Cysteinyl leukotriene-dependent \([\text{Ca}^{2+}]_i\) responses to angiotensin II in cardiomyocytes

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Liu, Pinggang, Derek A. Misurski, and Venkat Gopalakrishnan. Cysteinyl leukotriene-dependent \([\text{Ca}^{2+}]_i\) responses to angiotensin II in cardiomyocytes. Am J Physiol Heart Circ Physiol 284: H1269–H1276, 2003. First published December 12, 2002; 10.1152/ajpheart.00303.2002.—With the use of fura 2 measurements in multiple and single cells, we examined whether cysteinyl leukotrienes (CysLT) mediate angiotensin II (ANG II)-evoked increases in cytosolic free \([\text{Ca}^{2+}]_i\) in neonatal rat cardiomyocytes. ANG II-evoked CysLT release peaked at 1 min. The angiotensin type 1 (AT_1) antagonist losartan, but not the AT_2 antagonist PD-123319, attenuated the elevations in \([\text{Ca}^{2+}]_i\), and CysLT-sensitive responses evoked by ANG II. Vasopressin and endothelin-1 increased \([\text{Ca}^{2+}]_i\), but not CysLT levels. The 5-lipoxygenase (5-LO) inhibitor AA-861 and the CysLT-selective antagonist MK-571 reduced the maximal \([\text{Ca}^{2+}]_i\). Release, responses to ANG II not to vasopressin and endothelin-1. While MK-571 reduced the responses to leukotriene D_4 (LTD_4), the dual CysLT antagonist BAY-u9773 completely blocked the \([\text{Ca}^{2+}]_i\) elevation to both LTD_4 and LTC_4. These data confirm that ANG II-evoked increases, but not vasopressin- and endothelin-1-evoked increases, in \([\text{Ca}^{2+}]_i\) involve generation of the 5-lipoxygenase metabolite CysLT. The inositol (1,4,5)-trisphosphate \([\text{Ins}(1,4,5)\text{P}_3]_i\) antagonist 2-aminoethoxydiphenylborate attenuated the \([\text{Ca}^{2+}]_i\), responses to ANG II and LTD_4. Thus AT_1 receptor activation by ANG II is linked to CysLT-mediated \([\text{Ca}^{2+}]_i\) release from \([\text{Ins}(1,4,5)\text{P}_3]_i\)-sensitive intracellular stores to augment direct ANG II-evoked \([\text{Ca}^{2+}]_i\) mobilization in rat cardiomyocytes.

doendothein-1; intracellular free calcium; vasopressin

THE PRESENCE OF ALL COMPONENTS of the renin-angiotension system in neonatal rat cardiomyocytes (NRC) is consistent with its role in maintaining cardiovascular homeostasis (3, 5). Angiotensin II (ANG II) regulates cardiac contractility and growth via stimulation of angiotensin type 1 (AT_1) receptors (3). Stimulation of AT_1 receptors leads to \([\text{Ca}^{2+}]_i\) mobilization through the activation of phospholipase C (PLC), resulting in the generation of inositol (1,4,5)-trisphosphate \([\text{Ins}(1,4,5)\text{P}_3]_i\) and diacylglycerol (12, 27, 31, 32). The mechanisms governing the regulation of ANG II-evoked increases in cytosolic free calcium concentration \([\text{Ins}(1,4,5)\text{P}_3]_i\) levels are not fully understood. Besides enhancing the \([\text{Ca}^{2+}]_i\), level, ANG II elicits complex intracellular signaling events that include the production of superoxide anions and the activation of several kinases as well as the alteration of cyclic nucleotides and nitric oxide levels (31). In addition, ANG II activates phospholipase A_2 and D, resulting in elevation of arachidonic acid (AA)-derived metabolites (6, 15, 19, 22, 24, 36). In rat vascular smooth muscle cells, the hypertrophic responses to ANG II are suggested to be at least partially linked to generation of noncyclooxygenase-derived AA metabolites (6, 22). Recently, we and others have demonstrated that ANG II-evoked vasoconstrictor responses in rat aortic rings and perfused rat mesenteric vascular bed were reduced by 2-[12-hydroxydocos-5,10-diynyl]3,5,6-trimethyl-p-benzoquinone (AA-861), a selective blocker of 5-lipoxygenase (5-LO), or 3-[[3-2-(7-chloro-2-quinilinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl][thio]methyl][thio][E] sodium salt (MK-571), a selective cysteinyl leukotriene (CysLT) 1 antagonist (29, 30). These data suggested that ANG II may enhance the production of the AA-derived 5-LO metabolite CysLT. However, whether ANG II promotes CysLT production in vascular smooth muscle cells and cardiomyocyte has not been investigated.

