Restoration of diabetes-induced abnormal local Ca\textsuperscript{2+} release in cardiomyocytes by angiotensin II receptor blockade

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Yaras N, Bilginoglu A, Vassort G, Turan B. Restoration of diabetes-induced abnormal local Ca\textsuperscript{2+} release in cardiomyocytes by angiotensin II receptor blockade. Am J Physiol Heart Circ Physiol 292: H912–H920, 2007. First published September 29, 2006; doi:10.1152/ajpheart.00824.2006.—Stimulation of local renin-angiotensin system and increased levels of oxidants characterize the diabetic heart. Downregulation of ANG II type 1 receptors (AT\textsubscript{1}) and enhancement in PKC activity in the heart point out the role of AT\textsubscript{1} blockers in diabetes. The purpose of this study was to evaluate a potential role of an AT\textsubscript{1} blocker, candesartan, on abnormal Ca\textsuperscript{2+} release mechanisms and its relationship with PKC in the cardiomyocytes from streptozotocin-induced diabetic rats. Cardiomyocytes were isolated enzymatically and then incubated with candesartan or a nonspecific PKC inhibitor bisindolylmaleimide I (BIM) for 6–8 h at 37°C. Both cardiomyocytes and BIM applied on diabetic cardiomyocytes significantly restored the altered kinetic parameters of Ca\textsuperscript{2+} transients, as well as depressed Ca\textsuperscript{2+} loading of sarcoplasmic reticulum, basal Ca\textsuperscript{2+} level, and spatiotemporal properties of the Ca\textsuperscript{2+} sparks. In addition, candesartan and BIM significantly antagonized the hyperphosphorylation of cardiac ryanodine receptor (RyR2) and restored the depleted protein levels of both RyR2 and FK506 binding protein 12.6 (FKBP12.6). Furthermore, candesartan and BIM also reduced the increased PKC levels and oxidized protein thiol level in membrane fraction of diabetic rat cardiomyocytes. Taken together, these data demonstrate that AT\textsubscript{1} receptor blockade protects cardiomyocytes from development of cellular alterations typically associated with Ca\textsuperscript{2+} release mechanisms in diabetes mellitus. Prevention of these alterations by candesartan may present a useful pharmacological strategy for the treatment of diabetic cardiomyopathy.

Heart; candesartan; Type 1 diabetes; thiol oxidation

CHRONIC DIABETES ALTERS the structure and function of the human heart, and individuals with diabetes mellitus usually develop a specific cardiac dysfunction known as diabetic cardiomyopathy (37). Several mechanisms involved in the development of cardiomyopathy have been postulated, including alterations in intracellular ion homeostasis and glucose metabolism and enhanced oxidative stress. Although alteration of Ca\textsuperscript{2+} signaling via changes in critical processes that regulate intracellular Ca\textsuperscript{2+} has become a hallmark of this type of cardiomyopathy, controversies currently going on, relate to specific alterations in Ca\textsuperscript{2+} signaling pathways contributing to the cardiac defects in diabetes (7, 20). Recently, we reported that these defects result partially from altered local Ca\textsuperscript{2+} signaling due to a dysfunction of cardiac PKA-mediated ryanodine receptor Ca\textsuperscript{2+} release channel (RyR2) (51).

Several mechanisms have been proposed to explain how all of the pathologies involved in the progression of diabetic cardiomyopathy might result from hyperglycemia. Increased PKC isomorph expression and increased polyol pathway flux are two main hypotheses presented to describe how hyperglycemia might cause all of the diabetic complications (6). Furthermore, it has been demonstrated that hyperglycemia activates the local renin-angiotensin system (RAS) and enhanced RAS activity in diabetes (33, 40). In addition, it is known that ANG II has direct effects on cardiomyocytes through the ANG II type 1 (AT\textsubscript{1}) receptor (9–11). Thus, stimulation of the AT\textsubscript{1} receptor generates oxygen-derived free radicals, which have detrimental effects on the cardiovascular system (28). The AT\textsubscript{1} receptor has been shown to be coupled to several postreceptor signaling pathways, including NADPH oxidase (33). Furthermore, there is a significant amount of evidence demonstrating that increased PKC activity contributes to cardiovascular complications associated with diabetes and that elevated PKC activity has been found in diabetic hearts (25). PKC is known to phosphorylate a number of proteins that are directly involved in cardiac excitation-contraction coupling (8, 24, 25). Therefore, upregulation of cardiac RAS and increased PKC activity in diabetic animals suggest the importance of these pathways in the development of cardiac complications.

Defects in cardiac intracellular Ca\textsuperscript{2+} have been proposed as one possible contributory factor to the depressed myocardial contractile activity in diabetic cardiomyopathy (1, 32). Most alterations were attributed to anomalous sarcoplasmic reticulum (SR) pump activity (15), SR-Ca\textsuperscript{2+} storage (5), and partly to alterations in RyR2 properties (3, 27, 35, 49, 51). Recently, it has been shown that some cardioprotective agents correct the defective FK506 binding protein 12.6 (FKBP12.6)-mediated stabilization of RyR2, leading to an improvement in cardiac function of heart failure (29, 50). Recently, Gassanov et al. (16) described marked increases in the ANG II-induced depressed parameters of Ca\textsuperscript{2+} sparks with candesartan. We and others have shown that chronic administration of ANG II receptor blockers could protect the heart from the development of cellular alterations typically related with diabetes (18, 29, 30, 33, 34). Although ANG II antagonism leads to an attenuation of the depressed SR-Ca\textsuperscript{2+}-ATPase (SERCA) and to an improvement in intracellular Ca\textsuperscript{2+} handling in heart failure (16,
29), it is not known whether this antagonism can correct the defective interaction of RyR2 and FKBP12.6 that occurs in the SR during the development of diabetes-induced cardiomyopathy (51).

