ANG II inhibits insulin-mediated production of PI 3,4,5-trisphosphates via a Ca\(^{2+}\)-dependent but PKC-independent pathway in the cardiomyocytes

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Ikushima M, Ishii M, Ohishi M, Yamamoto K, Oghara T, Rakugi H, Kurachi Y. ANG II inhibits insulin-mediated production of PI 3,4,5-trisphosphates via a Ca\(^{2+}\)-dependent but PKC-independent pathway in the cardiomyocytes. Am J Physiol Heart Circ Physiol 299: H680–H689, 2010. First published July 2, 2010; doi:10.1152/ajpheart.00220.2009.—Insulin resistance (IR) is a condition where different organs are refractory to insulin stimulation of glucose uptake. ANG II has been suggested to be involved in the development of IR in the heart. The precise mechanism by which this occurs is still unknown. Here we have used dynamic fluorescent imaging techniques to show that ANG II inhibits insulin production of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P\(_3\)] in cardiac myocytes. Fluorophore (Venus)-conjugated CAMP-dependent protein kinase C-spleen lectin homology domain, which specifically binds to PI(3,4,5)P\(_3\), was transfected in neonatal rat cardiac myocytes. Insulin induced a robust increase in the fluorescence intensity at the cell surface, which was diminished by application of ANG II. The inhibitory action of ANG II was antagonized by RNH-6270 (an angiotensin type 1 receptor antagonist) but not by PD-122370 (an angiotensin type 2 receptor antagonist). BAPTA-AM (Ca\(^{2+}\) chelator) largely attenuated the ANG II effect, whereas K-252b (PKC inhibitor) did not. Furthermore, an elevation of intracellular Ca\(^{2+}\) induced by ionomycin mimicked the ANG II effect. Therefore, it is suggested that ANG II antagonizes insulin-mediated production of PI(3,4,5)P\(_3\) via a Ca\(^{2+}\)-dependent but PKC-independent pathway in cardiac myocytes.

Insulin is essential for control of energy metabolism to maintain appropriate cellular homeostasis. A number of pathological factors severely attenuate the sensitivity to insulin in a variety of tissues, including skeletal muscle (43), adipocytes (6), and heart (1, 30, 39). This abnormal condition, called “insulin resistance” (IR), is known to be a predisposition or even a direct genesis of various common diseases, such as diabetes mellitus and atherosclerosis. In the heart, IR is reported to be critically involved in dysfunction of cardiac myocytes and closely related to some cardiac disorders (17, 30, 39).

The downstream signaling of insulin stimulation has been extensively studied (15, 23, 38). The stimulation of insulin receptor induces activation of phosphatidylinositol 3-kinase (PI 3-kinase) via insulin receptor substrate-1 (IRS-1). The active form of PI 3-kinase then generates phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P\(_3\)] from phosphatidylinositol 4,5-bisphosphate on the membrane (10). PI(3,4,5)P\(_3\) can interact with the pleckstrin homology (PH) domain of a Ser/Thr kinase cAMP-dependent protein kinase (Akt)/PKB, which phosphorylates various downstream target molecules.

Recent studies have suggested that activation of ANG II type 1 receptor, AT\(_1\), attenuates insulin signaling, and thus contributes to the pathogenesis of IR in various tissues (7, 36). Stimulation of AT\(_1\) leads to activation of phospholipase C-\(\beta\) (PLC-\(\beta\)) (9) via a subtype of heterotrimeric G proteins, \(G_\text{q}\) (28, 35), and thus triggers phosphoinositide signaling cascades, which finally result in the elevation of intracellular Ca\(^{2+}\) concentration and activation of protein kinase C. A recent report showed that ANG II inhibits the insulin-mediated phosphorylation of Akt, a downstream signaling of PI(3,4,5)P\(_3\), through PKC-\(\alpha\) in vascular smooth muscle cells (VSMC) (22). The interaction between insulin and ANG II in cardiac myocytes, however, has not been well studied.

