Role of Ca\(^{2+}\)/calmodulin-dependent protein kinase II in the regulation of the cardiac L-type Ca\(^{2+}\) current during endothelin-1 stimulation

Kimiaki Komukai,1 Jin O-Uchi,2,3 Satoshi Morimoto,1,2 Makoto Kawai,1 Kenichi Hongo,1 Michihiro Yoshimura,1 and Satoshi Kurihara2

1Division of Cardiology and 2Department of Cell Physiology, The Jikei University School of Medicine, Tokyo, Japan; and 3Aab Cardiovascular Research Institute, Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York

Submitted 4 December 2009; accepted in final form 18 March 2010

ENDOTHELIN-1 (ET-1) IS KNOWN as a strong vasoconstrictive peptide. Production of ET-1 is increased under pathophysiological conditions in the cardiovascular system, such as heart failure (30), acute myocardial infarction (21), and hypertension (26). This peptide also directly affects cardiac muscle and shows a positive inotropic effect. However, the intracellular mechanism of the inotropic effect remains unclear. ET-1 increases intracellular pH by PKC-inositol hydrolysis and activates protein kinase C (PKC) (11, 12, 28). Therefore, ET-1 increases the L-type Ca\(^{2+}\) current (I\(_{Ca}\)), which is also one of the key determinants of cardiac muscle contraction, although the effect of ET-1 on I\(_{Ca}\) is controversial (5, 12, 29, 31, 32, 36).

There are two distinct endothelin receptor subtypes: ET\(_A\) and ET\(_B\). Stimulation of the ET\(_A\) receptor induces vasoconstriction in smooth muscle cells, while that of ET\(_B\) receptor induces vasorelaxation through the nitric oxide production in endothelial cells. The ET\(_A\) receptor is thought to be dominant in cardiac muscle. The ET\(_A\) receptor is one of the G protein-coupled receptors, and it couples with G\(_\text{q}\), which is the same as AT\(_1\) receptor and \(\alpha_{1A}\)-adrenoceptor. \(\alpha_{1A}\)-Adrenoceptor stimulation increases the I\(_{Ca}\) via the activation of PKC and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in rat ventricular myocytes (22, 23). Therefore, we hypothesized that ET-1 increases I\(_{Ca}\) via the same mechanism as \(\alpha_{1A}\)-adrenoceptor stimulation.

MATERIALS AND METHODS

This study was performed in accordance with the Guideline on Animal Experimentation of the Jikei University School of Medicine, and study protocol was approved by the Animal Care Committee of The Jikei University School of Medicine (approval reference number: H19-049).

Preparations. Single ventricular myocytes were freshly prepared from adult male Wistar rats (300–400 g), as described previously (19). Briefly, the rats were anesthetized with inhalation of ether, followed by intraperitoneal injection of pentobarbital. The heart was quickly removed, cannulated, and retrogradely perfused with Tyrode’s solution containing 2 mM Ca\(^{2+}\) and protease. The cells were stored in Tyrode’s solution containing 1 mM Ca\(^{2+}\) at room temperature (\(\approx\)25°C).

Measurement of the I\(_{Ca}\). The perforated patch-clamp technique was used to measure the I\(_{Ca}\) (18). The patch-clamp amplifier EPC-7Plus (HEKA Electronik, Lambrecht/Pfalz, Germany) was controlled by a personal computer through an analog-to-digital interface (ITC-16, Instrutech, Long Island, NY). The data acquisition was performed using the Pulse Pulsatife software program (HEKA Electronik) (22, 23). Electrodes were made from glass capillaries (Harvard Apparatus, Kent, UK) using an electric puller Model P-97 (Sutter Instrument, Novato, CA). The tip of the electrode was filled with pipette solution (see below for composition) and then was back filled with pipette solution containing 200–400 μg/ml amphotericin B. The pipette solution contained 1 mM CaCl\(_2\) to ensure against the accidental rupture of the patch membrane. Measurements were started after confirmation of the stable capacitive current. For measurement of the I\(_{Ca}\), the holding potential was set to −40 mV to inactivate Na\(^{+}\) current, and 200-ms depolarization pulse to 0 mV was applied every 10 s. The amplitude of the current was defined as the difference between peak inward current and the current remaining at the end of pulses. The
amplitude of \(I_{Ca}\), recorded with the perforated patch is stable (no significant rundown and/or up) for up to 15 min (22, 23). The holding current and remaining current were not altered throughout the experiments. A series of test pulses to between \(-30\) and \(+60\) mV was applied for obtaining current-voltage relationship. All experiments were performed with the Tyrode’s solution containing 1 mM Ca\(^{2+}\) as the extracellular solution at room temperature (\(\approx 25^\circ\)C).