To get further insight and to evaluate a potential direct role of ANG II activation in diabetic heart dysfunction, we examined direct effects of an AT1 receptor blocker, candesartan, on cardiac intracellular Ca\(^{2+}\) metabolism in terms of global Ca\(^{2+}\) release (electrically stimulated Ca\(^{2+}\) transients) and local Ca\(^{2+}\) release by RyR2 modulated by PKA (Ca\(^{2+}\) sparks) and also their possible relation with activated PKC in cardiomyocytes isolated from streptozotocin (STZ)-induced diabetic rats. Our results demonstrated that ANG II antagonism significantly restores the defective interaction of FKBP12.6 and RyR2 in addition to its restoring effects on activated PKC and oxidized protein thiols in these diseased cardiomyocytes.

**MATERIALS AND METHODS**

**Experimental groups.** Diabetes was induced by a single intraperitoneal injection of STZ (50 mg/kg body wt and dissolved in 0.1 M citrate buffer, pH 4.5) in adult male Wistar rats (200–250 g), whereas rats for the control group received citrate buffer alone. A week after injection of STZ, blood glucose levels were measured and only the rats having blood glucose levels at least three times higher than the preinjection levels were used later on. All rats had free access to standard rat chow and water. All animals were used 5 wk after STZ or citrate buffer injections. All experiments were approved by the Ankara University School of Medicine and performed in accordance with its ethics guidelines for the care and use of laboratory animals.

**Cell isolation.** Cell isolation was performed as described previously (51). Hearts were rapidly removed following anesthetization of the rats with pentobarbital sodium (30 mg/kg body wt). The hearts were cannulated on a Langendorff apparatus and perfused through the coronary arteries with a Ca\(^{2+}\)-free solution containing (in mM) 145 NaCl, 5 KCl, 1.2 MgSO\(_4\), 1.4 Na\(_2\)HPO\(_4\), 0.4 NaH\(_2\)PO\(_4\), 5 HEPES, and 10 glucose at pH 7.4, bubbled with O\(_2\) at 37°C. Hearts were perfused for 3–5 min to remove the remaining blood, and this was followed by perfusion with the same solution containing 1 mg/ml collagenase (collagenase A, Boehringer) for 30–35 min. Ventricles were then removed and minced into small pieces and gently massaged through a nylon mesh, and dissociated cardiomyocytes were washed with the collagenase-free solution. Subsequently, Ca\(^{2+}\) was increased in a graded manner to a concentration of 1 mM with three steps. Cells were kept in this solution at 37°C until used the same day.

**Cytosolic Ca\(^{2+}\) sparks measurement.** Cytosolic Ca\(^{2+}\) sparks measurement was performed as described previously (51). Cardiomyocytes were placed into the recording chamber setting over the stage of an inverted microscope equipped with a laser scanning confocal microscope (200-M, LSM-Pascal, Zeiss, Germany). In the experiments, ×40 (numerical aperture = 1.3) oil immersion objectives were used for imaging cardiomyocytes located over cover glass base of the recording chamber. A 488-nm laser line from an argon laser (25 mW) was used to excite the Ca\(^{2+}\)-sensitive dye, fluo-3, and emitted fluorescence collected at 505 nm. Changes in intracellular free Ca\(^{2+}\) were recorded in line-scan mode [spatial (x) vs. temporal (t), 1.9 ms per line]. To prevent photobleaching and cell damage, the laser line was kept at 4–6% of maximal intensity.

Image analysis was performed by using LSM Image Examiner. The F value for fluorescence intensity of image was calculated by averaging pixels except potential spark areas. Then ΔF/F image was created by using this F value. Ca\(^{2+}\) sparks were manually detected and converted to temporal lines by averaging fluorescence intensity of two to three pixels, aligning the peak of fluorescence intensity over time. The signals were filtered using a Butterworth digital filter. The temporal profiles were then fitted to gamma function to analyze time to peak (TP), peak amplitude of fluorescence intensity (PA), and decrease time to half-maximum (DT\(_{50}\)).

To assess the effects of candesartan (dissolvable form of candesartan cilexetil), an AT\(_1\) receptor blocker, or of the nonspecific PKC inhibitor bisindolylmaleimide I (BIM) (30) on the parameters of Ca\(^{2+}\) sparks in isolated cardiomyocytes, a fraction of diabetic and control cardiomyocytes were incubated with either candesartan (1 μM) or BIM (100 nM) or buffer solution for 6–8 h at 37°C. BIM was dissolved in DMSO; however, final dilution of DMSO in experimental solutions was <0.01%.