In this study, we examined the effects of insulin and ANG II on PI(3,4,5)P\(_3\) in cardiac myocytes with dynamic visualization of PI(3,4,5)P\(_3\). PI(3,4,5)P\(_3\) is the product generated by insulin-mediated activation of PI 3-kinase and thus serves as a good indicator of insulin-mediated signaling cascades (5, 25, 27). For this purpose, we exploited a real-time imaging technique using a fluorophore-conjugated Akt-PH domain, which specifically binds to PI(3,4,5)P\(_3\) in living cells. In result, we detected that ANG II competes insulin-mediated PI(3,4,5)P\(_3\) production in a Ca\(^{2+}\)-dependent mechanism in cardiac myocytes. Application of BAPTA-AM (Ca\(^{2+}\) chelator), but not K-252b (PKC inhibitor), blocked the effect of ANG II. Ionomycin but not phorbol ester mimicked the effect of ANG II. Therefore, different from VSMC, ANG II may antagonize insulin action via a Ca\(^{2+}\)-dependent and PKC-independent pathway in cardiac myocytes.

MATERIALS AND METHODS

Reagents and plasmid. Insulin, ANG II, wortmannin, ionomycin, phorbol 12-myristate 13-acetate (PMA), and PD-123319 were all purchased from Sigma-Aldrich (St. Louis, MO). K-252b was purchased from BIOMOL International. 2-[\(N\)-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) was purchased from Sigma-Aldrich. 2-

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study protocol was approved by our institution. Primary cultures of neonatal rat cardiac myocytes were prepared from the hearts of 1- to 2-day-old Wistar rats (Nippon Dobutsu, Osaka, Japan) as previously described (32). In brief, hearts were quickly removed from deeply anesthetized neonatal rats, minced, and digested with 0.125% trypsin (Becton-Dickinson, Sparks, MD) and 0.025% collagenase (Sigma-Aldrich) at 37°C for 1 h. Myocyte-enriched culture was achieved by preplating for 60 min to minimize the contamination of fibroblasts. Nonattached cells were then suspended in Hanks’ medium-199 (M-199) (PAA Laboratories, Linz, Austria), supplemented with 10% FCS (Hyclone, Logan, UT), and cultured at 37°C in 99.5% air and 0.5% CO2 for 1 day. The culture medium was changed to M-199 containing gentamycin and kanamycin (25 mg/l each) and then maintained for 2 days for the purpose of serum deprivation. On the next day, the medium was changed again and used for gene transfection. We performed immunostaining with α-actinin to confirm whether the isolated cells were cardiac myocytes. More than 95% of isolated cells were cardiac myocytes (data not shown).

**Gene transfection.** We applied Nucleofector (Amaxa Biosystems) for transient transfection of the Akt-PH-Venus gene. In brief, cardiac myocytes in medium 2 × 10^6 cells/ml were spun for 1 min at 340 g, and the cell pellet was resuspended in 100 μl Nucleofector solution. We mixed 100 μl of cell suspension with 2 μg of the Akt-PH-Venus plasmid, placed the sample in Nucleofector, and transfected the gene. Experiments evaluating insulin-mediated production of PI(3,4,5)P3 under several conditions were performed within 24–48 h postnucleofection. Transfection efficiency of primary neonatal rat cardiac myocytes during this time period was ~50%, which was evaluated with cell numbers that were significantly positive with a fluorescence microscope.

**Confocal microscopy imaging.** Dynamic imaging was performed using laser scanning confocal microscopy (model LSM510; Carl Zeiss, Jena, Germany). The Axiovert 100 M microscope is equipped with a ×40 oil immersion objective (numeric aperture ~1.3). The Venus (a variant of yellow fluorescent protein) channel used the 488-nm line of an argon laser for excitation, and the emission wavelength was collected by a 500- to 535-nm bandpass filter for Venus.

**Western blotting.** Cell lysate was subjected to SDS-PAGE and was electrophoretically transferred to a nitrocellulose membrane (42). The membranes were then exposed to primary antibodies for Akt or phosphorylated (p)-Akt (dilution at 1:1,000; Cell Signaling Technology) overnight at 4°C. After incubation with the peroxidase-linked secondary antibody (dilution at 1:2,000; Cell Signaling Technology) for 1 h at room temperature, immunoreactive proteins were visualized by ECL PLUS reagent (Amersham Life Sciences).

