Endothelin-1 enhances nuclear Ca\(^{2+}\) transients in atrial myocytes through Ins(1,4,5)P\(_3\)-dependent Ca\(^{2+}\) release from perinuclear Ca\(^{2+}\) stores

Jens Kockskämper\(^{1,2,*}\), Lea Seidlmayer\(^1\), Stefanie Walther\(^1\), Kristian Hellenkamp\(^1\), Lars S. Maier\(^1\) and Burkert Pieske\(^{1,2}\)

\(^{1}\)Department of Cardiology and Pneumology, University Medicine Göttingen, Germany
\(^{2}\)Department of Cardiology, Medical University Graz, Auenbruggerplatz 15, A-8036 Graz, Austria

*Author for correspondence (e-mail: jens.kockskamper@meduni-graz.at)

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Summary

Nuclear Ca\(^{2+}\) plays a key role in the regulation of gene expression. Inositol (1,4,5)-trisphosphate [Ins(1,4,5)P\(_3\)] might be an important regulator of nuclear Ca\(^{2+}\) but its contribution to nuclear Ca\(^{2+}\) signalling in adult cardiomyocytes remains elusive. We tested the hypothesis that endothelin-1 enhances nuclear Ca\(^{2+}\) concentration transients (CaTs) in rabbit atrial myocytes through Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release from perinuclear stores. Cytoplasmic and nuclear CaTs were measured simultaneously in electrically stimulated atrial myocytes using confocal Ca\(^{2+}\) imaging. Nuclear CaTs were significantly slower than cytoplasmic CaTs, indicative of compartmentalisation of intracellular Ca\(^{2+}\) signalling. Endothelin-1 elicited a preferential (10 nM) or a selective (0.1 nM) increase in nuclear versus cytoplasmic CaTs. This effect was abolished by inhibition of endothelin-1 receptors, phospholipase C and Ins(1,4,5)P\(_3\) receptors. Fractional Ca\(^{2+}\) release from the sarcoplasmic reticulum and perinuclear stores was increased by endothelin-1 at an otherwise unaltered Ca\(^{2+}\)-load. Comparable increases of cytoplasmic CaTs induced by \(\beta\)-adrenoceptor stimulation or elevation of extracellular Ca\(^{2+}\) could not mimic the endothelin-1 effects on nuclear CaTs, suggesting that endothelin-1 specifically modulates nuclear Ca\(^{2+}\) signalling. Thus, endothelin-1 enhances nuclear CaTs in atrial myocytes by increasing fractional Ca\(^{2+}\) release from perinuclear stores. This effect is mediated by the coupling of endothelin receptor A to PLC-Ins(1,4,5)P\(_3\) signalling and might contribute to excitation-transcription coupling.

Key words: Endothelin, Inositol (1,4,5)-trisphosphate, Calcium, Nucleus, Myocyte

Introduction

Regulation of the nuclear Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_n\)) plays a key role in the regulation of gene expression. In cardiac myocytes, there is a transient rise in the cytoplasmic [Ca\(^{2+}\)]\(_c\) during each heartbeat caused by Ca\(^{2+}\) influx through voltage-dependent sarcolemmal Ca\(^{2+}\) channels during the action potential and the ensuing Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) through ryanodine (Ry) receptor Ca\(^{2+}\) release channels. This cytoplasmic [Ca\(^{2+}\)]\(_c\) transient (CaT) serves to induce contraction and thus couples the electrical to the mechanical activity of the heart (excitation-contraction coupling). The increase in cytoplasmic [Ca\(^{2+}\)]\(_c\) is thought to transmit passively to the nucleus by Ca\(^{2+}\) diffusion through nuclear pore complexes (Genka et al., 1999; Tanaka et al., 1996a; Tanaka et al., 1996b). Thus, each cytoplasmic CaT elicits a nuclear CaT. According to this scheme, the nuclear [Ca\(^{2+}\)]\(_n\) in cardiac myocytes is regulated passively and is entirely dependent on changes in the cytoplasmic [Ca\(^{2+}\)]\(_c\). Whether the nuclear [Ca\(^{2+}\)]\(_n\) can also be regulated actively and, if so, by which mechanisms, remains unknown.

