Angiotensin II Ca\(^{2+}\) signaling in rat afferent arterioles: stimulation of cyclic ADP ribose and IP\(_3\) pathways

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Fellner, Susan K., and William J. Arendshorst. Angiotensin II Ca\(^{2+}\) signaling in rat afferent arterioles: stimulation of cyclic ADP ribose and IP\(_3\) pathways. Am J Physiol Renal Physiol 288:F785–F791, 2005. First published December 21, 2004; doi:10.1152/ajprenal.00372.2004.—ANG II induces a rise in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) in vascular smooth muscle (VSM) cells via inositol triphosphate receptor (IP\(_3\),R) activation and release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR). The Ca\(^{2+}\) signal is augmented by calcium-induced calcium release (CICR) and by cyclic adenosinediphosphate ribose (cADPR), which sensitizes the ryanodine-sensitive receptor (RyR) to Ca\(^{2+}\) to further amplify CICR. cADPR is synthesized from $\beta$-nicotinamide adenine dinucleotide (NAD\(^{+}\)) by a membrane-bound bifunctional enzyme, ADPR cyclase. To investigate the possibility that ANG II activates the ADPR cyclase of afferent arterioles, we used inhibitors of the IP\(_3\)R, RyR, and ADPR cyclase. Afferent arterioles were isolated from rat kidney with the magnetized microsphere and sieving technique and loaded with fura-2 to measure [Ca\(^{2+}\)]\(_i\). In Ca\(^{2+}\)-containing buffer, ANG II increased [Ca\(^{2+}\)]\(_i\) by 75% and 81%, respectively. Nicotinamide and Zn\(^{2+}\) were known inhibitors of the VSM calcium-induced Ca\(^{2+}\) release; ryanodine; vascular smooth muscle; cADPR activates a RyR (40, 54) and, in conjunction with calmodulin, sensitizes a RyR to Ca\(^{2+}\), thereby augmenting CICR (14, 23, 34).

Evidence for the presence of an ADPR cyclase and for a role of cADPR in Ca\(^{2+}\) signaling has been established in a large number of mammalian cell types, including pancreatic islet cells, cardiac myocytes, trachea, intestinal longitudinal muscle, lymphocytes, sympathetic neurons, salivary and lacrimal gland cells, and hepatocytes (24, 36). Only recently, however, has a role for cADPR been investigated in vascular smooth muscle (VSM). In membrane preparations of aorta (13, 53), renal microvessels (39), coronary arteries (31, 52, 55), and pulmonary artery (16), evidence for ADPR cyclase activity has been demonstrated. Measurement of changes in [Ca\(^{2+}\)]\(_i\), in response to ANG II, has been made in permeabilized renal VSM cells (39). To our knowledge, there have been no Ca\(^{2+}\) studies examining the activation of the cADPR pathway in intact fresh afferent arterioles and no studies exploring the effect of ANG II on this particular pathway in VSM of any origin.

The ADPR cyclase of VSM has several unique properties that distinguish it from the CD38 ADPR cyclase of nonvascular cells. In contrast to the CD38 enzyme of sea urchin eggs, aplysia, and HL-60 cells, in which Zn\(^{2+}\) enhances the activity of the enzyme, Zn\(^{2+}\) inhibits the cyclase of rat aortic VSM cells (13). Nitric oxide (NO) inhibits the VSM enzyme, whereas it is stimulatory in macrophages, neurons, pancreatic cells, and sea urchin eggs (52). Recently, it has been shown that oxidative stress increases [Ca\(^{2+}\)]\(_i\) in fresh bovine coronary VSM cells through a pathway that involves cADPR (55) and that NO inhibits ADPR cyclase in coronary artery VSM (52).

Only one study investigated the effect of ANG II on the activity of ADP-ribosyl cyclase (27). This laboratory found that in membrane preparations of neonatal but not older cardiac myocytes, ANG II increased cyclase activity in a dose-dependent fashion (27). The mechanism by which ANG II stimulated an increase in ADPR cyclase activity is unknown, but these investigators speculated that a G protein-coupled process is involved (27). Because of the crucial importance of afferent arterioles in regulating glomerular filtration and sodium balance, we investigated the effects of ANG II on Ca\(^{2+}\) signaling in freshly isolated afferent arterioles using inhibitors of IP\(_3\)R, RyR, and ADPR cyclase.

