from skeletal muscle, myocardium, and other tissues (18, 32, 36, 55, 57). The presence of RyR in MC microsomes was confirmed by Western blot analysis (Fig. 6).

We found that cADPR inhibits [ $^3$ H]ryanodine binding to MC microsomes. This observation would suggest that cADPR and ryanodine compete for binding to a similar site on the ryanodine receptor channel (19). In other systems, variable effects of cADPR on [ $^3$ H]ryanodine binding have been described. In cardiac SR vesicles and in lymphocytes, cADPR enhances [ $^3$ H]ryanodine binding (9, 31, 51). In parotid cells and, as described here in MC, cADPR competes with [ $^3$ H]ryanodine for binding to the ryanodine receptor channel (68). Recent data suggest that cADPR indirectly interacts with the ryanodine receptor channel, through interactions with one or more accessory proteins (46, 65), such as the FK506 binding protein 12.6 (53).



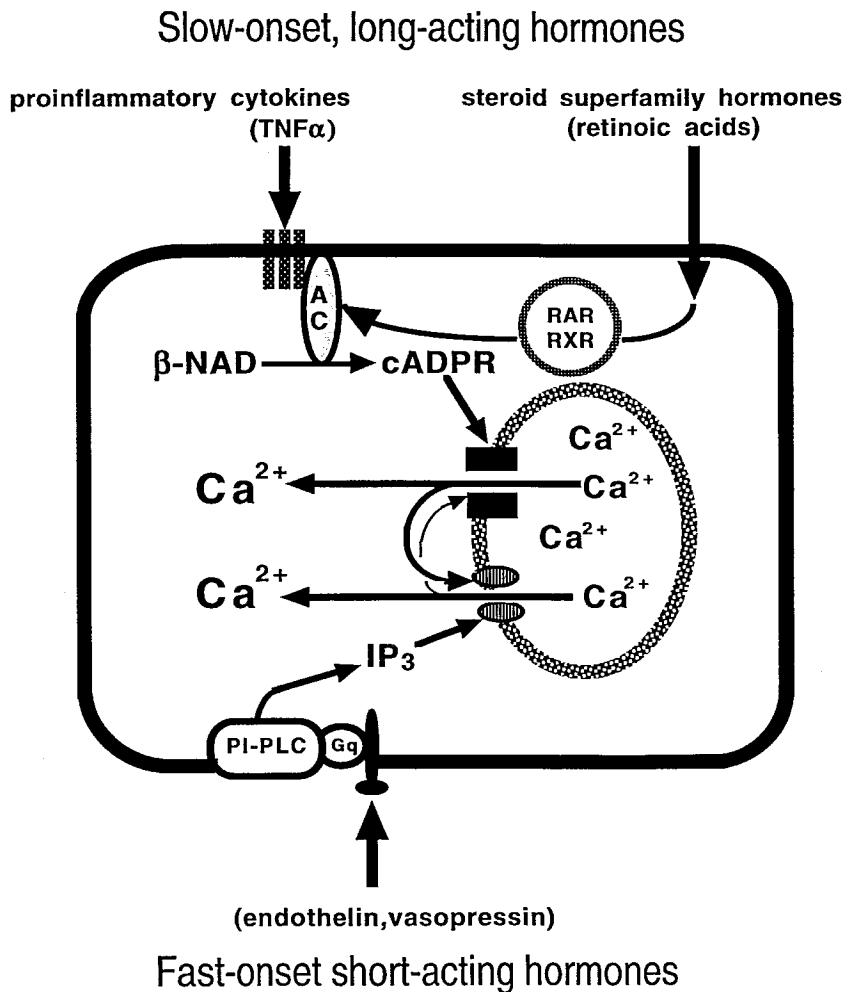


Fig. 10. Schematic depiction of cADPR→RyR→Ca<sup>2+</sup> and IP<sub>3</sub>→IP<sub>3</sub>R→Ca<sup>2+</sup> signaling pathways in MC and their crosstalk via Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR). Slow-onset, long-acting hormone, e.g., atRA (via nuclear receptors RAR/RXR), or cytokine TNF-α, by binding to its receptor, upregulates ADP-ribosyl cyclase (AC) activity and increases synthesis of cADPR from β-NAD. Elevated cADPR, by interacting with RyRs, elicits Ca<sup>2+</sup> release via CICR. Fast-onset, short-acting hormones, by binding to receptor, activate phosphatidylinositol-phospholipase C (PI-PLC) and increase generation of IP<sub>3</sub>, which triggers Ca<sup>2+</sup> release through IP<sub>3</sub> receptor channel (IP<sub>3</sub>Rs). Ca<sup>2+</sup> released from RyRs sensitizes IP<sub>3</sub>Rs to action of IP<sub>3</sub>; conversely, Ca<sup>2+</sup> released through IP<sub>3</sub>Rs can accentuate the cADPR-elicited Ca<sup>2+</sup> release through RyRs.

By RT-PCR, we have identified RyR-1, -2, and -3 in MC. By Western blot analysis, MC express relatively high levels of RyR-1, with minimal expression of RyR-2. High levels of RyR-1 expression have been described in skeletal muscle and smooth muscle, RyR-2 expression in cardiac muscle (5) and pancreatic acinar cells (42), and RyR-3 expression in brain, smooth muscle cells, and other tissues (21, 47, 48, 61, 68). Future studies, using RyR-3-specific antibodies, are needed to define the level of RyR-3 expression in MC.