In addition to Ry receptors, cardiac myocytes also express inositol (1,4,5)-trisphosphate [Ins(1,4,5)P\(_3\)] receptors, albeit at much lower densities (Lipp et al., 2000). Interestingly, the expression of Ins(1,4,5)P\(_3\) receptors is greater in atrial than in ventricular myocytes (Lipp et al., 2000), suggesting a more prominent role for Ins(1,4,5)P\(_3\) in the atrial myocardium. Atrial Ins(1,4,5)P\(_3\) receptors are found in the cytoplasm as well as in the perinuclear area (Bare et al., 2005; Mackenzie et al., 2002; Yamada et al., 2001). G-protein-coupled receptors activating the phospholipase C (PLC)-Ins(1,4,5)P\(_3\) cascade thus have the potential to modulate the cytoplasmic and nuclear [Ca\(^{2+}\)]\(_c\) through Ins(1,4,5)P\(_3\)-dependent Ca\(^{2+}\) release from the SR and nuclear envelope, respectively. Elegant studies by Remus et al. have demonstrated directly, using fluorescent Ins(1,4,5)P\(_3\) biosensors, that endothelin-1 (EDN1, hereafter referred to as ET) causes time-dependent increases in both cytoplasmic and nuclear Ins(1,4,5)P\(_3\) concentration ([Ins(1,4,5)P\(_3\)]) (Remus et al., 2006). It has not been shown so far, however, whether these increases in [Ins(1,4,5)P\(_3\)] cause relevant alterations in cytoplasmic and nuclear [Ca\(^{2+}\)] through Ins(1,4,5)P\(_3\)-dependent Ca\(^{2+}\) release. In particular, alterations in nuclear CaTs in beating adult cardiac myocytes following activation of the PLC-Ins(1,4,5)P\(_3\) cascade have not been demonstrated to date. Such a mechanism would offer the myocyte a means to regulate nuclear [Ca\(^{2+}\)] independently from the action-potential-induced cytoplasmic CaT during excitation-contraction coupling and, therefore, would constitute the basis for the concept of cardiac excitation-transcription coupling (Wu et al., 2006). Therefore, we characterised ET-induced changes in cytoplasmic and nuclear CaTs in electrically stimulated adult rabbit atrial myocytes and evaluated the possible involvement of PLC-Ins(1,4,5)P\(_3\)
signalling and Ins(1,4,5)P3-dependent Ca2+ release in the regulation of nuclear [Ca2+].

Results
Identification and characterisation of nuclear CaTs in electrically stimulated atrial myocytes
As we sought to detect ET-induced changes in cytoplasmic and nuclear Ca2+ regulation, changes in the cytoplasmic and nuclear [Ca2+] had to be measured simultaneously with sufficient spatial and temporal resolution. Fig. 1A illustrates a series of two-dimensional confocal images of an atrial myocyte during an electrically stimulated CaT. The myocyte exhibited quite uniform resting fluorescence (a), indicating that Fluo-4 was distributed mainly in the cytoplasm. Following stimulation, intracellular [Ca2+] started to increase in the subsarcolemmal space and then propagated to the centre of the cell (b-k). This pattern is typical for atrial myocytes lacking T-tubules (Kockskamper et al., 2001; Mackenzie et al., 2001). During the inward spread of the CaT, an ellipsoidal region in the centre of the myocyte was characterised by a delayed increase in fluorescence (f-k). During the decay of the CaT, the same region exhibited higher fluorescence than the surrounding parts of the cell (m-q). Thus, both rise and decay of the CaT were delayed in this central region, which presumably represented the nucleus, as suggested by previous studies on cardiomyocyte Ca2+ signalling (Genka et al., 1999; Tanaka et al., 1996a; Tanaka et al., 1996b).

For a detailed kinetic analysis, CaTs of subcellular regions corresponding to the whole cytoplasm (‘wc’, black), the subsarcolemmal regions (‘ss’, green), the nucleus (‘nuc’, red) and two adjacent central areas of identical size (‘ct’, blue) were obtained, and these are plotted in Fig. 1B (left). The subsarcolemmal CaTs had the largest amplitudes and peaked first. The two central areas exhibited CaTs with lower amplitudes. They peaked ~60 mseconds later than the subsarcolemmal CaTs, but their decay kinetics were almost identical to the subsarcolemmal decay. As the CaT from the whole cytoplasm represents the sum of all cytoplasmic sites, it exhibited spatiotemporal characteristics that were intermediate between those of the subsarcolemmal and central sites. By contrast, the nuclear CaT displayed the lowest amplitude and peaked 83 mseconds later than the central CaTs. Fluorescence in the nucleus still rose when the central CaTs had already started to decline (vertical line). Furthermore, during the decaying phase, fluorescence in the nucleus became greater and the kinetics of decay slower than in each of the cytoplasmic areas. Average values of peak systolic [Ca2+], time-to-peak and the time constant τ for decay are shown in Fig. 1B (right). Subsarcolemmal CaTs had significantly higher peaks and shorter times-to-peak than central CaTs, whereas their time constants for decay were identical. Nuclear CaTs, by contrast, exhibited significantly lower peaks, longer times-to-peak and larger time constants for decay than both central and subsarcolemmal areas.
Similar results were obtained using conventional line-scan imaging (data not shown).