**Measurement of cytosolic Ca\(^{2+}\) transient.** Intracellular Ca\(^{2+}\) transients were measured by fura-2 fluorescence at room temperature (21 ± 2°C) as described previously (51). The cells were incubated at 37°C for 50 min with 4 μM fura-2 AM for loading and then washed with fresh buffer. Fluorescence was recorded using a PTI Ratiomaster microspectrophotometer and FELIX software (Photon Technology International). Cells were excited at 340/380 nm, and emission was measured at 510 nm. The fluorescence ratio of the emitted lights on excitation at 340 and 380 nm (F\(_{340/380}\)) was calculated and used as an indicator of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)). F\(_{340/380}\) was measured at a frequency of 10 Hz. The composition of the bath solution used in [Ca\(^{2+}\)]\(_i\) measurement was as follows: (in mM) 130 NaCl, 4.8 KCl, 1.2 MgSO\(_4\), 1.5 CaCl\(_2\), 1.2 KH\(_2\)PO\(_4\), 10 HEPES, and 10 glucose (pH 7.4). Following the monitoring of [Ca\(^{2+}\)]\(_i\) for 20 s at rest, field-stimulation pulses of 20–30 V with 10-msec duration were applied at 0.2-Hz frequency. PA (difference between basal and peak F\(_{340/380}\) ratios), TP, and DT\(_{50}\) were determined from Ca\(^{2+}\) transients evoked by field stimulation. Background fluorescence measured from a cell-free field was substraction from all recordings before calculation of ratios.

Caffeine-induced Ca\(^{2+}\) transients were induced by bath application of 10 mM caffeine on cardiomyocytes 30 s after stopping field stimulation of the cells with electrical pulses (for 2 min at 0.2-Hz stimulation frequency) to ensure stable SR-Ca\(^{2+}\) load. Caffeine responses were measured in buffer and in both candesartan (1 μM)- and BIM (100 nM)-incubated cardiomyocytes from diabetic and control group rats for 6–8 h at 37°C.

**Western blot analysis.** Isolated cardiomyocytes were homogenized with a motor-driven Teflon-to-glass homogenizer in cold Tris-HCl buffer containing (in mM) 50 Tris-HCl (pH 7.4), 200 NaCl, 20 NaF, 1.0 Na\(_3\)VO\(_4\), 1 dithiothreitol, and protease inhibitors (complete tablet from Roche) and centrifuged at 1,000 g for 30 min at 4°C. Supernatant was centrifuged at 30,000 g for 30 min, and the pellet was then washed out via centrifugation at 30,000 g for 1 h at 4°C. Pellet was suspended with the homogenization buffer, and protein amount was measured by Bradford method as described previously (51).

The samples (100 μg protein) were subjected to 5% SDS-PAGE for RyR2 assay and 10% SDS-PAGE for both FKBP12.6 and actin assays and then transferred electrophoretically to nitrocellulose membrane. Immunoblotting was performed by using antibodies against RyR2 and phosphorylated RyR2 (kind gift of Drs. A. Marks and X. Wei). Western blot conditions were 1:5,000 and 1:3,000, respectively, and FKBP12.6 vs. actin (Santa Cruz), and then enhanced chemiluminescence (ECL) was performed. The samples (30 μg protein) were subjected to 10% SDS-PAGE for PKC assays and then transferred electrophoretically to nitrocellulose membrane. Immunoblotting was performed by using antibodies against PKC (Santa Cruz), and then ECL was performed as mentioned previously (51). Briefly, nitrocellulose membranes were incubated 1 h at 4°C in PBS (20 mM Na\(_2\)HPO\(_4\)-Na\(_2\)HPO\(_4\), pH 7.6, containing 154 mM NaCl, 3% bovine serum albumin, and 8% nonfatty dry milk). Blots were washed several times with PBS containing 0.1% Tween and were then incubated with antiserum at room temperature for 1–2 h by shaking. Blots were then washed several times with PBS, incubated with horseradish peroxidase-labeled anti-rabbit IgG (Santa Cruz, CA) (dilution, 1:10,000) for 1 h at room temperature. Blots were washed several times with PBS and then incubated
with ECL Western blotting reagent (Amersham, Vienna, Austria) for 1 min and exposed to X-ray film for 45–90 s.

**Determination of sulfhydryl groups in isolated cardiomyocytes.** Cardiomyocytes from both control and diabetic groups were first washed with a HEPES-buffered solution containing (in mM) 123 NaCl, 5.4 KCl, 1.7 MgCl₂, 1.8 CaCl₂, 10 HEPES, and 10 glucose (pH 7.4). The cells were then divided into two groups: untreated group (cells kept in the HEPES-buffered solution) and treated group [cells incubated in the same solution supplemented with either candesartan (1 μM) or BIM (100 nM) under the same conditions]. After 6- to 8-h incubation at 37°C, the cells from each group were washed with the HEPES-buffered solution and used for estimation of total and acid-soluble sulfhydryl (SH) groups. These estimations were done with Ellman’s reagent as described elsewhere (44). The cells were thawed and lysed in 0.2 M Tris·HCl buffer (pH 8.1) containing 2% sodium dodecyl sulfate. For the determination of total SH groups, 0.05-ml aliquots of cell lysates were mixed with 0.8-ml distilled water and 0.1 ml of 2 mM 5,5'-dithiobis-(2-nitrobenzoic acid), waiting 20 min for color development. Absorbance of the supernatants was read at 412 nm. After correction of the absorbance with sample and reagent blanks, concentrations of SH groups in each sample were calculated by employing an extinction coefficient of 1.31 mM⁻¹mm⁻¹. To determine the acid-soluble SH groups, 0.7 ml aliquots of cell lysates were mixed with 0.35 ml of 20% trichloroacetic acid and then centrifuged at 13,000 g for 10 min. The precipitates were then washed with 0.2 ml of 20% trichloroacetic acid in a similar manner. Later, the supernatants were adjusted to pH 8 with NaOH. The SH content of the supernatants was measured as described for total SH measurements.