METHODS

All studies were performed in compliance with the guidelines and practices of the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee.

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Preparation of fresh afferent arterioles. We used the magnetized polystyrene microsphere sieving technique as previously described in our laboratory (20) to isolate afferent arterioles (<20-μm diameter) from 5- to 6-wk-old Sprague-Dawley rats maintained in the Chapel Hill Colony. Phosphate-buffered saline (PBS), with the following composition (in mM) 137 NaCl, 4.1 KCl, 0.66 KH2PO4, 3.4 Na2HPO4, 2.5 NaHCO3, 1.0 MgCl2, and 5 glucose, was adjusted daily to pH 7.4 at 4°C, 23°C, and 34°C. The vessel segments in PBS containing 0.1% BSA were treated with collagenase type IV (Worthington, 197 U/mg, 5–10 μg/ml) for 18 min at 34°C. Arterioles were loaded with fura 2-AM (2 μM) and 0.1% BSA for 45 min at 23°C in the dark. After the arterioles were washed twice with PBS, the suspension was kept on ice. Ca2+ (1.1 mM) was added shortly before analysis of an arteriole. For studies of arterioles in Ca2+-free buffer, the vessels were kept in buffer containing 10−7 M Ca2+ only during the fura incubation and then they were washed with Ca2+-free buffer before analysis.

Vessels were stimulated with ANG II (3 × 10−7 M) (18). The concentrations of antagonists we chose were based on published results: 8-(N,N-diethylamino) octyl 3,4,5-trimethoxybenzoxate (TMB-8) (44, 47), 2-aminoethoxydiphenyl borate (2-APB) (41, 42), ryanodine (7, 18), Zn2+ (13), and nicotinamide (46). A dose-response analysis was performed for 8-Br cADPR (51). Arterioles were incubated with inhibitors for at least 1 min before initiation of an experimental measurement.

Measurement of [Ca2+]i. We measured [Ca2+]i, as previously described (20). Afferent arterioles were identified by their morphology and measured diameter of 15–20 μm. Additionally, we required visualization of microspheres in the lumen to exclude the possibility that the vessel was an efferent arteriole. The microspheres (4.0–4.5 μm) do not pass beyond the glomerular capillaries. An arteriole was centered in a small window of the optical field that was free of glomeruli or tubular fragments.

Although endothelial cells are present in these nonperfused afferent arterioles, we and others assumed that VSM cells provide the major source of fura-2 fluorescence owing to the greater bulk of VSM cells compared with endothelial cells (9). Studies in deendothelialized renal microvessels showed that ANG II-induced Ca2+ increases were not different from those of control arterioles (43). Furthermore, all reagents were added to the bath and therefore there was no immediate intraluminal exposure of endothelial cells to agonists and antagonists. We showed previously that freshly isolated single preglomerular VSM cells and afferent arterioles respond similarly, both qualitatively and quantitatively, to endothelin (20).

The VSM cells were excited alternately with light of 340- and 380-nm wavelength from a dual-excitation wavelength Delta-Scan equipped with dual monochrometers and a chopper [Photon Technology International (PTI)] as previously described (17–19). After passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. Signal intensity was acquired, passing signals through a barrier filter (510 nm), fluorescence was detected by a photomultiplier tube. Signal intensity was acquired, and quantitatively, to endothelin (20).

Statistics. Data are presented as means ± SE. Each data set was derived from afferent arterioles originating from at least three separate experimental days, two rats (4 kidneys) per experiment. Individual arterioles were studied only once and then discarded. In experiments with inhibitors, values for ANG II peak responses were included only for the experimental days in which a particular inhibitor was employed. Paired data for arterioles before and after ANG II stimulation were tested with Student’s paired t-test. Unpaired t-tests were employed for comparisons of responses between groups.