CysLT include leukotriene D_4 (LTD_4), C_4 (LTC_4), and E_4 (LTE_4). They exert their actions via activation of at least two pharmacologically defined G protein-coupled receptors (CysLT_1 and CysLT_2) that are linked to PLC-mediated \([\text{Ca}^{2+}]_i\) mobilization (8, 16). In addition to their well-known bronchiolar smooth muscle spasmogenic effect, these inflammatory mediators have been shown to enhance contractile responses in smooth muscle preparations or \([\text{Ca}^{2+}]_i\) levels in their target cells (4, 10, 20, 21, 23, 29, 30). Recently, with the use of a variety of techniques including in situ hybridization, Northern blotting, and RT-PCR, the presence of CysLT_1 and/or CysLT_2 transcripts in cardiac tissue has been identified (8, 10, 16, 21). CysLT has been shown to exert potent effects on the heart, contributing to heart failure (2, 10). Leukotriene A_4 hydrolase plays a critical role in the generation of CysLT, and its expression is elevated in the heart of ANG II-induced hypertensive rats, suggesting that ANG II may promote cardiac CysLT production (9). Moreover, low concentrations of

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CysLT, specifically LTD₄ and LTC₄, have been shown to promote a positive inotropic effect in the rat heart (11). Despite these reports, there are no studies to demonstrate that CysLT, namely, LTD₄ and LTC₄, promotes Ca²⁺ mobilization and that ANG II-evoked Ca²⁺ mobilization is linked to CysLT generation in cardiomyocytes. In the present study, using adherent NRC in primary cultures, we attempted to address these issues.

MATERIALS AND METHODS

Cardiomyocyte cultures. The care and use of animals conforms to the regulations stipulated by the University Animal Care Committee. The details of isolation and primary culture of ventricular myocytes from newborn (3 days old) Sprague-Dawley rats have been previously described (14, 34, 35). After removal of mesenchymal cells, the cell suspension was layered on either six-well culture plates for CysLT assay or on glass coverslips for fluorescence measurement. Bromodeoxyuridine (0.1 mM) was included in the medium to ensure selective suppression of mesenchymal cells to facilitate purification of myocyte-rich (>95%) primary cultures of NRC (14).

Determination of [Ca²⁺]ᵢ, using fura 2 fluorescence in adherent NRC. After NRC grown on glass coverslips attained confluence (3 days), the cells were maintained in serum-free medium for 24 h. The cells were washed twice in Krebs-HEPES buffer [composed of (in mM) 145 NaCl, 5 KCl, 1.8 CaCl₂·2H₂O, 1.2 MgCl₂·6H₂O, 10 glucose, and 10 HEPES with 0.2% BSA; pH 7.4]. Cells were loaded in the dark with fura 2-AM (final concentration 5 μM) for 30 min, followed by three buffer washes. Coverslips were inserted into a microcuvette containing 500 μl buffer at 37°C. The excitation signals (340/380 nm) were determined using a fluorimeter designed to monitor fura 2 fluorescence (JASCO CAF-100 Ca²⁺ Analyzer, Japan Spectroscopic; Tokyo, Japan). Details of calibration and determination of basal and agonist-evoked increases in [Ca²⁺]ᵢ levels have been described previously (14, 34). The concentration-peak [Ca²⁺]ᵢ response (CR) curves to each agonist (ANG II, arginine vasopressin (AVP), endothelin (ET-1), and LTC₄ or LTD₄) were evaluated using fresh coverslips of fura 2-loaded cells for each challenge. CR determinations for LTC₄ and LTD₄ were determined in the buffer medium devoid of BSA. The CR determinations to agonists were also performed in the presence of optimal concentration(s) of the 5-L0 inhibitor AA-861 (either 10 or 30 μM (29, 30, 33)), the CysLT₁ antagonist MK-571 (100 nM (16, 26, 29, 30)), the dual CysLT₁/CysLT₂ antagonist BAY-u9773 (100 nM (8)), the AT₁-selective antagonist losartan (1 μM (3, 12, 27, 28)), the AT₂-selective antagonist PD-123319 (1 μM (3, 12, 7, 27, 28, 32)), and the cell-permeant Ins(1,4,5)P₃ blocker 2-aminoethoxydiphenyl borate [2-APB; 50 μM (18)]. The concentrations of all these agents were carefully chosen to ensure their selectivity of inhibition/blockade as validated by previous reports. Each agent was maintained in the cuvette for 3 min before the agonist challenge. In select experiments, the fura 2-loaded cells on coverslips were washed and placed in Ca²⁺-free buffer with 1 mM EGTA (pH 7.4) in the cuvette for 15 min before the agonist challenge and fluorescence measurements.