**Chemicals and statistical evaluation of data.** All chemicals used were purchased from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany) except for fura-2 AM and fluo-3, which were purchased from Molecular Probes (Eugene, OR), and collagenase A, which was purchased from Boehringer-Mannheim (Roche Diagnostics, Mannheim, Germany). Candesartan was a gift from AstraZeneca (AstraZeneca R&D Mölndal, Sweden).

Groups were tested and compared using ANOVA and Tukey post hoc test. P values < 0.05 were taken as significant, and significance levels are given in the text. Data are presented as means ± SE throughout the text.

**RESULTS**

**General effects.** Diabetic animals had significantly high glucose levels (~4-fold) compared with control animals at the end of the 5-wk experimental period (105 ± 3 and 421 ± 5 mg/dl control vs. diabetics). During this period following STZ injection, they stopped gaining weight (219.2 ± 9.2 vs. 196.8 ± 7.9 g), whereas control animals continued to gain weight (211.4 ± 6.9 vs. 240.2 ± 7.8 g). Besides the measurement of heart weight of each animal, we also estimated cell capacitance of the isolated cardiomyocytes and compared these values between the groups. Mean cell capacitances of the control and diabetic rats were similar as previously reported by us (189.9 ± 12.2 and 180.6 ± 13.6 pF, respectively) (30).

**Candesartan restores diabetes-induced altered parameters of Ca²⁺ sparks of cardiomyocytes.** Since Ca²⁺ sparks represent SR-Ca²⁺ release from clusters of RyR2, we examined the effects of AT1 receptor blocker candesartan (+Can) on the characteristics of spontaneously arising Ca²⁺ sparks in cardiomyocytes isolated from STZ-induced diabetic (DM) and control (Con) rat hearts. Representative line-scan images of the candesartan- or buffer-incubated cardiomyocytes from diabetic rats and buffer-incubated control rats (individual Ca²⁺ spark image x vs. t) are displayed in Fig. 1. Representative temporal profiles of the Ca²⁺ sparks are shown in Fig. 1, A–D. The spatial (x) and (t) time courses were extracted as the mean of all recorded spatial or temporal pixels centered at the peak of the ΔF/F of the Ca²⁺ sparks. These transients were then fitted by a gamma distribution function to obtain objective kinetic and spatial values of the events (30).

Quantitative data for Ca²⁺ spark characteristics are summarized in Fig. 2. The Ca²⁺ spark amplitude (PA; as indicated by the ratio of peak fluorescence over baseline fluorescence) was not affected by candesartan (0.56 ± 0.02 for Con; 0.54 ± 0.02 for Con + Can; 0.51 ± 0.02 for DM; and 0.53 ± 0.02 for DM + Can) (Fig. 2A, right). On the other hand, TP Ca²⁺ fluorescence (rise time) and half-decay time (DT50) were 10.35 ± 0.43 and
of the sparks, Ca$^{2+}$, AT$_1$ receptors, and PKC activity in diabetic rats (41).

Currents in diabetic rats, suggesting a role of increases in
cytoskeleton with a PKC inhibitor could restore depressed potassium
sparks. Time-to-peak amplitude (TP), half-decay time (DT$_{50}$), and peak am-
plitude (PA) of Ca$^{2+}$ sparks are measured in cardiomyocytes from Con and
DM groups ($n_{rat} = 4$, $n_{cell} = 30$, and $n_{spark} = 152$ vs. $n_{rat} = 4$, $n_{cell} = 25$, and
$n_{spark} = 145$ in DM vs. Con), and Can (1 $\mu$M)- or BIM (100 nM)-incubated
cardiomyocytes from DM group ($n_{rat} = 5$, $n_{cell} = 44$, and $n_{spark} = 185$ vs.
$n_{rat} = 4$, $n_{cell} = 25$, and $n_{spark} = 133$ in Can vs. BIM). B: spatial distribution
doing the spikes, Ca$^{2+}$ sparks width at half their maximal amplitude (FWHM).
C: comparison of Ca$^{2+}$ sparks frequency (10 vs. 12 cardiomyocytes from Con
vs. DM groups). *$P < 0.05$ vs. Con; †$P < 0.05$ vs. DM.

25.36 $\pm$ 1.05 ms in candesartan-incubated diabetic cells com-
pared with 12.88 $\pm$ 0.50 ($P < 0.05$) and 32.90 $\pm$ 1.29 ms ($P <
0.05$) in buffer-incubated diabetic cells and compared with 9.08 $\pm$
0.30 ($P < 0.05$) and 20.78 $\pm$ 0.87 ms ($P < 0.05$) in buffer-
incubated control cells (Fig. 2A, left). Consistent with these
findings, significant differences were obtained for the mean fre-
cquency of Ca$^{2+}$ sparks width at half maximum (FWHM). The
spatial spread (FWHM) was significantly prolonged in the diabe-
tic group with respect to the control group (3.16 $\pm$ 0.20 $\mu$m vs.
2.43 $\pm$ 0.07 $\mu$m), and incubation of these diabetic cardiomyocytes
with candesartan could restore significantly (2.62 $\pm$ 0.12 $\mu$m)
(Fig. 2B), whereas there was no significant effect on the
controls (2.35 $\pm$ 0.10 vs. 2.43 $\pm$ 0.07 $\mu$m).