RESULTS

[Ca2+]i response to ANG II. Afferent arterioles in Ca2+-containing PBS responded to ANG II with a sharp peak response in 1–3 s. The mean baseline [Ca2+]i, was 64 ± 4, the peak 189 ± 10, and the plateau 100 ± 7 nM (n = 48, P < 0.01 for both peak and plateau values vs. baseline; Fig. 1). Thus the net peak increase in [Ca2+]i, after ANG II stimulation for all control experiments was 125 ± 10 nM.

Effect of inhibitors of the IP3R. To assess the contribution of G protein-coupled ANG II receptor stimulation of phospholipase C (PLC), formation of IP3, and IP3R-mediated release of [Ca2+]i, from the SR, we employed two inhibitors of the IP3R, TMB-8 and 2-APB (42, 47). Because these studies were conducted in Ca2+-containing buffer, inhibition of IP3R-mediated Ca2+ mobilization may also affect Ca2+ entry mechanisms (e.g., voltage-gated Ca2+ and store-operated Ca2+ entry channels) as well. Approximately one-half to two-thirds of the initial peak Ca2+ responses to ANG II in afferent arterioles have been shown to arise from mobilization mechanisms (5, current study, vide infra). Afferent arterioles were stimulated with ANG II in the absence (n = 11) or presence (n = 8) of TMB-8 (10−5 M; P < 0.01 for ANG II ± TMB-8 vs. baseline). A representative tracing (Fig. 2A) shows that the peak response to ANG II was attenuated by TMB-8-mediated inhibition of the IP3R. Group averages (Fig. 2C) demonstrate that the net ANG II-stimulated increase in [Ca2+]i, with TMB-8 present was 40 ± 4 nM and with ANG II alone 152 ± 18 nM in this group of experiments (74% inhibition, P < 0.01). In a similar fashion, afferent arterioles were pretreated with 2-APB (33 μM) to antagonize IP3R (Fig. 2B). In this group of experiments, ANG II alone increased [Ca2+]i, by 180 ± 18 nM (n = 11) and in the presence of 2-APB (n = 13) by 35 ± 4 nM (81% inhibition, P < 0.01 for ANG II + 2-APB vs. baseline, and <0.01 for ANG II alone vs. ANG II + 2-APB; Fig. 2C). Neither TMB-8 nor 2-APB had any influence on baseline [Ca2+]i, values (P > 0.5 for both agents). These data confirm that ANG II-stimulated IP3 production and activation of the
Effect of inhibitors of the RyR. We postulated that blockade of RyRs should abolish CICR (4) and the ability of cADPR to enhance the Ca\(^{2+}\) sensitivity of RyRs (24, 36). Ryanodine in high concentrations locks the RyR in a closed state (7, 48). Previously, we showed that a low concentration of ryanodine (2–3 \(\mu\)M) activates the RyR to mobilize Ca\(^{2+}\) from the SR (18). In contrast, a high concentration of ryanodine (100 \(\mu\)M) had no effect on baseline [Ca\(^{2+}\)]\(_i\) (21). In the current study, we found that ryanodine (100 \(\mu\)M) did not alter basal [Ca\(^{2+}\)]\(_i\) in afferent arterioles but inhibited the subsequent ANG II response by 69%. As Fig. 3C shows, the average increase in [Ca\(^{2+}\)]\(_i\) following stimulation with ANG II alone was 163 ± 27 nM (\(n = 12\)) and with ANG II following ryanodine was 50 ± 10 nM (\(n = 8, P < 0.01\)).

We tested the ability of 8-Br cADPR, a cell-permeant antagonist of the RyR (36), to attenuate the Ca\(^{2+}\) response to ANG II. In a dose-response analysis, 8-Br cADPR at concentrations of 50 and 100 \(\mu\)M was equally inhibitory (ANG II-induced increases in [Ca\(^{2+}\)]\(_i\) were 38 ± 5 and 31 ± 5 nM, respectively, \(P < 0.05\) vs. baseline) but were twice as potent as a concentration of 10 \(\mu\)M (ANG II response 62 ± 7 nM). Therefore, we pooled the data for the two higher concentrations. 8-Br cADPR did not alter baseline values of [Ca\(^{2+}\)]\(_i\) (\(P > 0.3\)). A representative tracing is shown in Fig. 3B. In this group of experiments, ANG II alone increased [Ca\(^{2+}\)]\(_i\) by 152 ± 17 nM (\(n = 11\)), but in the presence of 8-Br cADPR, the net increase was only 36 ± 5 nM (76% inhibition, \(n = 16, P < 0.01\) for ANG II alone vs. ANG II + 8-Br cADPR; Fig. 3C). Together, these two inhibitors of RyRs demonstrate the importance of cADPR in conjunction with RyRs for amplifying the [Ca\(^{2+}\)]\(_i\) signal initiated by IP\(_3\). That 8-Br cADPR blocks the ANG II-induced Ca\(^{2+}\) increase suggests that ANG II is linked to the production of cADPR.