Western immunoblot analysis. Membrane, cytosolic, and nuclear/filament fractions were prepared from cardiomyocytes using differential centrifugation (23), and only membrane proteins were used for this analysis. Membrane protein from rat brain was obtained using ProteoExtract Native Membrane Protein Extraction Kit (Calbiochem, La Jolla, CA). Protein concentration was determined by bicinchoninic acid methods. Each protein was separated by SDS-PAGE and analyzed by Western immunoblotting (22, 23). Immunoreactive bands were visualized by the ECL-plus detection kit (GE Healthcare UK, Amersham, UK).

Solutions, chemicals, and antibodies. Tyrode’s solution used during isolation and measurement of the \(I_{Ca}\) contained the following (in mM): 136.9 NaCl, 5.4 KCl, 0.5 MgCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 5 HEPES, and 5 glucose (pH 7.40 adjusted by NaOH). The composition of the pipette solution for the \(I_{Ca}\) measurements was as follows (in mM): 130 CsCl, 10 NaCl, 0.5 MgCl\(_2\), 1 CaCl\(_2\), and 5 HEPES (pH 7.20 adjusted by CsOH).

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise mentioned. A 50 mg/ml stock solution of amphotericin B was freshly prepared with dimethylsulfoxide (DMSO; Dojindo Laboratories, Kumamoto, Japan) and then was diluted with pipette solution for the extracellular solution. One-millimolar stock solutions of the PKC inhibitor, chelerythrine (Calbiochem), was freshly prepared with dimethylsulfoxide (DMSO; Dojindo Laboratories, Kumamoto, Japan) and then was diluted with pipette solution for the extracellular solution. One-millimolar stock solutions of the ET receptor antagonists did not alter the basal \(I_{Ca}\) regulation were investigated using pharmacological tools. BQ-123 (9) and BQ-788 (10) are selective antagonistic peptides for the ETA and ETB receptors, respectively. After 10-min pretreatment with BQ-123 or BQ-788, ET-1 was applied in the continuous presence of BQ derivatives. Ten-minute application of these receptor antagonists did not alter the basal \(I_{Ca}\) amplitude (data not shown). In the presence of 1 mM BQ-123, ET-1 did not alter the \(I_{Ca}\); current at 15 min after application of ET-1 was 92.6 \(\pm\) 3.6% of the value before ET-1 application (\(N = 5\); \(P = \) 0.05 vs. baseline). Statistical analysis. The data are expressed as means \(\pm\) SE. The \(t\)-test was performed for two data sets, while one-way ANOVA (followed by post hoc Tukey test) was performed for multiple comparisons. Statistical significance was defined as a \(P\) value of <0.05.

RESULTS

Effect of ET-1 on the \(I_{Ca}\). Application of 10 nM ET-1 increased the \(I_{Ca}\), as shown in Fig. 1A. In contrast to \(\alpha\)-adrenoceptor stimulation (22), negative effect (a decrease in the \(I_{Ca}\)) was not apparent. After 15 min, ET-1 significantly increased the \(I_{Ca}\) from 1.21 \(\pm\) 0.17 to 1.53 \(\pm\) 0.19 nA (28.8 \(\pm\) 6.8% increase compared with the current before stimulation, \(P = 0.002\), \(N = 6\)), without changing the shape of current-voltage relationship. The \(I_{Ca}\) reached a steady state 15 min after the application of ET-1 (Fig. 1B). The current measured 15 min after application (1.53 \(\pm\) 0.19 nA) and that 20 min after application (1.54 \(\pm\) 0.18 nA) of ET-1 were not statistically different (\(P = 0.767\), \(N = 6\)). Therefore, the effect of ET-1 was observed after 15-min application in the following experiments.