Spontaneous Ca$^{2+}$ sparks frequency was significantly higher
in diabetic cardiomyocytes (0.051 $\pm$ 0.006 s$^{-1}$-$\mu$m$^{-1}$) than in
the control cells (0.032 $\pm$ 0.003 s$^{-1}$-$\mu$m$^{-1}$), whereas they were
significantly reduced after candesartan incubation
(0.042 $\pm$ 0.005 s$^{-1}$-$\mu$m$^{-1}$) (Fig. 2C). No significant difference
for spark frequency was present between incubated and non-
incubated cardiomyocytes from the respective control groups
(0.035 $\pm$ 0.004 s$^{-1}$-$\mu$m$^{-1}$ vs. 0.032 $\pm$ 0.003 s$^{-1}$-$\mu$m$^{-1}$).

**BIM restores diabetes-induced altered parameters of Ca$^{2+}$
sparks.** It has been demonstrated that incubation of cardiomy-
ocyes with a PKC inhibitor could restore depressed potassium
currents in diabetic rats, suggesting a role of increases in
ANG II, AT$_1$ receptors, and PKC activity in diabetic rats (41).
Therefore, we incubated isolated diabetic cardiomyocytes with
BIM for 6–8 h to assess the role of cardiac PKC (a known
downstream mediator of AT$_1$ receptor action) in the altered
parameters of the Ca$^{2+}$ sparks obtained in the cardiomyocytes
from the diabetic rats. Incubation of diabetic cardiomyocytes
with BIM, similar to candesartan incubation, significantly
restored the prolonged TP and DT$_{50}$ values, whereas it had no
significant effect on the diminished amplitude of Ca$^{2+}$ sparks
(Fig. 2A). BIM incubation of these diabetic cardiomyocytes
could significantly restore spontaneous Ca$^{2+}$ sparks frequency
(0.039 $\pm$ 0.005 s$^{-1}$-$\mu$m$^{-1}$) and the FWHM (2.59 $\pm$ 0.13 s$^{-1}$-
$\mu$m$^{-1}$) with respect to the controls as well (Fig. 2, B and C).
Moreover, incubation of the cardiomyocytes with BIM from
the control group did not affect these parameters significantly
(data not shown).

**Candesartan restores global Ca$^{2+}$ release in diabetic rat
heart.** Fig. 3A shows original recordings of Ca$^{2+}$ transients
elicted in the control and the diabetic rat cardiomyocytes
(incubated with buffer or candesartan). The averaged PA of

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**Fig. 2.** Can or BIM restores diabetes-induced altered parameters of Ca$^{2+}$ sparks of the cardiomyocytes. **A:** averaged spatial and temporal values of Ca$^{2+}$ sparks. Time-to-peak amplitude (TP), half-decay time (DT$_{50}$), and peak am-
plitude (PA) of Ca$^{2+}$ sparks are measured in cardiomyocytes from Con and
DM groups ($n_{rat} = 4$, $n_{cell} = 30$, and $n_{spark} = 152$ vs. $n_{rat} = 4$, $n_{cell} = 25$, and
$n_{spark} = 145$ in DM vs. Con), and Can (1 $\mu$M)- or BIM (100 nM)-incubated
cardiomyocytes from DM group ($n_{rat} = 5$, $n_{cell} = 44$, and $n_{spark} = 185$ vs.
$n_{rat} = 4$, $n_{cell} = 25$, and $n_{spark} = 133$ in Can vs. BIM). **B:** spatial distribution
doing the spikes, Ca$^{2+}$ sparks width at half their maximal amplitude (FWHM).
**C:** comparison of Ca$^{2+}$ sparks frequency (10 vs. 12 cardiomyocytes from Con
vs. DM groups). *$P < 0.05$ vs. Con; †$P < 0.05$ vs. DM.

**Fig. 3.** Effect of Can incubation on the parameters of intracellular Ca$^{2+}$
transients from DM rats. **A:** intracellular Ca$^{2+}$ transients recorded from
different groups of cells are given with different colors as indicated.
**B:** changes in PA of the fluorescence Ca$^{2+}$ transients ($\Delta F_{340/380}$ = $F_{340}$ - $F_{380}$) vs. $F_{340/380}$ basal ), TP, and DT$_{50}$ of the DM ($n_{rat} = 5$, and $n_{cell} = 35$) and the
Con groups ($n_{rat} = 5$, and $n_{cell} = 44$). Traces were obtained from
cardiomyocytes of Con, DM, and Can (1 $\mu$M)- or BIM (100 nM)-incubated
cells (4 h). Ca$^{2+}$ transients were induced by field stimulation (20–30 V
with 10-ms duration at 0.2 Hz). **C:** changes of basal intracellular [Ca$^{2+}$]$^{i}$ in
the DM and Con groups with or without Can or BIM incubations. Values
are expressed as means $\pm$ SE of 48–60 cells from at least 5 animals. $D$: caffeine-induced peak intracellular Ca$^{2+}$ transients elicited in cardiomyocytes.
Bar graph of $\Delta F_{340/380}$ values of the 2 groups are expressed as means $\pm$ SE of
20–27 cells from at least 4 animals. *$P < 0.05$ vs. Con; †$P < 0.05$ vs. DM.
The averaged diastolic value of $\Delta F_{340/380}$ was significantly recovered in candesartan-incubated cardiomyocytes from diabetic rats compared with buffer-incubated diabetic cells (0.24 ± 0.01 vs. 0.25 ± 0.01, respectively; Fig. 3B). The TP amplitude of $Ca^{2+}$ transients of candesartan-incubated diabetic cells (0.183 ± 0.004 s) was significantly restored compared with buffer-incubated diabetic cells (0.249 ± 0.006 s), whereas the DT50 was significantly shortened in candesartan-incubated diabetic group with respect to buffer-incubated diabetic group (0.62 ± 0.02 vs. 0.68 ± 0.02 s) (Fig. 3B). Candesartan incubation of the cells from the control group could significantly affect neither PA nor TP and DT50. The averaged diastolic value of $\Delta F_{340/380}$ was 0.436 ± 0.006 in candesartan-incubated diabetic cells, and this value was significantly smaller than in the buffer-incubated diabetic cells (0.46 ± 0.01). This is indicative that the basal [Ca$^{2+}$]$_i$ level is larger in the diabetic groups with respect to the controls (0.42 ± 0.01), and candesartan incubation could restore this value to the levels of the control. Candesartan incubation of the cells from control group did not change the diastolic value of $\Delta F_{340/380}$ (0.43 ± 0.01 vs. 0.42 ± 0.01) (Fig. 3C).