To verify whether the ellipsoidal region in the centre of the myocyte characterised by a delayed CaT indeed represented the nucleus, myocytes were first loaded with Fluo-4 and electrically stimulated to record the CaT. Afterwards, stimulation was switched off and the cells were loaded additionally with the fluorescent nucleic acid dye Syto-16. Fig. 1C (left) illustrates an image of a pair of myocytes obtained during the decay of the CaT. Fig. 1C (right) shows the same cells after cessation of stimulation and loading with Syto-16. Clearly, Syto-16 stained two regions in the cell centres that were essentially identical to the regions of increased Fluo-4 fluorescence during the CaT decay. Similar observations were made in five other cells. These results confirm that the ellipsoidal regions with delayed CaTs indeed represent the nuclei of atrial myocytes.

Taken together, these data indicate that cytoplasmic and nuclear CaTs in atrial myocytes can be measured simultaneously and that the nuclear CaTs are characterised by distinct kinetics. This suggests that the nucleus represents a cell compartment with its own $[Ca^{2+}]$-regulating properties.

The relative amplitude of the nuclear CaT varies between atrial myocytes.

Within a given atrial myocyte, the amplitude of the nuclear CaT relative to the cytoplasmic CaT was constant (data not shown). A comparison between myocytes, however, revealed that the amplitude of the nuclear CaT relative to the cytoplasmic CaT was quite variable (Fig. 2). Fig. 2A shows three pairs of cytoplasmic (black) and nuclear (red) CaTs from three different atrial myocytes. In each case, the amplitudes were normalised to the amplitude of the cytoplasmic CaT. The nuclear CaT could be smaller, equal to or higher than the cytoplasmic CaT. The kinetics of the nuclear CaTs, however, were consistently slower than the kinetics of the cytoplasmic CaTs, and this was true for all atrial myocytes studied ($n=95$). Fig. 2B shows a histogram of the distribution of the ratio of systolic $[Ca^{2+}]$ in the nucleus to systolic $[Ca^{2+}]$ in the cytoplasm obtained from a total of 95 atrial myocytes. In most cells (75%), this ratio was <1. However, in 25% of the atrial myocytes studied, the nuclear to cytoplasmic ratio was >1. On average, the peak F/F₀ (see Materials and Methods) in the nucleus amounted to 88±2% of the peak F/F₀ in the cytoplasm ($n=95$). The fact that different atrial myocytes exhibited variable relative amplitudes of the nuclear CaT provides further evidence for the notion that the nucleus is capable of regulating $[Ca^{2+}]$ independently from cytoplasmic $[Ca^{2+}]$. The reason for the differences in relative nuclear CaT amplitude between cells, however, is not known at present. We speculate that it might be related to variations of the $Ca^{2+}$ content of perinuclear and SR $Ca^{2+}$ stores and/or variations of the $Ca^{2+}$ release from these stores. In the context of this study, it is important to note that neither the absolute nor the relative amplitude of the nuclear CaT affected the ability of ET to enhance nuclear CaTs (see Fig. 5).

ET augments cytoplasmic and nuclear CaTs and alters their kinetics.