**Measurement of glucose uptake.** To measure glucose uptake in rat cardiomyocytes (H9c2 cells), a nonradioactive assay was performed using a fluorescent D-glucose analog, 2-NBDG, as previously described (19, 41). Briefly, the cells plated on a 96-well plate at a density of 5,000 cells/well were serum depleted for 24 h. Next, the cells were incubated with the indicated drugs for 10 min, and 100 μM 2-NBDG

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**Fig. 1. Effects of ANG II on insulin signaling in cardiac myocytes.** **A:** rat neonatal cardiac myocytes transfecting a fluorophore (Venus)-conjugated pleckstrin homology (PH) domain of cAMP-dependent protein kinase (Akt)/PKB were observed using a laser confocal microscope. Wortmannin prevents phosphatidyl-inositol trisphosphate production. **a,** in the basal state; **b,** cardiac myocyte after insulin (100 nM) application for 10 min; c, cardiac myocytes following wortmannin (100 nM) application for 10 min. **B:** fluorescence of Venus concentrated on application of insulin and ANG II. a, Cardiac myocyte in the basal state; b, cardiac myocyte after insulin (100 nM) application for 10 min; c, cardiac myocyte after ANG II (100 nM) application for 10 min. **C:** Western blotting of Akt or phosphorylated (p)-Akt in the cardiac myocyte on the application of insulin and ANG II. Panels on right indicate semiquantitative assessment of fluorescence. Surface intensity = intensity (10% from both edges)/total intensity. Scale bar indicates 10 μm.
was administrated to each well. After 10 min incubation, the cells were washed with PBS two times, and fluorescence was measured using a microplate reader (ARVOsx; PerkinElmer).

Statistical analysis. Statistical analysis was performed by Student’s t-test. Statistical analysis for glucose uptake was performed by ANOVA, followed by Fisher’s protected least-significant difference test using StatView (Abacus Concepts, Berkeley, CA). Results are expressed as means ± SE. A value of P < 0.05 was regarded significant.

RESULTS

Insulin-mediated PI(3,4,5)P3 production on the cell membrane of cardiac myocytes. To visualize insulin signaling and to test the effects of ANG II on it in living cardiac myocytes, we used a real-time imaging technique that monitors the dynamics of PI(3,4,5)P3, a membrane phospholipid generated by the insulin-signaling pathway. PH domain of Akt/PKB has been reported to strongly and specifically bind PI(3,4,5)P3 (16, 31, 34). We therefore monitored the intensity/distribution of the fluorescence of a bright fluorophore, Venus, conjugated to the Akt-PH domain. Because of the improved speed and efficiency of maturation, the signal intensity of Venus is not affected by intracellular pH and not quenched by chloride ion. Therefore, the fluorophore is regarded as a powerful tool for tracking the movement of various substances in living cells (24).

We detected the fluorescence of the Venus-PH domain on the plasma membrane as well as in the cytosolic region in the control condition (Fig. 1A). This may indicate that a certain amount of PI(3,4,5)P3 is constitutively present on the plasma membrane, even in the resting state. To evaluate the distribution of PI(3,4,5)P3 in the cell, we vertically scanned the cell along a single line, as shown in Fig. 1A, and measured the intensity of fluorescence as the sum of pixels on the line (Fig. 1A, bottom). Next, we divided the sum of pixels within 10% from both edges with the total number of pixels on the line. We referred to this value as “percent surface intensity” (%SI). This value was used as a semiquantitative indicator reflecting the distribution of the fluorescence, i.e., PI(3,4,5)P3, on the plasma membrane.

Application of insulin to the bath prominently enhanced the fluorescent signal at the cellular surface and its vicinity, although decreasing it in the cytosol (Fig. 1, Aa and Ab). This alteration gradually reached a plateau within 5 min, and %SI increased from 24 to 50 in this example (Fig. 1A, right). The increase of %SI was 95.2 ± 10.3% (n = 10, P < 0.0001). This translocation of the fluorescence of the Venus-PH domain from the cytosol to the plasma membrane region may be interpreted as indicating that insulin stimulates production of PI(3,4,5)P3 in the close vicinity of plasma membrane in cardiac myocytes.