Effects of BIM on the altered parameters of the Ca$^{2+}$ transients seen in diabetes were also tested in the present study. We observed that incubation of diabetic cardiomyocytes with BIM for 6–8 h significantly restored the prolongation in both TP and DT50 values of the diabetic cardiomyocytes. Additionally, BIM incubation caused a significant recovery of the diminished Ca$^{2+}$ transient amplitude in the diabetic cardiomyocytes (Fig. 3A–C).

Effect of candesartan on SR-Ca$^{2+}$ content of diabetic rat cardiomyocytes. It is well known that caffeine application causes a sudden and transient increase in intracellular Ca$^{2+}$ in cardiomyocytes due to a Ca$^{2+}$ release from SR. The size of the caffeine-induced Ca$^{2+}$ release has been used to assess the SR-Ca$^{2+}$ load of the diabetic cardiomyocytes. We observed that caffeine-induced peak of intracellular Ca$^{2+}$ transient was smaller in the diabetic cardiomyocytes compared with control cells without any significant change in the kinetic parameters. Candesartan or BIM incubation of the cardiomyocytes could counteract the reduction in caffeine responses (in terms of the amplitude of the transients) of Ca$^{2+}$ release from SR in the diabetic rats (Fig. 3D), whereas no significant effect was observed in the controls.

Candesartan inhibits PKC activation in diabetic rat cardiomyocytes. To confirm that the increased PKC activity has an important role in the pathogenesis of the heart complications in diabetes, we estimated, using Western blot analysis, total PKC protein content in the cytosolic and membrane fractions of the cardiomyocytes from both control and diabetic rats before and after candesartan incubation. Total PKC content in the membrane increased markedly in diabetic hearts, whereas it decreased markedly in the cytosolic part (Fig. 4, A and B). Nonetheless, candesartan significantly attenuated these changes in both cytosolic and particulate fractions. The ratio of the protein content of total PKC in the membrane to that in the cytosolic fraction was increased very markedly in the diabetic hearts; candesartan incubation caused a significant decrease in this value compared with control values (Fig. 4C). Western blot analysis was also performed with BIM incubation. As can be seen from Fig. 4, we obtained significant recovery in the parameters of PKC similar to the data with candesartan. Neither candesartan nor BIM incubations of the cardiomyocytes from control rats have a significant effect on PKC content (Fig. 4C).
any significant effect in the parameters of the controls (data not shown).

Effects of candesartan or BIM on RyR2 in diabetic rat cardiomyocytes. We previously hypothesized and showed that the alterations in Ca\(^{2+}\)-release behavior observed at the global and local spark level in STZ-induced diabetic rat cardiomyocytes were due to a defect in RyR2 function following its hyperphosphorylation and release of FKBP12.6 (51). The phosphorylation level of RyR2 in candesartan- or BIM-incubated (6–8 h, at 37°C) diabetic cardiomyocytes was evaluated by using specific antibodies directed against RyR2 and phosphorylated RyR2. Total RyR2 in the diabetic group was significantly recovered following candesartan or BIM incubation as estimated from the Western blot bands, whereas no effect on control cells was observed (data not given) (Fig. 5A). The significant phosphorylation of RyR2 in diabetic rat heart was significantly preserved with treatment with both candesartan and BIM at a level of about 50% (Fig. 5B). The amount of FKBP12.6, which was decreased by about 40% in the diabetic rat heart compared with control rat heart, was significantly preserved by treatment of cardiomyocytes with either candesartan or BIM (Fig. 5C).

AT1 receptor blockage effects occur through reduction of oxidized protein thiols. It is well known that metabolic shifts brought about by diabetes increase production of reactive oxygen species (ROS) and nitrogen species (see review in Ref. 47). In addition, in our previous study we, like others, have shown that oxidative stress is involved in the etiology of diabetes-induced downregulation of transient outward K\(^+\) currents. Moreover, Shimoni et al. (43) have recently shown that the attenuation of transient outward K\(^+\) currents with diabetes-related oxidative stress through generated ANG II increased superoxide ion levels and their recovery following ACE inhibition through a reduction in oxidative stress. In this regard, our results indicated that the effects of AT1 receptor blocker occurred mainly through the alteration in the oxidative state of
protein thiols (Table 1). Total and acid-soluble SH concentrations were measured in the isolated cardiomyocytes. The results, summarized in Table 1, demonstrate that both total and soluble SH levels of diabetic cardiomyocytes were significantly decreased with respect to the controls. Both candesartan and BIM incubation of these diabetic cardiomyocytes could markedly preserve these values to the levels of the controls.