In cardiac myocytes, ET activates G-protein-coupled receptors to stimulate the PLC-Ins(1,4,5)P₃ signalling cascade (Endoh et al., 1998; Sugden, 2003). Previous studies have shown that ET can increase global CaTs in atrial myocytes of various species, including rat (Bootman et al., 2007; Mackenzie et al., 2002), cat (Zima and Blatter, 2004), mouse (Li et al., 2005) and human (Meyer et al., 1996). In rabbit atrial myocytes, we found that ET elicited a time- and concentration-dependent increase in intracellular CaTs ($n=58$, [ET] ranging from 0.1 to 50 nM). Maximal increases were observed 10-20 minutes after application of 10 nM ET (data not shown). Therefore, ET-induced changes in cytoplasmic and nuclear CaTs were studied 10 minutes after application of the peptide and compared with those of pre-ET controls. The effects of ET on cytoplasmic and nuclear CaTs are illustrated in Fig. 3. Fig. 3A shows two-dimensional fluorescence images of an atrial myocyte during individual CaTs before (left, control) and during exposure of the cell to 10 nM ET (right). ET increased both the cytoplasmic and nuclear $[Ca^{2+}]$. To quantify and compare the increases in cytoplasmic and nuclear $[Ca^{2+}]$, regions of interest corresponding to the whole cytoplasm and the nucleus, respectively, were analysed. Fig. 3B shows four consecutive CaTs from the cytoplasm (black) and the nucleus (red) before and during ET exposure. Clearly, ET increased CaTs in both regions. Notably, however, the increase in nuclear CaTs was larger than in cytoplasmic CaTs. Average values are shown in Fig. 3C ($n=24$). ET increased systolic $[Ca^{2+}]$ in the cytoplasm from 6.75±0.44 F/F₀ to 8.74±0.66 F/F₀ or to 130±5% of the control and in the nucleus from 6.07±0.75 F/F₀ to 8.94±1.12 F/F₀ or to 148±5% of the control ($P<0.01$). The ratio of nuclear to cytoplasmic peak $[Ca^{2+}]$ (nuc/cyto ratio) was elevated from 0.85±0.05 to 0.97±0.06 or to 115±3% of the control ($P<0.01$).

ET also altered the kinetics of the CaT (Fig. 3D, $n=24$). The time-to-peak CaT was significantly increased in the cytoplasm from 134±6 ms to 163±7 ms ($P<0.01$) but was unaltered in the nucleus (~220 mseconds). This reduced the delay between peak $[Ca^{2+}]$ in the cytoplasm and nucleus from 84±9...
Endothelin enhances nuclear Ca\(^{2+}\). The time constant for Ca\(^{2+}\) decay was decreased both in the cytoplasm and nucleus from 236±25 mseconds to 205±17 mseconds (\(P<0.05\)) and from 350±32 mseconds to 262±20 mseconds (\(P<0.01\)), respectively. Thus, ET prolonged the time-to-peak in the cytoplasm, reduced the difference between peak [Ca\(^{2+}\)] in the cytoplasm and nucleus and accelerated Ca\(^{2+}\) decay in the cytoplasm and nucleus.

Fig. 4 illustrates the effects of ET on cytoplasmic and nuclear CaTs when recorded using conventional confocal line-scan imaging. Compared with our Nipkow disc-based 2D imaging system, line-scan (1D) imaging offers higher temporal resolution (0.77 mseconds per scan line versus 8.33 mseconds per 2D image) but provides less spatial information. To record part of the cytoplasmic and part of the nuclear CaT simultaneously with this technique, the scan line was positioned perpendicular to the longitudinal axis of the myocyte crossing the nucleus, as illustrated in the schematic drawing of the cell in Fig. 4A. The line-scan images and the cytoplasmic (black) and nuclear (red) CaTs obtained from the regions shown next to the line-scan images are presented in Fig. 4A before (top) and following (bottom) ET exposure. Similar to what was found using 2D imaging (Fig. 3), ET increased cytoplasmic and nuclear CaTs and altered their kinetics in a specific manner. In a total of five myocytes (Fig. 4B), ET increased systolic [Ca\(^{2+}\)] in the cytoplasm by 22±9% and in the nucleus by 46±7% (\(P<0.01\)). Time-to-peak was significantly increased in the cytoplasm from 46±7 mseconds to 99±16 ms (\(P<0.05\)) but remained unchanged in the nucleus (156±9 ms versus 164±23 ms, \(P=\text{not significant}\)). Finally, the time constant for decay was unaltered in the cytoplasm (193±23 mseconds versus 186±21 mseconds; \(P=\text{not significant}\)) and decreased from 372±43 mseconds to 264±21 mseconds in the nucleus (\(P<0.01\)). These results compare well with the data obtained by 2D confocal imaging (see Fig. 3) and confirm that ET augments CaTs preferentially in the nucleus.