Single cell fura 2 imaging. NRC grown on glass coverslips (Delta T Dish 0.15 nm, Bioptechs; Butler, PA) were maintained in DMEM for 3 days and changed to serum-free medium for 24 h. The cells were washed in Krebs-HEPES buffer and subjected to fura 2 loading and washing. The dish was mounted on the stage of an inverted Olympus I ×70 epifluorescence microscope fitted with the specification of UApo20×/340.5 objective that has the capability to monitor the apochromat-reflected light fluorescence of fura 2. The fura 2 fluorescence images were acquired using a fast monochromatic integral 125-W xenon light source (SpectraMaster Monochromator, Life Science Resources, Perkin Elmer; Gaithersburg, MD) with a shutter speed for monitoring alternate 340- and 380-nm excitation signals every 200 ms. Ratiometric signals at 340/380 nm were acquired at a rate of 3 images/s. The emission signal at 510 nm was collected using a charge-coupled device camera (Astrocam; Cambridge, UK). The digitized signals were stored and processed using UltraVIEW Imaging System software (Wallac Imaging, Perkin Elmer). The digitized signals were stored and processed using UltraVIEW Imaging System software (Wallac Imaging, Perkin Elmer). ANG II (50 nM) was added to the coverslips after images were acquired for the first 30 s to determine basal fura 2 fluorescence. Interacting agents were added to fura 2-loaded cells for 3 min before imaging. The respective Ca²⁺-saturated and Ca²⁺-free 340- to 380-nm fluorescence ratio values were determined using the Ca²⁺-ionophore bromo- A-23187 (50 μM) and then by quenching with 50 μl of Tris (50 mM) + EGTA (100 mM) solution (pH 8.5) at the end of each experiment.

Materials and supplies. Single cell fura 2 imaging. NRC grown on glass coverslips (Delta T Dish 0.15 nm, Bioptechs; Butler, PA) were maintained in DMEM for 3 days and changed to serum-free medium for 24 h. The cells were washed in Krebs-HEPES buffer and subjected to fura 2 loading and washing. The dish was mounted on the stage of an inverted Olympus I ×70 epifluorescence microscope fitted with the specification of UApo20×/340.5 objective that has the capability to monitor the apochromat-reflected light fluorescence of fura 2. The fura 2 fluorescence images were acquired using a fast monochromatic integral 125-W xenon light source (SpectraMaster Monochromator, Life Science Resources, Perkin Elmer; Gaithersburg, MD) with a shutter speed for monitoring alternate 340- and 380-nm excitation signals every 200 ms. Ratiometric signals at 340/380 nm were acquired at a rate of 3 images/s. The emission signal at 510 nm was collected using a charge-coupled device camera (Astrocam; Cambridge, UK). The digitized signals were stored and processed using UltraVIEW Imaging System software (Wallac Imaging, Perkin Elmer). ANG II (50 nM) was added to the coverslips after images were acquired for the first 30 s to determine basal fura 2 fluorescence. Interacting agents were added to fura 2-loaded cells for 3 min before imaging. The respective Ca²⁺-saturated and Ca²⁺-free 340- to 380-nm fluorescence ratio values were determined using the Ca²⁺-ionophore bromo-A-23187 (50 μM) and then by quenching with 50 μl of Tris (50 mM) + EGTA (100 mM) solution (pH 8.5) at the end of each experiment. Experimental values are the software program employed provided the absolute [Ca²⁺]ᵢ values for each cell using the Gryniewicz equation.

Total CysLT measurement. NRC grown on six-well culture plates for 3 days (~0.3 – 0.5 × 10⁶ cells/well) were maintained for the last 24 h in 2 ml of medium devoid of serum. The medium was replaced with Krebs-HEPES buffer before stimulation with ANG II (100 nM) for varying time intervals (from 5 to 360 s). CR determinations for ANG II (100 μM–1 μM)-evoked increases, AVP (100 pM–1 μM)-evoked increases, and ET-1 (100 μM–1 μM)-evoked increases in CysLT levels were determined 1 min after the addition of the respective agonists. All these assays were performed in duplicate. The responses to ANG II were also determined in the presence of AA-861 (10 μM), losartan (1 μM), or PD-123319 (1 μM). A 450–μl aliquot of culture medium was stored in siliconized tubes at −80°C. Total CysLT levels were determined within 10 days of storage by enzyme immunoassay following the protocol provided by Cayman Chemicals (Ann Arbor, MI). Total CysLT levels were assayed spectrophotometrically (405 nm for measurement of acetylcholinesterase activity) as outlined in the kit using an Anthos HT1 96-well microplate reader (Anthos Labtech Instruments; Salzburg, Austria). The lowest detection limit was 4.0 pg/ml and the 50% (B/F) ratio was 40 pg/ml. The intra- and interassay coefficients of variation were 7.3 ± 2.5% and 5.8 ± 2.9%, respectively. Cells were counted, and the data were normalized to express the values as CysLT per milliliter per million cells. Materials and supplies. Fura 2-AM and pluronic acid F-127 were from Molecular Probes (Eugene, OR). Culture media, DMEM, serum, and trypsin were from GIBCO-BRL (Life Technologies; Grand Island, NY). AVP, ANG II, and ET-1 were from Bachem (Torrance, CA). Bromodeoxyuridine, AA-861, PD-123319, 2-APB, and analytic grade salts for the preparation of Krebs buffer were purchased from Sigma Chemical (Oakville, Ontario, Canada). BAY-u9773 was obtained from BioMol Research Laboratories (Plymouth Meeting, PA). MK-571 and losartan were provided by Merck-Frosst Canada. LTC₄, LTD₄, and enzyme immunoassay kits for the estimation of CysLTs were obtained from Cayman Chemicals. Experimental values are reported as means ± SEM of a minimum of five separate experiments performed on different days using different batches of NRC. Comparison of means was performed with ANOVA (Super ANOVA software). Simultaneous multiple comparisons were assessed using Scheffe’s F-test, and the concentration of