**DISCUSSION**

The major novel result of our study is that candesartan applied on cardiomyocytes isolated from STZ-induced diabetic rats exerts a protective action against anomalous Ca\(^{2+}\) homeostasis that is attributable to reduced RyR2 phosphorylation level and recovery of protein levels of both RyR2 and FKBP12.6. Furthermore, candesartan downregulates PKC translocation to the membranes. Similar recoveries are also obtained by treating the diabetic cells with a PKC inhibitor, BIM. In this regard, the present data demonstrate that activation of AT\(_1\) receptor plays a major role in inducing functional hyperphosphorylation of RyR2 via modulation with PKA, as well as an upregulation of the PKC and increase in ROS generation, although our study did not clarify all the intermediate steps.

All components of the RAS are present in the cardiac tissue, and ANG II is generated in the heart. However, ANG II generation depends largely, if not completely, on kidney-derived renin. The significant effects of application of candesartan, an AT\(_1\) antagonist, on isolated cardiomyocytes imply that the AT\(_1\) receptors are activated. This could seem surprising at first, since the cardiomyocytes were enzymatically dissociated at least 1 h before the application of the antagonist. However, diabetic cardiomyocytes produce ANG II (14, 43). Most probably, ANG II could be released and will act in an autocrine fashion, similar to a manner in neonatal cardiomyocytes in which ANG II was shown to be released 100-fold under stretching (ref. 38; see review in Ref. 7a). Also, one cannot exclude the fact that in the diabetic heart, AT\(_1\) receptors may exhibit a relatively stable binding to ANG II or would be constitutively active.

Enhanced RAS action in tissues and related signaling pathways have been consistently demonstrated to contribute to cardiovascular disease in diabetes, including diabetic cardiomyopathy (12, 34, 39). We previously reported that, in a rat model of STZ-induced diabetes, a prominent abnormal Ca\(^{2+}\) leak occurs through the RyR2, owing to a partial loss of RyR2-bound FKBP12.6 and PKA-mediated hyperphosphorylation of RyR2 (51). Both protein kinases, PKA and PKC, are expressed in cardiac muscle (22), and they strongly controlled cardiac inotropic activity (17, 19, 45). PKA and PKC play a central role in transducing signal and potentiating intracellular cross talk by phosphorylating diverse substrates, including receptors, enzymes, and transcription factors. They control the cardiac pump function and might be activated by various mediators including ANG II (13). The marked recovery in the characteristics of Ca\(^{2+}\) sparks with candesartan may be attributable in part due to an indirect effect of ANG II on PKA activation (34). These findings suggest that, in diabetic rat hearts, a hyperadrenergic signal is transmitted into the cell, resulting in a hyperphosphorylation of RyR2, which triggers a defective interaction of FKBP12.6 with RyR2. Hence, candesartan, by acting on AT\(_1\), would reduce RyR2 hyperphosphorylation. In a very recent review article, Yano et al. (50) discussed the stabilization of RyR2 as a new therapeutic paradigm in heart failure due to the effects of ANG II receptor blockers that are suppressing the hyperadrenergic state, thereby reversing PKA-mediated hyperphosphorylation of RyR2, restoring the stoichiometry of its macromolecular complex, and inhibiting the Ca\(^{2+}\) leak from SR. This hypothesis is further supported by our data. We have shown that with candesartan incubation, the cardiomyocytes could prevent diabetes-induced hyperphosphorylation of RyR2 and defect in the interaction of FKBP12.6 with RyR2. This prevention can further cause a recovery in abnormal Ca\(^{2+}\) leak from SR and a significant recovery in intracellular Ca\(^{2+}\) overload.

It is known that stimulation of AT\(_1\) receptors in diabetic heart results in a marked activation in PKC activity (8, 14, 26). Our competitive studies with an AT\(_1\) receptor blocker candesartan and a nonspecific inhibitor BIM revealed that there is a close relationship between increased levels of ANG II receptors, enhanced PKC activity, and abnormal Ca\(^{2+}\) release in diabetic heart. Furthermore, AT\(_1\) receptor stimulation induces the generation of oxygen-derived free radicals, which can have detrimental effects on heart function (8, 25, 28). Recently published data support this idea (23, 31). They reported the role of ROS in the amplification of PKC signaling and ANG II activation in high glucose-induced cell dysfunction. With these findings in mind, it can be suggested that PKC may play important roles in the alterations of both local and global Ca\(^{2+}\) releases in cardiomyocytes from diabetic rat hearts.

The heart, like many other organs, has a local RAS (9–11, 14), and the activation of RAS is shown to contribute to diabetic cardiomyopathy (12). Supporting this idea, Gassanov et al. (16) have recently demonstrated that ANG II could induce marked changes in the characteristics of Ca\(^{2+}\) sparks in human atrial myocytes and that candesartan (1 μM) could cause significant recovery in these parameters within 12 h of incubation. Moreover, other studies with both AT\(_1\) receptor blockers and antioxidants showed significant augmentation in diabetes-induced cardiac cell dysfunctions (41–43). It is important to note that hyperglycaemia can lead to oxidative stress by a wide variety of mechanisms, including activation of RAS, and activation of PKC also leads to oxidative stress (25, 26). In the present study, we have demonstrated that both total and soluble SH levels in diabetic cardiomyocytes were significantly decreased with respect to control. Both candesartan and BIM

### Table 1. Effects of Can and BIM on sulfhydryl contents of cardiomyocytes isolated from Con and DM rat hearts

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>DM</th>
<th>DM + Can</th>
<th>DM + BIM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free sulfhydryl, μmol/mg wet wt</td>
<td>1.74±0.02</td>
<td>0.95±0.22*</td>
<td>1.67±0.12†</td>
<td>1.64±0.14†</td>
</tr>
<tr>
<td>Total sulfhydryl, μmol/mg wet wt</td>
<td>3.30±0.46</td>
<td>1.56±0.06*</td>
<td>2.40±0.10†</td>
<td>2.84±0.29†</td>
</tr>
</tbody>
</table>

Values are means ± SE; *n = 4 rats. Cardiomyocytes from control (Con) and diabetic (DM) groups were incubated with 1 μM candesartan (Can) or 100 nM bisindolylmaleimide I (BIM) for 6–8 h at 37°C. *P < 0.05 vs. Con; †P < 0.05 vs. DM.