The ability of ET to enhance nuclear CaTs is independent of the amplitude of the nuclear CaT

The absolute as well as the relative amplitude of the nuclear CaT varied between cells (see Fig. 2). This raised the question regarding whether the effect of ET on nuclear CaTs might be dependent on either the absolute or relative amplitude of the nuclear CaT before ET stimulation. Fig. 5 shows the ET-induced increase in nuclear CaTs from 24 atrial myocytes as a function of the absolute amplitude (expressed as systolic F/F\(_{0}\); Fig. 5A) and the relative amplitude of the nuclear CaT (expressed as the ratio of systolic nuclear to systolic cytoplasmic F/F\(_{0}\); Fig. 5B) before ET exposure. There was no clear correlation between the ET effect on nuclear CaTs and nuclear CaT amplitudes in either case, indicating that the ability of ET to enhance nuclear CaTs was independent of the initial nuclear CaT amplitude.

Increases in cytoplasmic CaTs are not sufficient to enhance nuclear CaTs

The ET-induced enhancement of nuclear CaTs might have been caused by specific nuclear actions of ET or simply be secondary
to increases in cytoplasmic CaTs. To examine this possibility, we varied the amplitude of cytoplasmic CaTs either by variations in extracellular [Ca\(^{2+}\)] or by exposure of the myocytes to the β-adrenoceptor agonist isoproterenol (ISO). To facilitate direct comparison, cells were treated with ISO concentrations (2-10 nM) eliciting increases in cytoplasmic CaTs that were almost identical to the ET-induced increases [ISO: 126±5% (n=10) versus ET: 130±5% (n=24), P=not significant, Fig. 6B]. Fig. 6A illustrates cytoplasmic (black) and nuclear (red) CaTs of two atrial myocytes challenged with either ET (10 nM) or ISO (10 nM). Both ET and ISO elicited a comparable increase in the cytoplasmic CaT. Only ET, however, was able to cause a relatively larger increase in the nuclear CaT. ISO, by contrast, did not elicit an increase in the nuclear CaT, despite the augmentation of the cytoplasmic CaT. Similar results were obtained when the cytoplasmic CaT was elevated by increasing extracellular [Ca\(^{2+}\)] from 2 mM to 4 mM (data not shown). Average results of the three protocols are presented in Fig. 6B,C. The normalised increases in systolic [Ca\(^{2+}\)] in the cytoplasm (black) and the nucleus (red) evoked by ET, ISO and 4 mM extracellular Ca\(^{2+}\) are shown in Fig. 6B, whereas the respective alterations in the nuclear:cytoplasmic ratio are displayed in Fig. 6C. Fig. 5. The ET-induced enhancement of nuclear CaTs depends neither on the basal systolic [Ca\(^{2+}\)] in the nucleus nor on the ratio of nuclear to cytoplasmic peak [Ca\(^{2+}\)]. The ET-induced increase in peak [Ca\(^{2+}\)] in the nucleus as a function of basal systolic [Ca\(^{2+}\)] in the nucleus (A) or the ratio of nuclear to cytoplasmic systolic [Ca\(^{2+}\)] (B) before ET exposure. The data are from 24 atrial myocytes challenged with ET.
Selective increases of nuclear CaTs by low concentrations of ET

Fig. 7A illustrates cytoplasmic (black) and nuclear (red) CaTs of a myocyte challenged with 0.1 nM ET. Before exposure to ET, the amplitudes of cytoplasmic and nuclear CaTs were essentially identical. Following application of ET, cytoplasmic CaTs remained unchanged. Nuclear CaTs, however, clearly rose above baseline levels (dashed red line). Similar observations were made in five additional myocytes. The average data for changes in cytoplasmic and nuclear CaTs elicited by 0.1 nM ET are presented in Fig. 7B. Cytoplasmic CaTs were unaffected by 0.1 nM ET. Ten minutes after exposure to 0.1 nM ET, the systolic [Ca2+] in the cytoplasm was essentially identical (104±4%, P=not significant) to that of the initial control. By contrast, nuclear CaTs were significantly increased by this low concentration of ET. After 10 minutes, the systolic [Ca2+] in the nucleus was increased to 109±4% (P<0.05) of the initial control. Consequently, ET (0.1 nM) significantly increased the nuclear:cytoplasmic ratio by 5±1% (Fig. 7B, right). The results show that, at 0.1 nM, ET increased nuclear CaTs selectively without elevating cytoplasmic CaTs.