The plateau level of fluorescence was maintained constant during continuous presence of insulin at least for 40 min (data not shown). Treatment of the cardiac myocytes with wortman-nin (100 nM), a blocker for PI 3-kinase, caused rapid redistribution of the fluorescence from the surface to the cytosol (Fig. 1Ac) (33, 37), and the %SI decreased to the level less than the control. The decrease of %SI was 46.1 ± 3.9% (n = 5, P <

Fig. 2. ANG II antagonizes insulin signal via the angiotensin type 1 (AT1) receptor. ANG II inhibits production of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3] by insulin through AT1 receptor but not the angiotensin type 2 (AT2) receptor. Insulin (100 nM) was added at 30 min after RNH-6270 or PD-123319 application in the transfected myocyte medium, followed by ANG II (100 nM). A and B: effect of RNH-6270 (10 mM) and PD-123319 (10 mM). a, In the basal state; b, cardiac myocyte after insulin (100 nM) application for 10 min; c, cardiac myocyte following ANG II (100 nM) application for 10 min. Panels on right indicate quantitative assessment of fluorescence. Scale bar indicates 10 μm.
0.0001). This suggests that PI 3-kinase continued to produce PI(3,4,5)P3 as long as insulin was present.

Inhibitory effect of ANG II on insulin-mediated production of PI(3,4,5)P3 and phosphorylation of Akt through AT1. We next examined the effect of ANG II on insulin-mediated translocation of the Venus-PH domain in cardiac myocytes (Fig. 1B). Insulin again caused the translocation of the Venus-PH domain from the cytosol to the plasma membrane region (Fig. 1B, a and b), and the %SI increased from 20 to 40 in this myocyte. ANG II (100 nM) further added to the bath reduced the fluorescence on the membrane region within 10 min almost to the initial level, and the %SI decreased to 25 in this example (Fig. 1Bc, right). The decrease of %SI after ANG II stimulation was 36.2 ± 3.9% (n = 6, P < 0.0001).

We next analyzed the phosphorylation of Akt/PKB (p-Akt) in cardiac myocytes induced by insulin by the Western blotting technique using an antibody specific to p-Akt. Because the phosphorylation of Akt requires the presence of PI(3,4,5)P3, the p-Akt should be a good indicator for the active state of insulin signaling (3). Little signal of p-Akt was visible under control conditions (Fig. 1C, bottom, lane 1). The intensity of the band of p-Akt clearly increased in the presence of insulin (Fig. 1C, bottom, lane 2). When we treated the myocytes with the mixture of insulin (100 nM) and ANG II (100 nM), we detected a much less amount of p-Akt (Fig. 1C, bottom, lane 3). This result may indicate that ANG II inhibited the insulin-mediated production of PI(3,4,5)P3 and thus decreased the amount of p-Akt. This is consistent with the fluorescent data using the Venus-Akt-PH domain in Fig. 1B. It is of note that neither insulin nor ANG II changed the amount of total Akt (Fig. 1C, top).

ANG II is known to exert its specific action by binding to either AT1 receptor or angiotensin type 2 receptor (AT2) that are coupled to G proteins (12, 20). When we preincubated the cells with a selective antagonist for AT1 receptor, RNH-6270 (10 mM), ANG II could not interfere with the effect of insulin on PI(3,4,5)P3 production on the plasma membrane (Fig. 2A, a–c). The %SI only marginally decreased from 40 to 35 in this example. Similar results were obtained in five different cardiac myocytes. The decrease of %SI was 4.5 ± 6.0% (n = 6, P = 0.49). On the other hand, ANG II could still block the insulin-mediated redistribution of the Venus-PH domain in the presence of PD-123319 (10 mM), a blocker specific for AT2 (Fig. 2B, a–c). The %SI decreased from 40 to 18 in the example of Fig. 2B. The decrease of %SI was 32.9 ± 4.9% (n = 5, P < 0.003). These results strongly suggest that the inhibitory reaction of ANG II on insulin-mediated production of PI(3,4,5)P3 occurs through AT1 receptor, but not through AT2.