Inhibitors of the ADPR cyclase. Thus a major question in trying to understand the relationship between agonist stimulation of a G protein-coupled receptor to initiate the sequence of IP\(_3\) generation, activation of the IP\(_3\)R, release of Ca\(^{2+}\) from the SR, and participation of the RyR to augment the Ca\(^{2+}\) signal is what is the communication to the membrane ADPR cyclase to direct formation of cADPR? We tested the effect of nicotinamide and Zn\(^{2+}\), well-studied inhibitors of the ADPR cyclase of VSM (13, 46), on Ca\(^{2+}\) signaling in response to ANG II. Nicotinamide (3 mM) pretreatment of afferent arterioles did not change baseline values of [Ca\(^{2+}\)]\(_i\) but reduced the [Ca\(^{2+}\)]\(_i\) response to ANG II by 66% (Fig. 4A). In this group of experiments, ANG II alone increased [Ca\(^{2+}\)]\(_i\) by 102 ± 18 nM (\(n = 7\)); in the presence of nicotinamide, the response was reduced to 35 ± 6 nM (\(n = 9, P < 0.01\); Fig. 4C).

As noted above, Zn\(^{2+}\) inhibits the VSM ADPR cyclase, whereas it stimulates the cyclase of nonvascular cells (13). Because Zn\(^{2+}\) may inhibit voltage-gated Ca\(^{2+}\) entry channels (32) and because voltage-gated Ca\(^{2+}\) entry is a major entry pathway in afferent arterioles (6, 10, 29), our studies assessing the action of Zn\(^{2+}\) on the ADPR cyclase were done in Ca\(^{2+}\)-free buffer. A typical tracing (Fig. 4B) shows that the response to ANG II is attenuated in Ca\(^{2+}\)-free PBS and is markedly inhibited by Zn\(^{2+}\) (3 mM). In nominally Ca\(^{2+}\)-free PBS, ANG II increased [Ca\(^{2+}\)]\(_i\) by 77 ± 30 nM (a value that is 62% of the
Fig. 3. Effect of antagonists of the ryanodine receptor on ANG II-induced elevation of \([\text{Ca}^{2+}]_i\), in afferent arterioles. A: representative tracing of the lack of a response to ryanodine (100 nM) and subsequent inhibition of the ANG II response. B: typical tracings of the response to ANG II in the presence or absence of 8-Br cADPR. C: summary data showing the inhibitory effects of ryanodine \((n = 7)\) and 8-Br cADPR \((n = 11)\) on the peak increases of \([\text{Ca}^{2+}]_i\) to ANG II (*\(P < 0.01\) for both inhibitors vs. ANG II alone).

Fig. 4. Effect of inhibitors of the adeninediphosphate ribose (ADPR) cyclase on ANG II-stimulated peak changes in \([\text{Ca}^{2+}]_i\), in afferent arterioles. A: typical tracings of the responses to ANG II in the presence or absence of nicotinamide in \([\text{Ca}^{2+}]_i\)-replete buffer. B: representative tracings of the responses of afferent arterioles in \([\text{Ca}^{2+}]_i\)-free buffer to ANG II in the presence or absence of \(\text{Zn}^{2+}\). C: summary data showing the inhibitory effects of nicotinamide \((n = 7)\), *\(P < 0.01\) vs. ANG II alone) and \(\text{Zn}^{2+}\) \((n = 7), **\(P = 0.02\) vs. ANG II alone).
response of afferent arterioles to ANG II in Ca\textsuperscript{2+}-replete PBS). In the presence of Zn\textsuperscript{2+}, the response to ANG II was decreased to 25 ± 10 nM (n = 7 for both, P < 0.05 for Zn\textsuperscript{2+} + ANG II vs. baseline and <0.03 for ANG II vs. ANG II + Zn\textsuperscript{2+}; Fig. 4C). Zn\textsuperscript{2+} had no effect on baseline [Ca\textsuperscript{2+}]\textsubscript{i}. Taken together, the nicotinamide and Zn\textsuperscript{2+} data suggest that there is a pathway used by ANG II that increases the activity of the ADPR cyclase and that is independent of the IP\textsubscript{3} pathway.