Effect of ET receptor subtype-specific stimulation on the \(I_{Ca}\). mRNA of both ETA and ETB receptors has been detected in isolated adult rat ventricular myocytes by Western immunoblotting, with the commercially available antibodies against ETA and ETB. As shown in Fig. 2A, ETA was detected in rat membrane protein from ventricular myocytes, whereas the immunoreactive band against ETB was absent. Next, the functional roles of ET-receptor subtypes on \(I_{Ca}\) regulation were investigated in isolated adult rat ventricular myocytes by Western immunoblotting, with the commercially available antibodies against ETA and ETB. As shown in Fig. 2A, ETA was detected in rat membrane protein from ventricular myocytes, whereas the immunoreactive band against ETB was absent. Next, the functional roles of ET-receptor subtypes on \(I_{Ca}\) regulation were investigated in isolated adult rat ventricular myocytes by Western immunoblotting, with the commercially available antibodies against ETA and ETB. As shown in Fig. 2A, ETA was detected in rat membrane protein from ventricular myocytes, whereas the immunoreactive band against ETB was absent. Next, the functional roles of ET-receptor subtypes on \(I_{Ca}\) regulation were investigated in isolated adult rat ventricular myocytes by Western immunoblotting, with the commercially available antibodies against ETA and ETB.

Fig. 1. Effect of endothelin-1 (ET-1) on L-type Ca\(^{2+}\) current (\(I_{Ca}\)). A: representative time-dependent effect of 10 nM ET-1 on the \(I_{Ca}\). Inset: original records of the \(I_{Ca}\) at the points indicated. B: mean time-dependent effect of ET-1. \(N = 6\); *\(P < 0.05\) vs. the value before application of ET-1. C: mean current-voltage relationship of the \(I_{Ca}\) before (○) and 20 min after (●) application of ET-1. \(N = 6\). Values are means \(\pm\) SE. NS, nonsignificant.
Therefore, the positive effect of ET-1 on the $I_{Ca}$ was via ET$_A$-receptor stimulation in rat ventricular myocytes.

**Role of PKC and CaMKII in the regulation of $I_{Ca}$ during ET-1 stimulation.** ET-1 induces phosphoinositide hydrolysis and activates PKC (11, 12, 28). PKC is also involved in the regulation of $I_{Ca}$ during ET-1 stimulation in rat ventricular myocytes (5). The involvement of PKC in the regulation of the $I_{Ca}$ during ET-1 stimulation was investigated using chelerythrine, a synthetic PKC inhibitor (6). The application of 5 µM chelerythrine decreased the $I_{Ca}$, and the $I_{Ca}$ reached another steady state 10 min after the application. Chelerythrine abolished the effect of ET-1 (Fig. 3, A and B). The current measured 15 min after the application of ET-1 in the presence of chelerythrine was 86.1 ± 6.0% of the value before ET-1 application ($N = 5$; $P = 0.001$ vs. ET-1 alone). These findings confirmed that ET-1 increased the $I_{Ca}$ via PKC activity in rat ventricular myocytes.

The role of CaMKII in the regulation of the $I_{Ca}$ was investigated during ET-1 stimulation. KN-93 is a synthetic CaMKII inhibitor and KN-93 (500 nM) selectively inhibits CaMKII without affecting other protein kinases (27). The application of 500 nM KN-93 decreased the $I_{Ca}$, and the $I_{Ca}$ reached another steady state 15 min after the application (22). ET-1 stimulation did not cause an increase in the $I_{Ca}$ in the continuous presence of KN-93, (Fig. 3, C and E). The current measured 15 min after the application of ET-1 was 95.1 ± 11.7% of the value before ET-1 application ($N = 4$). The effect of ET-1 on the $I_{Ca}$ in the presence of KN-92, an inactive analog of KN-93, was also measured because KN-93 would have a nonspecific effect on membrane current (1). ET-1 increased the $I_{Ca}$ in the presence of KN-92, as it did in the absence of KN derivatives ($N = 4$; Fig. 3D). The relative values 15 min after application of ET-1 in the presence of KN-93 and KN-92 were statistically different ($P = 0.011$; Fig. 3E). The role of CaMKII in the regulation of the $I_{Ca}$ during ET-1 stimulation was also confirmed using autocamtide-2 related inhibitory peptide (AIP), another CaMKII inhibitor. Before making the seal, the cell was pretreated with AIP (10 µM) for 1 h and then was used in the continuous presence of AIP in a perfusion solution. AIP also blocked ET-1-induced increase in the $I_{Ca}$ as in the case of KN-93 ($N = 3$, Fig. 4).