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LEUKOTRIENES MEDIATE ANG II-EVOKED [Ca²⁺],

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agonist required to produce 50% of the maximal response (EC₅₀) and the maximal increase in [Ca²⁺]ı (Eₘ₉ₐₓ values attained for each agonist were derived from log CR curves.

RESULTS

The basal [Ca²⁺]ı levels in normal Ca²⁺ buffer were 90 ± 12 nM in multiple cells (n = 54) and 121 ± 18 nM in single cells (n = 23), and the differences in resting levels observed by both methods were not significant. None of the interacting agents (AA-861, MK-571, BAY- u9773, 2-APB, losartan, or PD-123319) affected the basal fura 2 fluorescence ratio. A representative tracing of ANG II-evoked increases in the fura 2 fluorescence ratio performed on the same day in the presence or absence of either AA-861 (10 µM) or MK-571 (100 nM) is shown in Fig. 1. The addition of ANG II led to a rapid concentration-dependent increase in the ratio of fura 2 fluorescence in multiple cells, with maximal increases observed between 30 s and 1 min; at 2 min after stimulation, the fluorescence ratio decreased to a steady state above the basal level. The CR curves to ANG II determined in either normal Ca²⁺ (1.8 mM) or Ca²⁺-free buffer is shown in Fig. 2. The addition of AA-861 (10 µM) or MK-571 (100 nM) led to a significant reduction in ANG II-evoked increases in peak [Ca²⁺]ı; values (P < 0.01) in both normal and Ca²⁺-free buffer. Moreover, the addition of AA-861 or MK-571 did not affect either the time to attain the peak response or the time for reduction in peak Ca²⁺ to steady-state levels for varying concentrations of ANG II (Fig. 1). The effect of blockade on ANG II responses was similar when AA-861 was increased to 30 µM. Although the

**Fig. 2.** Effects of the 5-lipoxygenase (5-LO) inhibitor AA-861 and cysteinyl leukotriene (CysLT₁)-selective antagonist MK-571 on peak cytosolic free Ca²⁺ concentration ([Ca²⁺]ı) responses to ANG II in NRC. Primary cultures of NRC were stimulated with increasing concentrations of ANG II in either the absence (control; ○) or presence of AA-861 (10 or 30 µM; ●) or MK-571 (100 nM; □) in Krebs buffer (pH 7.4) at 37°C. ANG II concentration-peak [Ca²⁺]ı response (CR) curves were determined with either Ca²⁺ being present (1.8 mM; left) or absent (0 mM Ca²⁺ + 1 mM EGTA; pH adjusted to 7.4; right) in the buffer. Each CR curve was determined eight times using different batches of NRC. *P < 0.05 and **P < 0.01 compared with AA-861- and MK-571-treated cells.

Table 1. ANG II, AVP, and ET-1 evoked increases in peak [Ca²⁺]ı in either the presence or absence of the 5-LO inhibitor AA-861 or the CysLT₁-selective antagonist MK-571 in NRC maintained at 37°C

<table>
<thead>
<tr>
<th></th>
<th>1.8 mM Ca²⁺</th>
<th>0 mM Ca²⁺ + 1.0 mM EGTA</th>
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<tbody>
<tr>
<td></td>
<td>EC₅₀, nM</td>
<td>Eₘ₉ₐₓ, nM</td>
</tr>
<tr>
<td>ANG II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.6 ± 4.6</td>
<td>498 ± 30</td>
</tr>
<tr>
<td>10 µM AA-861</td>
<td>13.9 ± 6.1</td>
<td>344 ± 15*</td>
</tr>
<tr>
<td>30 µM AA-861</td>
<td>15.1 ± 3.5</td>
<td>313 ± 28*</td>
</tr>
<tr>
<td>100 nM MK-571</td>
<td>16.8 ± 7.2</td>
<td>370 ± 22*</td>
</tr>
<tr>
<td>AVP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.9 ± 7.3</td>
<td>210 ± 11</td>
</tr>
<tr>
<td>30 µM AA-861</td>
<td>21.5 ± 6.6</td>
<td>199 ± 14</td>
</tr>
<tr>
<td>100 nM MK-571</td>
<td>20.4 ± 7.8</td>
<td>212 ± 13</td>
</tr>
<tr>
<td>ET-1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.3 ± 1.4</td>
<td>253 ± 18</td>
</tr>
<tr>
<td>30 µM AA-861</td>
<td>7.2 ± 2.1</td>
<td>232 ± 21</td>
</tr>
<tr>
<td>100 nM MK-571</td>
<td>8.4 ± 2.3</td>
<td>228 ± 19</td>
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</table>