The functional competence of RyRs for Ca<sup>2+</sup> release from MC is evidenced by measurements of *in vitro* release of <sup>45</sup>Ca<sup>2+</sup> from preloaded MC microsomes (Fig. 8). The natural second messenger cADPR (19, 40), as well as the pharmacological RyR agonists caffeine (36, 57) and 4-CmC (18, 32, 57), all elicited <sup>45</sup>Ca<sup>2+</sup> release from MC microsomes to the same extent (Fig. 8A). The <sup>45</sup>Ca<sup>2+</sup>-releasing effects of all these agents were prevented by the RyR blocker ruthenium red (Fig. 8, A and B), and the <sup>45</sup>Ca<sup>2+</sup>-releasing effect of cADPR was blocked by a selective inhibitor, 8-Br-cADPR (64). ADPR, an inactive hydrolytic metabolite of cADPR (14, 19, 40), was without effect on <sup>45</sup>Ca<sup>2+</sup> release (Fig. 8B). All these observations evince that <sup>45</sup>Ca<sup>2+</sup> release through RyRs in MC is sensitive to stimulation by cADPR, a novel second messenger.

In experiments on intact monolayers of MC, we observed that the RyR agonist caffeine enhanced the increment in cytoplasmic Ca<sup>2+</sup> level that was elicited by vasopressin. Conceivably, by activating RyRs and CICR, caffeine sensitized the IP<sub>3</sub>Rs to the Ca<sup>2+</sup>-releasing action of IP<sub>3</sub> generated in response to vasopressin and produced an enhanced rise of Ca<sup>2+</sup> (Fig. 9A). atRA+TNF-α also enhanced vasopressin-mediated increases in cytoplasmic Ca<sup>2+</sup> (Fig. 9B). These findings are consistent with the notion that RyRs can be activated in the intact MC and that there may be crosstalk between the functionally distinct cADPR and IP<sub>3</sub> signaling pathways. However, the exact mechanism by which this occurs remains to be clarified in future studies.

Because the cADPR→RyR→Ca<sup>2+</sup>-release signaling system and the IP<sub>3</sub>→IP<sub>3</sub>R→Ca<sup>2+</sup>-release signaling system do coexist in MC, it should be considered whether these two pathways interact with each other and regulate MC functions in a coordinated, integrated manner. A major point of mutual interaction is the fact that both RyRs (19, 14) and IP<sub>3</sub>Rs (12, 22) can behave as CICR and thereby may influence release of Ca<sup>2+</sup> via each other. We thus propose, as a working hypothesis (Fig. 10), that slow-onset, long-acting "adaptive" cytokines and hormones, which act via upregulating the

ADP-ribosyl cyclase→cADPR→RyR→CICR pathway, regulate and control primarily the ambient, steady-state  $\text{Ca}^{2+}$  levels in cytoplasm. The level of cytoplasmic  $\text{Ca}^{2+}$  determines sensitivity of  $\text{IP}_3$ Rs to the  $\text{Ca}^{2+}$ -releasing effect of  $\text{IP}_3$  that is generated in response to fast-acting hormones, such as vasoactive peptides. In short, via this mechanism, long-acting adaptive hormones would modulate the responsiveness of MC to fast-acting hormones and autacoids. Conversely,  $\text{Ca}^{2+}$  released in response to  $\text{IP}_3$  may contribute to enhance activity of RyRs as positive feedback to CICR (Fig. 10).

Conceivably, hormones that act via the cADPR→RyR→ $\text{Ca}^{2+}$ -release signaling pathway can regulate other cellular functions of MC in addition to contractility. As recently reported, RyRs and  $\text{IP}_3$ Rs can be differentially distributed within one cell (68):  $\text{IP}_3$ Rs were detected close to plasma membranes, whereas RyRs were located in the perinuclear region (68). cADPR has been shown to elicit release of  $\text{Ca}^{2+}$  through RyRs into the nucleus (23, 56), and as a result, nuclear  $\text{Ca}^{2+}$  levels elevated in response to cADPR can modulate expression of various genes via  $\text{Ca}^{2+}$ -dependent phosphorylation-dephosphorylation of transcription factors (e.g., CREB, CREM, SRF) (24). The cADPR→RyR→ $\text{Ca}^{2+}$ -release signaling pathway might also play a role in initiation of apoptosis. This possibility is suggested by observations that release of  $\text{Ca}^{2+}$  from intracellular stores by thapsigargin (66) or cyclopiazonic acid (7, 66) can cause apoptosis (9, 51, 63). Apoptosis initiated by thapsigargin or by other maneuvers was prevented or attenuated by dantrolene (51) and ruthenium red (51, 68), both blockers of RyRs.

In conclusion, we provide evidence that MC are endowed with a novel cADPR→RyR→ $\text{Ca}^{2+}$ -release signaling pathway and that biosynthesis of the initial step, second messenger cADPR, is upregulated by proinflammatory cytokines and steroid superfamily hormones. We surmise that the cADPR→RyR→ $\text{Ca}^{2+}$ -release regulatory pathway in MC is an essential, hitherto unrecognized, signaling system operant in the regulation of contractility and other functions of MC. It represents another layer of signaling machinery that governs normal glomerular MC functions and interacts, via crosstalk, with other signaling pathways. Finally, it can also play a pathogenic role in glomerular inflammation and may be a potential target for signal transduction pharmacotherapy.

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