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incubations of these diabetic cardiomyocytes could markedly preserve these values to the levels of control. With these findings kept in mind and the data related to recovery in the hyperphosphorylation level of RyR2 with both candesartan and BIM, it can be suggested that oxidative stress can play important roles in the alterations of both local and global Ca\(^{2+}\) releases in cardiomyocytes from diabetic rat hearts. This is strongly supported by the study of Bidasee et al. (4), which demonstrated significant increased disulfide bond formation and alterations in RyR2 functions in STZ-induced diabetic heart (4). The role of diabetes in the modification of SH groups of heart preparations has been reported earlier. In that study (32), ATPase activities of myofibrils and myofibrillar SH reactivity to \(\text{5,5}'\text{-dithiobis}(2\text{-nitrobenzoic acid})\) of isolated cardiomyocytes from diabetic rat hearts were measured. Both parameters were depressed and responded differentially to two different types of specific SH group modifiers. Therefore, the mechanism for the effect of candesartan on altered Ca\(^{2+}\) release mechanisms in heart during diabetes could involve, partially, marked recovery in modified SH groups into the cells. It is also well known that metabolic shifts brought about by diabetes increase production of ROS and nitrogen species (see review in Ref. 47). These species are also capable of reacting with several amino acid residues on proteins. Like glycation, modifications of amino acid residues by these species can alter the tertiary structure, and these changes, in turn, could alter the function of RyR2 (4). In addition, in our previous study we, like others, have shown that oxidative stress is involved in the etiology of diabetes-induced downregulation of cardiac function and that there is a close relationship between insulin deficiency, or impaired insulin signaling, and alterations in the cardiac function via depressed endogenous defense mechanisms (1, 41, 48).

Another possible mechanism to account for an AT\(_1\) receptor blockade-induced reversal of slowed relaxation of the intracellular Ca\(^{2+}\) transients in diabetic cardiomyocytes is restoration of the function of SERCA, which is diminished in STZ-induced diabetes (7, 25, 30). Activation of PKC has also been shown to decrease SR-Ca\(^{2+}\) uptake and to have a negative inotropic effect in rat cardiomyocytes (36). Decreased caffeine-induced intracellular Ca\(^{2+}\) transient in diabetic cardiomyocytes has been interpreted as decreased SR-Ca\(^{2+}\) load due to the depressed SERCA activity (7). In the light of these published data, our findings indicated that the decreased SR-Ca\(^{2+}\) load could also be restored via restoration of SERCA activity by candesartan treatment, besides its possible effect on RyR2 hyperphosphorylation. This hypothesis is supported by the study of Hanatani et al. (18) in which candesartan treatment prevented left ventricular dysfunction via normalization of the SERCA and Na\(^{+}\)-Ca\(^{2+}\) exchanger levels.

Our present data demonstrate that both PKA and PKC, either by separate ways or in a common pathway, can play important roles in the diabetes-induced alterations in Ca\(^{2+}\) homeostasis as well as ANG II-induced cell dysfunction in diabetes. We have shown that both candesartan and BIM have similar protective effects on these altered parameters related with Ca\(^{2+}\) homeostasis and finally with heart function from male rats. Our data are supported with several studies performed with different diseased heart models. Recently, Watanuki et al. (46) investigated alterations of expression and site-specific phosphorylation of phospholamban, and SERCA in STZ-induced diabetes, and showed that PKA-dependent phosphorylation might incompletely counteract the function of phospholamban as an inhibitor of SERCA activity in diabetes in which PKC expression and activity were enhanced. Altogether, our data point out the roles of PKA and PKC, either together or individually, via the activation of ANG II and oxidant stress in diabetes-induced heart dysfunction.

Viewed together, our observations are consistent with the concept that ANG II is upregulated in the heart during diabetes, and this causes several major alterations in the Ca\(^{2+}\) homeostasis of cardiomyocytes. This research extends our previous observations that chronic ANG II blockade protects the heart from the development of cellular alterations typically related with diabetes, such as prolonged action potentials or reduced Ca\(^{2+}\) transient and contraction (30). Both PKA and PKC are involved in these alterations either individually or together in the same pathway. It is clear that activities of both kinases are closely related to diabetes and also to oxidative stress status in the cell. Our results should help to identify a better basis to understand the molecular mechanisms of diabetes-induced heart dysfunction, the common clinical observations that use ANG II receptor blockers to improve prognoses among patients with diabetes, and other treatments. These agents appear to prevent severe disease complications from occurring, partially by ameliorating the defective FKBP12.6-mediated stabilization of the RyR2 as well as SERCA function. This new therapeutic strategy, targeting the intracellular Ca\(^{2+}\) homeostasis, should be promising for the treatment of diabetic cardiomyopathy.

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