Values are means ± SE; n = 8 experiments. ANG II, angiotensin II (10 pM–10 µM); AVP, arginine vasopressin (100 pM–10 µM); ET-1, endothelin-1 (10 pM–100 nM); [Ca²⁺]ı, cytosolic Ca²⁺ concentration; 5-LO, 5-lipoxygenase; CysLT₁, cysteinyl leukotriene; NRC, neonatal rat cardiomyocytes; Eₘ₉ₐₓ, maximal increase in [Ca²⁺]ı. The basal level of [Ca²⁺]ı was 90 ± 12 nM in normal Ca²⁺ buffer. AA-861 or MK-571 was maintained in the cuvette in the indicated buffer for a period of 3 min before the addition of the agonist. The Eₘ₉ₐₓ of ANG II or AVP was attained at 1 µM, whereas the Eₘ₉ₐₓ of ET-1 was reached at 100 nM. Note that stimulation with either AVP or ET-1 was not performed in Ca²⁺-free buffer because no significant changes in the EC₅₀ and Eₘ₉ₐₓ values were noted in normal Ca²⁺ buffer. *P < 0.05 and **P < 0.01 compared with the respective control group.
ination of either AA-861 (30 μM) or MK-571 (100 nM) failed to affect the CR curves to AVP or ET-1 (Table 1).

A representative tracing of ANG II-, LTD4-, and LTC4-evoked [Ca2+]i responses is shown in Fig. 3. Both LTD4 and LTC4 evoked rapid increases in peak [Ca2+]i levels (E_max: LTD4 >> LTC4) with a similar time course but much lower E_max compared with ANG II. The peak responses were attained at 40, 43, and 47 s after the addition of ANG II, LTD4, and LTC4, respectively. The plateau phase that sustained at levels slightly above their respective baseline values were reached between 120 and 180 s after the addition of respective agonist(s). There were no significant differences between them (Fig. 3). The inclusion of MK-571 significantly attenuated the E_max and increased the EC_{50} value for LTD4, but it failed to evoke a significant reduction in the weak [Ca2+]i signals evoked by LTC4. On the contrary, the inclusion of a nonselective CysLT antagonist, BAY-u9773, completely blocked the [Ca2+]i responses to both LTD4 and LTC4 (Fig. 4). The analysis of data obtained from several CR curves is summarized in Table 2. The Ins(1,4,5)P3 blocker 2-APB and the AT_{1}-selective antagonist losartan significantly attenuated the responses to ANG II, whereas PD-123319 had no effect on either basal or ANG II-evoked peak [Ca2+]i responses (Fig. 5, left). 2-APB also attenuated the maximal [Ca2+]i responses, with a significant reduction in E_max values for LTD4 (Fig. 5, right).

Single cell Ca^{2+} imaging studies revealed qualitatively similar results. A typical experiment performed with ANG II stimulation (50 nM) on three different coverslips loaded with fura 2 on the same day using the same batch of cardiomyocytes is shown in Fig. 6. Basal fluorescence levels before stimulation are shown in Fig. 6, A–C [control (A), AA-861 (B), and MK-571 (C)]. Figure 6, E and F, shows the effect of ANG II at 30 s in the presence of either AA-861 (E) or MK 571 (F). The responses are compared with images determined for ANG II in the absence of these interacting agents [control (Fig. 6D)]. Single cell fluorescence determination from several experiments (n = 5) gave the following absolute [Ca2+]i values: control 585 ± 34 nM, AA-861 400 ± 27 nM (P < 0.01), and MK-571 460 ± 21 nM (P < 0.01). Although single cell E_max values for ANG II were higher compared with data obtained with multiple cells, a similar pattern of blockade in the presence of AA-861 or MK-571 was evident. Between 2- and 3-min intervals, the cells had reached steady-state fluorescence close to the basal value, suggesting the fluorescence changes are consistent with [Ca2+]i changes, and that the results gathered were not due to photobleaching (data not shown).

The basal CysLT level in the culture medium was 9.7 ± 1.9 pg·ml^{-1}·10^6 cells^{-1}. The addition of ANG II led to a rapid, time-dependent increase in the CysLT

<table>
<thead>
<tr>
<th>Treatments</th>
<th>LTD4</th>
<th>LTC4</th>
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<tbody>
<tr>
<td>Control</td>
<td>17.0 ± 4.5</td>
<td>37.3 ± 5.6*</td>
</tr>
<tr>
<td>100 nM MK-571</td>
<td>37.3 ± 5.6*</td>
<td>52 ± 11†</td>
</tr>
<tr>
<td>100 nM BAY-u9773</td>
<td>57.5 ± 7.4*</td>
<td>17 ± 7†</td>
</tr>
<tr>
<td>Control</td>
<td>49.8 ± 16.5</td>
<td>107 ± 10</td>
</tr>
<tr>
<td>100 nM MK-571</td>
<td>42.6 ± 17.3</td>
<td>95 ± 12</td>
</tr>
<tr>
<td>100 nM BAY-u9773</td>
<td>63.8 ± 6.2</td>
<td>21 ± 9†</td>
</tr>
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</table>