Intracellular Ca2+ mobilization by ANG II is critically involved in inhibitory effects of ANG II on insulin-mediated PI(3,4,5)P3 production. Compared with VSMC, little is known about the mechanism of IR mediated by ANG II signaling in cardiac myocytes. To clarify the mechanism of ANG II inhibition of insulin-mediated PI(3,4,5)P3 production, we examined the role of inositol trisphosphate (IP3) and diacylglycerol (DAG), which are known to be produced by ANG II via activation of PLC-β. We first examined the role of intracellular Ca2+, which is released from endoplasmic reticulum by IP3.
When we preincubated the cells with BAPTA-AM, a chelator of intracellular Ca\textsuperscript{2+}, ANG II could no longer inhibit the effect of insulin on the distribution of Venus-PH domain (Fig. 3A, a–c, right). The decrease of %SI after ANG II stimulation was 8.2 ± 7.6% (n = 4, P = 0.36). Conversely, application of the Ca\textsuperscript{2+} ionophore ionomycin (100 nM) instead of ANG II abolished insulin-mediated translocation of the fluorescence signal to the plasma membrane region (Fig. 3B, a–c, right). The decrease of

Fig. 4. Calmodulin and calcineurin have no effect of antagonizing insulin signaling. W-7 (calmodulin antagonist) or cyclosporine A (CyA, calcineurin antagonist) application was undertaken 30 min before insulin application. A: W-7 application (50 μM). B: CyA application (1 μM). a and d, In the basal state; b, cardiac myocyte after insulin application (100 nM) for 10 min; c, cardiac myocyte following ionomycin (100 nM) application; e, cardiac myocyte after ANG II application (100 nM) for 10 min; f, cardiac myocyte following ionomycin (100 nM) application. Panels on right indicate quantitative assessment of fluorescence. Scale bar indicates 10 μm.
%SI was 35.8 ± 3.6% \((n = 5, P < 0.001)\). The time course of ionomycin action on insulin signaling resembled those of ANG II (see Fig. 1B). We also confirmed using the \(\text{Ca}^{2+}\) indicator fluo 3 that the application of ANG II caused the prominent \(\text{Ca}^{2+}\) mobilization in the cardiac myocytes (data not shown). Thus, it is suggested that an increase of intracellular \(\text{Ca}^{2+}\) by ANG II is critically involved in insulin-mediated PI(3,4,5)P\(_3\) production. W-7, a specific blocker for calmodulin, and cyclosporine, a blocker of calcineurin, did not influence inhibition of the %SI of Venus-PH domain by ionomycin (Fig. 4, A and B), suggesting that these downstream signals may not be involved in the ANG II effect.

**ANG II-induced activation of PKC is not essential for the inhibitory effect of ANG II on insulin-mediated PI(3,4,5)P\(_3\) production.** In vascular smooth muscles, it was shown that DAG-induced PKC activation by ANG II directly inhibits activation of IRS-1, which is considered to be a cause of ANG II-mediated IR (22). In addition, it is known that localized changes of intracellular \(\text{Ca}^{2+}\) determine the spatial and temporal targeting of PKC in smooth muscle cells (21). We therefore examined the effect of a PKC inhibitor, K-252b, on insulin-mediated PI(3,4,5)P\(_3\) production in cardiac myocytes (Fig. 5). Pretreatment of the cells with K-252b (15 min) did not affect the inhibitory effect of either ionomycin or ANG II on the insulin-mediated increase of the %SI of Venus-PH domain (Fig. 5, A and B, respectively). The decrease of %SI was 29.6 ± 1.3% after stimulation with ionomycin \((n = 3, P < 0.002)\) and 36.2 ± 3.3% after stimulation with ANG II \((n = 4, P < 0.002)\). Accordingly, it is likely that intracellular \(\text{Ca}^{2+}\) elevation interferes with the action of insulin in a PKC-independent manner and that activation of PKC is not involved in ANG II-induced inhibition of insulin-mediated PI(3,4,5)P\(_3\) production.