Simultaneous inhibition of ADPR cyclase and of IP\textsubscript{3}:R. To further document the relative contributions of activation of the IP\textsubscript{3}:R and of formation of cADPR, which sensitizes the RyR to Ca\textsuperscript{2+}, we employed inhibitors of both of these pathways. In this group of experiments, ANG II increased [Ca\textsuperscript{2+}]\textsubscript{i} by 100 ± 13 nM (n = 16). In afferent arterioles pretreated with nicotinamide and 2-APB, the increase in [Ca\textsuperscript{2+}]\textsubscript{i} following addition of ANG II was 18 ± 4 nM and with nicotinamide and TMB-8 17 ± 3 nM (n = 9 and 8, respectively, P < 0.01 vs. ANG II alone and P < 0.01 for ANG II + inhibitor vs. baseline; Fig. 5).

DISCUSSION

In the present study, we show that G protein-coupled receptor activation by ANG II leads to Ca\textsuperscript{2+} release from the SR via two different receptor/release channels, one sensitive to IP\textsubscript{3} and the other to ryanodine. We present new information that ANG II signaling in fresh afferent arterioles causes activation of the VSM ADPR cyclase and formation of cADPR. The mechanism by which this occurs awaits elucidation. Only one study, performed in neonatal rat cardiac myocytes, suggests that ANG II (possibly with involvement of a G protein) activates an ADPR cyclase to form cADPR, which, in turn, stimulates the RyR to release Ca\textsuperscript{2+} from the SR (27).

We were the first to demonstrate that fresh preglomerular VSM cells have a functional RyR (18) and that depletion of SR Ca\textsuperscript{2+} stores with ryanodine activates store-operated Ca\textsuperscript{2+} entry in both Wistar-Kyoto and spontaneously hypertensive rats (18, 19). Subsequently, other laboratories presented evidence for a role for a RyR in renal VSM. Stimulation of cultured renal VSM cells with RGD peptides induces Ca\textsuperscript{2+} waves that are completely blocked by closure of the RyR with ryanodine (20 μM) but not by the IP\textsubscript{3}:R antagonist xestospongin (8). In the isolated hydronephrotic kidney model, ANG II-induced arteriolar oscillations in afferent arterioles are blocked by ryanodine but not by 2-APB, an IP\textsubscript{3}:R inhibitor (50). In freshly dispersed VSM cells derived from third and fourth order renal vessels, the IP\textsubscript{3}:R and RyR-sensitive Ca\textsuperscript{2+} stores of the SR appear to communicate with each other, in contrast to the spatial organization of pulmonary arterial VSM cells in which the compartments are independent (30). Taken together, these studies confirm the important physiological role of RyRs in renal VSM.

In the present study, we examined ANG II-stimulated participation of cADPR by inhibiting the ADPR cyclase and by assessing the contribution of cADPR to the stimulation of RyRs. When RyR is locked in a nonconductive state with a high concentration of ryanodine (100 μM) (7, 48), the [Ca\textsuperscript{2+}]\textsubscript{i} response of afferent arterioles to ANG II is markedly reduced. Pretreatment of arterioles with the cell-permeant antagonist of cADPR, 8-Br cADPR, likewise substantially diminishes the response to ANG II. Thus ANG II appears to be involved in stimulating the formation of cADPR. Taken together, our data are consistent with the conclusion that the effect of cADPR on the RyR and the participation of cADPR in sensitizing the RyR to Ca\textsuperscript{2+} (CICR) are major components of the total [Ca\textsuperscript{2+}]\textsubscript{i} response to ANG II stimulation of afferent arteriolar VSM and suggest that Ca\textsuperscript{2+} release from the SR through the IP\textsubscript{3}:R accounts for less than one-third of the mobilization pathway.