Therefore, the positive effect of ET-1 on $I_{Ca}$ was performed via the CaMKII activity in rat ventricular myocytes.

**DISCUSSION**

The present study provides the evidence for the intracellular mechanisms by which ET-1 activates the $I_{Ca}$ in rat ventricular myocytes. The findings confirmed that 1) ET$_A$ receptor is expressed at protein level in the sarcolemma; and 2) ET$_A$-receptor stimulation activates the $I_{Ca}$. Moreover, findings confirmed that both PKC and CaMKII activities are involved in this mechanism, as in the case of $\alpha_1$A-adrenoceptor stimulation in rat ventricular myocytes (22, 23) (Fig. 4).

**Effect of ET-1 on the $I_{Ca}$** The effect of ET-1 on the $I_{Ca}$ varies, depending on the preparation, species, presence or absence of $\beta$-adrenoceptor, and especially the experimental conditions (5, 29, 32, 36). He et al. (5) showed ET-1 (10 nM) increases the $I_{Ca}$ using a perforated patch-clamp technique. Zeng et al. (36) showed ET-1 (10 nM) increases the open probability of the L-type Ca channel using the cell-attached
mode of single-channel recording. The present study also showed that the same concentration of ET-1 increased the $I_{Ca}$ using a perforated patch-clamp technique. In both cell-attached mode and perforated patch-clamp technique, intracellular Ca$^{2+}$ buffer is not used. Therefore, Ca$^{2+}$-dependent signaling can be observed by these methods. Our results suggest that the conflicting results of the effect of ET-1 on the $I_{Ca}$ are predominantly due to the involvement of CaMKII, a Ca$^{2+}$-dependent enzyme. The conflicting results of the $I_{Ca}$ measured with various patch-clamp methods are also reported during $\alpha_1$-adrenoceptor stimulation and acidosis, in which CaMKII is activated (18, 22, 23).

In some studies, ET-1 shows a negative effect on the $I_{Ca}$ via the pertussis toxin-sensitive pathway (7, 31, 35). The pertussis toxin-sensitive effect is accentuated in the presence of $\beta_2$-adrenoceptor stimulation. In the present study, a negative effect might not have been observed, due to the absence of $\beta_2$-adrenoceptor stimulation.

In rat ventricular myocytes, a lower concentration of ET-1 (100 pM) increases cell shortening without an increase in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}]_i$) (15). However, a higher concentration of ET-1 (100 nM) increases both the [Ca$^{2+}]_i$ and cell shortening (14). Because the $I_{Ca}$ is one of the most important determinants of [Ca$^{2+}]_i$, ET-1 appears to increase the [Ca$^{2+}]_i$ at the concentration used in this study. The plasma concentration of ET-1 is not as high as the concentration used in this study, but ET-1 is produced in the myocardium. As a result, it appears possible that ET-1 regulates cardiac myocytes in an autocrine-paracrine manner at higher concentrations than the plasma concentrations.

Fig. 3. Effect of chelerythrine and KN-93 on $I_{Ca}$ in the response to ET-1. A: representative time-dependent record of the $I_{Ca}$ in the presence of 5 μM chelerythrine, a PKC inhibitor. Inset: original records of the $I_{Ca}$ at the point indicated. B: mean time-dependent effect of ET-1 in the absence (●, N = 6) or presence (●, N = 5) of chelerythrine. *P < 0.05 vs. ET-1 alone. Values are means ± SE. C: representative time-dependent record of the $I_{Ca}$ in the presence of 500 nM KN-93, a Ca$^{2+}$/calmodulin-dependent protein kinase II inhibitor. Inset: original records of the $I_{Ca}$ at the point indicated. D: representative time-dependent record of the $I_{Ca}$ in the presence of 500 nM KN-92, an inactive analog of KN-93. Inset: original records of the $I_{Ca}$ at the point indicated. E: mean time-dependent effect of ET-1 in the presence of KN-93 (●, N = 4) or KN-92 (●, N = 4). *P < 0.05 vs. KN-92 + ET-1. Values are means ± SE.