We next examined the effect of direct activation of endogenous PKC with the phorbol ester PMA. Insulin-mediated PI(3,4,5)P\(_3\) production on the cell surface was only transiently inhibited by PMA (Fig. 6A). The transient decrease of %SI after PMA stimulation was 39.6 ± 5.7\% \((n = 5, P < 0.003)\). This effect was not observed after the application of an inactive form of PMA, 4\alpha\)-PMA (data not shown). These results indicate that the activation of PKC has the rapid and transient interfering effect on the insulin-mediated PI(3,4,5)P\(_3\) production. Chelating transient intracellular \(\text{Ca}^{2+}\) elevation by BAPTA-AM (Fig. 6B) did not block the effect of PMA, suggesting that this effect of PKC occurs independent of \(\text{Ca}^{2+}\) mobilization. The decrease of %SI after PMA stimulation was 34.6 ± 7.2\% \((n = 3, P < 0.04)\). These results suggest that the activation of PKC is insufficient to explain the sustained inhibitory effect of ANG II against the insulin-mediated PI(3,4,5)P\(_3\) production in cardiac myocytes.

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**Fig. 5. Intracellular \(\text{Ca}^{2+}\) mobilization reduces PI(3,4,5)P\(_3\) production mediated by insulin through a PKC-independent pathway.** A: K-252b (100 \(\mu\)M) pretreatment 15 min before insulin application. \(a\), in the basal state; \(b\), cardiac myocyte after insulin (100 \(\mu\)M) application for 10 min; \(c\), cardiac myocyte following ionomycin application (100 nM) for 10 min. B: K-252b (100 \(\mu\)M) pretreatment 15 min before insulin application. \(a\), in the basal state; \(b\), cardiac myocyte after insulin (100 nM) application for 10 min; \(c\), cardiac myocyte following ANG II application (100 nM) for 10 min. Panels on right indicate quantitative assessment of fluorescence. Scale bar indicates 10 \(\mu\)m.
Inhibitory effect of ANG II on insulin-mediated glucose uptake was reversed through a Ca\(^{2+}\)/H\(^{+}\)-dependent pathway, but not through a PKC-dependent pathway. We confirmed whether the inhibitory effects of ANG II on insulin induced PI(3,4,5)P\(_3\) production related to glucose uptake in cardiomyocytes. Insulin-mediated uptake of glucose in a clonal line of rat cardiomyocytes was measured using a fluorescent D-glucose derivative, 2-NBDG. ANG II inhibited insulin-mediated glucose uptake, which was reversed with BAPTA-AM, an intracellular Ca\(^{2+}\)/H\(^{+}\) chelator, but not with the PKC inhibitor K-2526 (Fig. 7). Changes in glucose uptake corresponded to changes in PI(3,4,5)P\(_3\) production with these reagents.

DISCUSSION

ANG II inhibition of insulin signaling has been taken as one of the important mechanisms for the genesis of IR in various cells, including adipocytes (4, 26) and VSMC (22). Activation of PKC and an increase in intracellular Ca\(^{2+}\) are reported to be involved in ANG II-induced IR in adipocytes (2, 40) and VSMC (22). Although it was also reported that ANG II inhibits insulin signaling in cardiac myocytes (7, 36), its intracellular mechanisms remain unknown.

Using real-time imaging techniques with a fluorophore conjugated domain of Akt/PKB we analyzed the time course of changes in PI(3,4,5)P\(_3\) concentration in the cardiac cell mem-
brane under various conditions. Both ANG II and ionomycin caused sustained inhibition of insulin-mediated Pi(3,4,5)P3 production. However, phorbol ester caused transient inhibition of insulin-mediated Pi(3,4,5)P3 production even though PKC activation was sustained under blocking the elevation of intracellular Ca2+ by BAPTA. These pharmacological investigations strongly suggest that ANG II-mediated inhibition of insulin signaling in cardiac myocytes is caused mainly by elevation of intracellular Ca2+ but not by activation of PKC.

We have confirmed that this inhibitory effect of ANG II on insulin-mediated Pi(3,4,5)P3 production and the intracellular pathways corresponded with those on insulin-mediated glucose uptake.