Values are means ± SE; n = 6 experiments. LTD4 and LTC4, leukotriene D4 (10 μM–100 μM) and C4 (10 μM–100 μM), respectively. The basal level of [Ca2+]i in the absence of either LTD4 or LTC4 stimulation was 90 ± 12 nM. MK-571 or BAY-u9773 was added 3 min before the addition of agonist to the cuvette. *P < 0.05 and †P < 0.01 compared with the respective control group.
level, reaching a maximum (19.8 ± 2.6 pg·ml⁻¹·10⁶ cells⁻¹) at 1 min, remaining at the same level until 5 min, and gradually decreasing thereafter. However, the increases in CysLT levels were significantly higher (15.7 ± 1.8 pg·ml⁻¹·10⁶ cells⁻¹) even at 30 s after the addition of ANG II (Fig. 7, left). The CR determinations for CysLT generation revealed that the ANG II effect was also concentration dependent. The $E_{\text{max}}$ for ANG II (100 nM)-evoked CysLT release was twofold higher than the basal level. In contrast to ANG II, the addition of ET-1 (100 nM) or AVP (100 nM) failed to evoke a significant increase in CysLT production. The inclusion of losartan or AA-861 led to a significant reduction in ANG II-evoked total CysLT release into the culture medium, whereas the addition of PD-123319 failed to affect either the basal or ANG II-evoked increases in CysLT production (Fig. 7, right). CysLT generation was significantly higher at 30 s, and the peak [Ca²⁺]ᵢ increase was reached at 40 s after the addition of ANG II (100 nM), suggesting that the increased CysLT generation evoked by ANG II may contribute to an elevation in [Ca²⁺]ᵢ (Figs. 3 and 7).

**DISCUSSION**

The present study provides several new observations: First, the 5-LO-derived metabolites of AA, CysLT, augment [Ca²⁺]ᵢ responses to ANG II in NRC. Second, the inclusion of the 5-LO inhibitor AA-861 (10 μM) led to significant attenuation of ANG II-evoked increases, but not ET-1- and AVP-evoked increases, in peak [Ca²⁺]ᵢ levels and CysLT release into the culture medium. Third, ANG II-evoked increases in [Ca²⁺]ᵢ levels and CysLT release in NRC are mediated by AT₁ receptor activation because losartan, an AT₁ antagonist, but not the AT₂-selective antagonist PD-123319, blocked the responses to ANG II. Fourth, both LTD₄ and LTC₄ evoke [Ca²⁺]ᵢ increase in these cells; however, the responses to these mediators were much lower than those evoked by ANG II. The CysLT₁-selective antagonist MK-571 significantly abolished the [Ca²⁺]ᵢ responses to LTD₄ but failed to inhibit the responses to LTC₄, whereas the CysLT₁/CysLT₂ antagonist BAY-u9773 completely blocked the [Ca²⁺]ᵢ responses to both LTD₄ and LTC₄. Finally, both ANG II- and CysLT-evoked increases in [Ca²⁺]ᵢ levels were markedly attenuated by 2-APB, an agent that blocks Ins(1,4,5)P₃-mediated Ca²⁺ release. These data confirm that both ANG II and CysLT recruit predominantly Ins(1,4,5)P₃-sensitive intracellular Ca²⁺ pools in NRC. Thus the CysLT pathway may serve as an additional amplifier pathway in sustaining direct AT₁-mediated intracellular Ca²⁺ mobilization evoked by ANG II in NRC.

**AT₁ and AT₂ subtypes on NRC.** The presence of two high-affinity binding sites for [¹²⁵I]-labeled ANG II has been demonstrated in membrane preparations of NRC (25). Rogers and colleagues (13, 15) proposed that the AT₁ subtype would promote [³H]Ins(1,4,5)P₃ accumulation via activation of PLC, whereas the AT₂ subtype would be linked to [³H]AA release via activation of phospholipase A₂, and that ANG II-induced alkalization was sensitive to blockade by AT₂-selective but not AT₁-selective antagonists. Others have suggested that AT₁-mediated hypertrophy by ANG II could be opposed by AT₂-mediated antihypertrophy mediated by S-nitrosothiols (6, 15, 19, 22, 24, 31, 36). The present study demonstrates that ANG II-evoked increases in CysLT and [Ca²⁺]ᵢ levels were abolished by losartan but not by PD-123319. Therefore, AT₁ receptor activation and the resultant PLC/Ins(1,4,5)P₃-mediated [Ca²⁺]ᵢ responses may also be partly mediated by AA-derived CysLT production in NRC. The focus of the present study was to examine the involvement of CysLT in the ANG II-mediated elevation in peak [Ca²⁺]ᵢ responses.