To examine the possibility that ANG II influences the activity of the ADPR cyclase, we used two inhibitors of this plasma membrane enzyme, nicotinamide (46) and Zn\textsuperscript{2+} (13). Nicotinamide does not actually inhibit the cyclase but rather forces the reaction in the reverse direction to form NAD\textsuperscript{+} rather than cADPR (33). Nicotinamide is not known to influence other components of ANG II signaling pathways. Because Zn\textsuperscript{2+} may inhibit voltage-gated Ca\textsuperscript{2+} entry (32), a major entry channel in afferent arterioles (6, 10, 29), the Zn\textsuperscript{2+} experiments were performed in Ca\textsuperscript{2+}-free buffer. Zn\textsuperscript{2+} may also inhibit the membrane Ca\textsuperscript{2+} ATPases (28). If this were a significant effect in renal VSM cells, one would expect an enhancement of the [Ca\textsuperscript{2+}]\textsubscript{i} response to ANG II rather than inhibition. Zn\textsuperscript{2+} is also an inhibitor of proton currents generated via NOX in phagocytic cells (11). Whether Zn\textsuperscript{2+} has an influence with the novel isoforms of NOX of VSM (2) has not been studied. Superoxide dismutase, which converts superoxide to H\textsubscript{2}O\textsubscript{2}, is inhibited by Zn\textsuperscript{2+} (15), an effect that would enhance rather than diminish a possible role for superoxide in ANG II signaling. The strong inhibitory effects of nicotinamide and Zn\textsuperscript{2+} in our studies provide evidence that ANG II stimulation of VSM of afferent arterioles ultimately results in activation of the ADPR cyclase to form cADPR.

An enzyme capable of forming cADPR was first described in homogenates of sea urchin eggs (38) and subsequently has been shown to be present in a wide variety of cell types (24, 25, 35). In mammals, a single bifunctional protein, CD38, can act as a cyclase or hydrolase for cADPR (37, 45). The ADPR cyclase of VSM appears to have several unique characteristics that distinguish it from CD38 (13). Of particular interest is the fact that the VSM enzyme is inhibited, rather than stimulated, by Zn\textsuperscript{2+} (13).
Mammalian ADPR cyclases of specific cell types are stimulated by a number of different agonists: for example, estrogen in myometrium (1), glucose in pancreatic beta cells (49), retinoic acid and triiodothyronine in aortic VSM cells (12), reactive oxygen species in bovine coronary VSM (55), ANG II in neonatal cardiac myocytes (27), tumor necrosis factor-α and interleukin-1β in glomerular mesangial cell (54), and acetylcholine and endothelin in airway smooth muscle (51). How this structurally diverse group of molecules mediates the same process, namely activation of the ADPR cyclase, has not yet been elucidated with certainty.

In summary, we show that ANG II stimulation of afferent arterioles increases [Ca2+]i via several distinct pathways. The classic G protein-coupled receptor activation that results in IP3 generation and release of [Ca2+]i from the SR likely provides an initial and transient burst of [Ca2+]i, which can activate CICR from RyR. We present new information demonstrating that the ANG II response is blocked by the specific cADPR antagonist, 8-Br cADPR, and by high concentrations of ryanodine, which closes RyR. At present, we do not know the pathway(s) that link activation of the ADPR cyclase to ANG II stimulation of afferent arteriolar VSM, studies of which await further investigation.

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REFERENCES


41. Missiaen L, Callewaert G, De Smedt H, and Parys JB. 2-Aminoethoxydiphenyl borate affects the inositol 1,4,5-trisphosphate receptor, the intracellular Ca^{2+} pump and the nonspecific Ca^{2+} leak from the non-mitochondrial Ca^{2+} stores in permeabilized A7r5 cells. Cell Calcium 29: 111–116, 2001.