On the contrary, in VSMC, the dominant signal to inhibit insulin-mediated Akt activation by ANG II is PKC-α but not intracellular Ca2+ (22). Therefore, the signal mechanism underlying ANG II inhibition of insulin signaling may vary according to cell types. Transient inhibition of insulin-mediated Akt activation by PMA in cardiomyocytes suggests activated PKC near the sarcolemma interfered with insulin signaling. However, this inhibitory effect of PMA was not sustained, and ANG II inhibition of insulin signaling was not suppressed by the PKC inhibitor in heart muscle cells. Taken together, we speculate that translocation of activated PKC to the sarcolemma is functional in VSMC but not in heart muscle cells.

Although we showed that the elevation of intracellular Ca2+ was involved in ANG II inhibition of insulin-mediated production of Pi(3,4,5)P3, we could not identify the signaling cascade following elevation of intracellular Ca2+: W-7, a specific blocker for calmodulin, and cyclopiazonine, a blocker of calcineurin, did not influence inhibition of the %SI of Venus-PH domain by ionomycin (Fig. 4, A and B), suggesting that neither calmodulin nor calcineurin may be involved in the ANG II effect in cardiac myocytes.

There may be three possibilities for the downstream mechanism. First, we speculate the existence of unspecified molecules that cross talk with intracellular Ca2+ mobilization specifically in cardiac myocytes. The second possibility is that the target protein of PKC-α in VSMC is expressed at very low levels or absent in cardiac myocytes. The third possibility is that the levels of PKC activation by ANG II in our study might be insufficient to inhibit insulin signaling like as previously reported in PKC-transfected L6 muscle cells (8). Regarding the first possible mechanism, Jang et al. (13) speculated the existence of Ca2+-dependent phosphatase because chelation of intracellular Ca2+ improved insulin sensitivity in high-fat-fed rats. Further studies are required to clarify the molecules that cross talk with intracellular Ca2+ mobilization in cardiac myocytes.

Insulin has close relationships with nutrition and energy metabolism. Between heart and vasculature, the importance of insulin signaling seems to be different because of different energy sources (11), i.e., free fatty acids for cardiac myocytes but glucose for VSMC. Although our studies were performed using nonfailing cardiomyocytes, the significance of insulin signaling in cardiac myocytes increases in the patients with heart failure because the development of heart failure induces crucial changes in energy source from fatty acid to glucose. It is reported that cardiac and plasma levels of ANG II increased in patients with heart failure (29). Taken together, clinical findings of usefulness of the renin-angiotensin blockers in patients with heart failure (18) may be explained in part through improvement of ANG II-mediated IR in cardiomyocytes of which energy source has changed to glucose. Exploration of the target molecules that cross talk with intracellular Ca2+ mobilization may lead to the further progress in the treatment of heart failure.

Study limitations. The first limitation of our study is that the analysis of the intensity of fluorophore is weak in quantification. We believe that %SI, which indicates the percent distribution of fluorophore in the edge site of whole fluorophore, is very useful to assess semiquantitatively the time course of changes in the distribution of fluorophore in response to pharmacological stimulation. Furthermore, the result of the quantitative analysis of glucose uptake was consistent with the semiquantitative result of the changes in Pi(3,4,5)P3 production.

The second limitation is that we could not make any conclusion about signaling pathways other than the ANG II-mediated pathway, such as the α-adrenergic receptor pathway that cross talks or overlaps with the insulin receptor signaling. Our previous experiment, however, indicates that the ANG II-mediated pathway plays a more important role than the α-adrenergic receptor pathway in hyperinsulinemia-induced cardiac hypertrophy of fructose-fed rats (14).

In summary, using the real-time imaging techniques, we have shown that ANG II may negatively regulate insulin-mediated Pi(3,4,5)P3 production via an increase of intracellular Ca2+ rather than via PKC activation through the stimulation of AT1 receptor in the cardiac myocytes. The real-time imaging techniques may hold enormous potential to explore new cell signaling. The present finding indicates that the cardiomyocyte-specific interaction between insulin signaling and ANG II could be a new target for improving the metabolism of cardiomyocytes.

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