**ANG II-CysLT interaction.** Previous studies have shown that ANG II-evoked vascular hypertrophy may be mediated by noncyclooxygenase-derived AA metabolites (6, 15, 19, 22, 24, 31, 36). The 5-LO inhibitor AA-861 or the CysLT₁ antagonist MK-571 reduced the vasoconstrictor responses to ANG II, suggesting that CysLT generation and the subsequent CysLT₁ receptor activation may mediate vasoconstriction to ANG II (29, 30). Therefore, it was important to provide direct evidence at the level of signal transduction for a link among ANG II, AT₁ receptor activation, CysLT generation, and CysLT receptor-mediated alterations in [Ca²⁺]ᵢ levels. It is well known that cell surface receptors for ET-1 are several orders of magnitude higher than the receptors for ANG II in NRC (3). Previously, we have...
shown that ANG II evoked much greater increases in
[
Ca^{2+}
]/H11001
than both AVP and ET-1 in NRC (34, 35). These
data suggest that additional mechanisms and signal
transduction events likely account for the much
greater increases in the [
Ca^{2+}
]/H11001
response to ANG II.
The present data confirm that ANG II increased CysLT
generation via AT1 receptor activation, which in turn
augments the [
Ca^{2+}
]/H11001
response to ANG II. In contrast,
AVP- and ET-1-evoked increases in [
Ca^{2+}
]/H11001
levels were
not reduced by the inclusion of either AA-861 or MK-
571. Moreover, both ET-1 and AVP failed to promote
CysLT production, suggesting that only ANG II pro-
motes CysLT production via AT1 receptor activation.
This is consistent with the view that ANG II is a
pleiotropic agonist that recruits multiple signaling
pathways to account for its potential role in the regu-
lation of cardiovascular function (31). Previously, oth-
ers have confirmed that ANG II-evoked increases in
[
Ca^{2+}
]/H11001
might play a more important role than protein
kinase C activation in hypertrophic responses to ANG
II in NRC (28). The present study demonstrates the
consistency in the time course response for CysLT
generation and the subsequent Ca^{2+} mobilization,
suggesting that CysLT generation evoked by ANG II
may serve as an additional amplifier pathway in sustain-
ing direct AT1-mediated Ca^{2+} mobilization in NRC.
Several studies in the past have established that ANG II
and AVP-evoked elevations in [
Ca^{2+}
]/H11001
in NRC stemmed
predominantly from the release of Ca^{2+} from the intra-
cellular stores and that removal of extracellular Ca^{2+}
led to only a partial reduction in the peak increases in
[
Ca^{2+}
]/H11001
evoked by ANG II and AVP (7, 12, 14, 32).
Moreover, thapsigargin, a sarcoplasmic reticulum (SR)
Ca^{2+} pump inhibitor, abolished the [
Ca^{2+}
]/H11001
response to

Fig. 6. Fura 2 Ca^{2+} imaging in a single NRC stimulated with ANG II (50 nM). Ca^{2+} images were acquired at a rate
of 3 images/s using three different coverslips loaded with fura 2 under identical conditions on the same day. A–C:
basal fura 2 fluorescence images (340-to-380-nm excitation ratio) acquired before the addition of ANG II in the
absence (A) or presence of AA-861 (B) or MK-571 (C). D–F: changes in fura 2 fluorescence at 30 s after the addition
of ANG II in the same cells. D: ANG II alone; E: ANG II in the presence of AA-861 (10 µM); F: ANG II in the
presence of MK-571 (100 nM). The aggregate [Ca^{2+}], values in single cells obtained were as follows (in nM): 110
(A), 124 (B), 117 (C), 710 (D), 470 (E), and 530 (F). At the end of 2 min, the fluorescence values were <200 nM in
all cells (data not shown). Similar response patterns were recorded in five separate experiments.
ANG II or AVP (12, 14). Therefore, the ANG II-evoked responses noted in Ca\(^{2+}\)-free medium or the significant attenuation of [Ca\(^{2+}\)]\(_i\) responses to ANG II seen in the presence of 2-APB are consistent with observations reported earlier. Thus, besides providing a possible explanation for the higher \(E_{\text{max}}\) to ANG II, the present study suggests for the first time that CysLT may play a role in ANG II-evoked contractility and/or hypertrophy in cardiomyocytes.