Fig. 4. Summary of the effect of ET-1 on the $I_{Ca}$ in various antagonists. All of the measurements were done 15 min after application of 10 nM ET-1, in the absence or continuous presence of antagonists. *P < 0.05 vs. ET-1 alone. #P < 0.05 vs. KN-92 + ET-1. Numerals in parentheses indicate the number of the experiments. Values are mean ± SE. Chele, chelerythrine; AIP, autacamtide-2 related inhibitory peptide.
Therefore, intracellular signaling induced by ET_A dominantly regulates the I_{Ca}, but ET_B-dependent intracellular signaling is not involved in this mechanism.

Role of PKC and CaMKII in the regulation of I_{Ca} during ET-1 stimulation. The present study showed that ET-1-induced increase in the I_{Ca} is both PKC and CaMKII dependent (Figs. 3 and 4). ET-1 couples to G_{q} protein, which induces phosphoinositide hydrolysis, and this signaling can activate PKC (11, 12, 28). There are several reports that show the coupling between PKC and CaMKII in vitro (33) and in vivo (22, 23, 24). α_{1}-Adrenoceptor-G_{q} phospholipase C (PLC) signaling activates CaMKII through PKC activation (22, 23). β-Adrenoceptor-Epac-PLC-ε signaling activates CaMKII through PKC-ε activity (24). The detailed mechanism of how PKC can couple to CaMKII has not yet been established; however, one possible mechanism is that PKC directly phosphorylates the autophosphorylation site (Thr^{268}) of CaMKII (33), which strongly regulates CaMKII activity. There are also several reports that ET-1 stimulation can activate CaMKII activity (20, 34). ET-1 increases phospholamban phosphorylation at CaMKII-specific phosphorylation site in murine hearts (20). ET-1 also induces CaMKII autophosphorylation in rabbit hearts (34). These reports strongly support the current hypothesis that ET-1 stimulation activates PKC and the activated PKC induces the phosphorylation of the CaMKII autophosphorylation site, which directly increases the CaMKII activity. Activated CaMKII would directly bind to L-type Ca^{2+} channels, phosphorylate the channel, and increase the open probability of the channel (4, 8).

There are several subtypes of PKC in cardiac myocytes (3, 23), but this study did not examine which subtype of PKC is responsible for CaMKII activation. ET-1 stimulation in the rat ventricular myocytes induces the translocation of PKC-δ and PKC-ε, but not PKC-α (3), as in the case of α_{1A}-adrenoceptor stimulation (23). Epac-PLC-ε signaling activates CaMKII through PKC-ε (24). These reports indicate that PKC-ε is the most likely candidate involved in the mechanism of I_{Ca} potentiation induced by ET-1, but further study is still required to elucidate this phenomenon.

In conclusion, the present study showed that ET-1 increased the I_{Ca} via the ET_{A}-receptor-PKC-CaMKII pathway. The activation of PKC and CaMKII by ET-1 would contribute to such pathophysiological conditions as cardiac hypertrophy and heart failure.

ACKNOWLEDGMENTS

The authors thank Naoko Tomizawa, Yoko Natake, and Yasuo Kimura for technical assistance.

GRANTS

This study was supported by KAKENHI (15790397 and 18590789 to K. Komukai, 19590833 to K. Hongo, and 19500357 to S. Kurihara), Ueda Memorial foundation (to K. Komukai), Japan Heart Foundation Young Investigator’s Research grant (to J. O-Uchi), Kato Memorial Foundation (J. O-Uchi), and the Vehicle Racing Commemorative Foundation (to K. Hongo and S. Kurihara). J. O-Uchi is a recipient of Kanace Foundation for the Promotion of Medical Science foreign study award (2008) and American Heart Association Founders Affiliate (FDA) Spring 09 Postdoctoral Fellowship grant (09POST2310079).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