CysLT and Ca\(^{2+}\) signaling. It is important to demonstrate that CysLT contributes to Ca\(^{2+}\) mobilization in NRC. There are no studies suggesting the presence of CysLT receptors in NRC. In human detrusor smooth muscle cells, it was reported that elevation in [Ca\(^{2+}\)]\(_i\) levels evoked by LTD\(_4\) were almost exclusively due to mobilization from intracellular Ca\(^{2+}\) stores (4). In other target cells, the LTD\(_4\)-evoked Ca\(^{2+}\) response was dependent on the release of Ca\(^{2+}\) from intracellular stores and enhanced Ca\(^{2+}\) influx (20, 23). The present study demonstrates that both ANG II- and CysLT-evoked increases in [Ca\(^{2+}\)]\(_i\) levels were significantly attenuated by 2-APB. In addition, both AA-861 and MK-571 caused a similar degree of blockade of the ANG II response in normal as well as Ca\(^{2+}\)-free buffer. Thus this is the first report to characterize CysLT-evoked increases in [Ca\(^{2+}\)]\(_i\) that may be mainly due to the release of Ca\(^{2+}\) from Ins(1,4,5)P\(_3\)-sensitive intracellular SR Ca\(^{2+}\) pools in NRC.

Human CysLT\(_1\) and CysLT\(_2\) receptors have been cloned and characterized only in recent years (8, 16). Several recent studies have proposed the presence of CysLT\(_1\) and CysLT\(_2\) transcripts in cardiac tissues (8, 10, 16, 21). Consistent with this observation, we noted that both LTD\(_4\) (MK-571 sensitive) and LTC\(_4\) (MK-571 resistant) evoked concentration-dependent [Ca\(^{2+}\)]\(_i\) responses in NRC. Moreover, the addition of BAY-u9773 completely blocked both LTD\(_4\) and LTC\(_4\)-evoked [Ca\(^{2+}\)]\(_i\) responses. These data suggest that NRC may possess both CysLT\(_1\)- and CysLT\(_2\)-specific binding sites that are linked to Ca\(^{2+}\) mobilization. Previously, it was reported that the affinity of LT\(_C\) for the CysLT\(_1\) receptor was roughly 10- to 350-fold lower than that of LT\(_D\) (8, 16). Overall, the rank order of affinities of CysLT for the CysLT\(_1\) and CysLT\(_2\) receptors is LT\(_D\) > LTC\(_4\) > LT\(_C\) and LT\(_D\) = LT\(_C\) > LTC\(_4\), respectively (8, 17). Our study demonstrates that LT\(_D\) induced a stronger [Ca\(^{2+}\)]\(_i\) response than LT\(_C\) and that BAY-u9773 but not MK-571 blocked LTC\(_4\)-induced [Ca\(^{2+}\)]\(_i\) responses. It is likely that LTD\(_4\)-evoked responses are mediated by CysLT\(_1\) and CysLT\(_2\) receptors, whereas LT\(_C\) interacts at CysLT\(_2\) receptors on NRC that are insensitive to blockade by MK-571. These observations are consistent with earlier findings showing that [\(^{3}\)H]LTC\(_4\)-specific binding to human lung tissues could not be displaced at concentration ranges up to 3 \(\mu\)M by either CysLT\(_1\) antagonists (zaflurilast and montelukast) or LT\(_D\) (1, 8, 16). Moreover, zaflurilast-resistant contractile responses to LT\(_C\) were observed in the guinea pig trachea when LT\(_C\) metabolism to LT\(_D\) was prevented (1). In LT\(_D\)-expressing clones, such as CHO-7A, CHO-8B3, and PC12 cells, both LT\(_C\) and LT\(_D\) exhibited dose-dependent increases in [Ca\(^{2+}\)]\(_i\) levels that were sensitive to blockade by BAY-u9773 (21). Peritoneal macrophages (which express both CysLT\(_1\) and CysLT\(_2\) receptors) responded substantially to 1 \(\mu\)M LT\(_D\) and only slightly to 1 \(\mu\)M LT\(_C\) (17). All these studies, together with our present data using NRC, support the notion that LT\(_D\)-evoked [Ca\(^{2+}\)]\(_i\) responses may be mediated by both CysLT\(_1\) and CysLT\(_2\) receptors, whereas the weaker [Ca\(^{2+}\)]\(_i\) response evoked by LT\(_C\) may be mediated by CysLT\(_2\) receptors.

Although several studies have suggested that CysLT evoke a negative inotropic effect, this has been attributed to profound coronary vasoconstriction mediated by CysLT\(_2\) receptors located on coronary arteries (10). Indeed, both LT\(_D\) and LT\(_C\) at low concentrations have been shown to exert a positive inotropic effect on the rat myocardium, and ANG II infusion has been shown to enhance leukotriene A\(_4\) hydrolase activity in the rat heart (9, 11). These findings support our observation of elevated [Ca\(^{2+}\)]\(_i\) levels evoked by CysLT in NRC. In fact, the elevation in the [Ca\(^{2+}\)]\(_i\) level has been suggested to play a more critical role than protein kinase C activation toward hypertrophy evoked by ANG II in NRC (27, 28). Taken together with our new findings, these data provide the impetus for a more detailed characterization of the interactions among ANG II, CysLT generation, and CysLT receptor-mediated increases in Ca\(^{2+}\) mobilization and their relative roles and contribution to cardiac hypertrophy.

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