The ET-induced increases in cytoplasmic and nuclear CaTs are mediated by ET receptors coupling to PLC-Ins(1,4,5)P3 signalling

To elucidate the signalling pathway underlying the ET-induced enhancement of nuclear CaTs, the following inhibitors were tested: BQ-123 (0.25 μM) – targeting the ET receptor A (EDNRA, hereafter referred to ETα); U-73122 (3 μM) – targeting PLC; and 2-aminoethoxydiphenylborate (2-APB; 3 μM) and xestospongin C (5 μM) – both targeting Ins(1,4,5)P3 receptors. Myocytes were incubated with xestospongin C (>40 minutes) before ET treatment, whereas the other inhibitors were applied acutely 10 minutes before ET exposure. BQ-123 (n=10) and 2-APB (n=16) did not affect basal CaTs both in the cytoplasm (BQ-123: 101±3% of the control; 2-APB: 99±3% of the control) and in the nucleus (BQ-123: 103±3% of the control; 2-APB: 99±3% of the control). U-73122 (n=8), by contrast, reduced basal CaTs to 82±6% of the control in the cytoplasm and to 82±6% of the control in the nucleus (BQ-123: 103±3% of the control; 2-APB: 99±3% of the control). ET (0.1 nM) increased cytoplasmic [Ca2+] to 130±5%, 126±5% and 114±3% of the initial control, respectively. Only ET, however, elicited a relatively larger increase in nuclear [Ca2+] (to 148±5% of the control). By contrast, both ISO and 4 mM extracellular Ca2+ caused increases in nuclear [Ca2+] (to 125±5% and 115±3% of the control, respectively) that were essentially identical to the increases in cytoplasmic [Ca2+]. These subcellular differences in the elevations of cytoplasmic and nuclear CaTs are also reflected in the nuclear:cytoplasmic ratio obtained during the peak of the transient (Fig. 6C). ET increased this ratio by +15±3%. In the presence of ISO or 4 mM extracellular Ca2+, however, it remained unaffected, indicating that there was no additional increase in nuclear [Ca2+] beyond the increase in cytoplasmic [Ca2+] under these conditions. Taken together, these data demonstrate that the enhancement of nuclear CaTs is a specific action of ET. It cannot be mimicked by elevations of cytoplasmic CaTs by either ISO or 4 mM extracellular Ca2+. Thus, elevations in cytoplasmic CaTs are not sufficient to increase nuclear CaTs to a larger extent than cytoplasmic CaTs.

Fig. 7. Low concentrations of ET augment nuclear CaTs in the absence of increases in cytoplasmic CaTs. (A) Original recording of cytoplasmic (black) and nuclear (red) CaTs of an atrial myocyte before and during exposure to 0.1 nM ET. (B) Changes of peak systolic [Ca2+] in the cytoplasm (left) and nucleus (right) induced by 0.1 nM ET. (C) Normalised ratio of nuclear to cytoplasmic [Ca2+] following application of 0.1 nM ET. In B and C, values are normalised to the initial control (=100%). Means±s.e.m. of six atrial myocytes (N.S.=not significant).

Fig. 8. Signalling pathway underlying the ET-induced enhancement of nuclear CaTs. (A) Original recordings of cytoplasmic (black) and nuclear CaTs (red) before and after application of 0.25 μM BQ-123 (left) or 3 μM 2-APB (right) and following additional application of 10 nM ET. (B) Means±s.e.m. of peak cytoplasmic and nuclear [Ca2+] following exposure to ET for 10 minutes in the absence and presence of BQ-123 (n=10), U-73122 (n=7), 2-APB (n=12) and xestospongin C (n=7).
unaffected. Fig. 8B shows average data of the changes in cytoplasmic and nuclear CaTs induced by exposure to 10 nM ET for 10 minutes in the absence and presence of BQ-123, U-73122, 2-APB and xestospongic C. Each inhibitor tested attenuated or abolished the ET-mediated increases in cytoplasmic and the relatively larger increases in nuclear CaTs. In addition, each inhibitor also abolished the ET-induced alterations in the kinetics of cytoplasmic and nuclear CaTs (data not shown). Thus, the intracellular signalling pathway causing the ET-induced increases in cytoplasmic and nuclear CaTs involved activation of ETα receptors that couple to PLC and Ins(1,4,5)P₃.

Increased fractional Ca²⁺ release from SR and perinuclear stores underlies the ET-induced increase in cytoplasmic and nuclear CaTs

The results obtained so far suggested that Ins(1,4,5)P₃-dependent Ca²⁺ release from SR and perinuclear stores contributed to the ET-induced increases in cytoplasmic and nuclear CaTs. To characterise in more detail the role of Ca²⁺ release from these intracellular Ca²⁺ stores for the ET-induced increases in cytoplasmic and nuclear CaTs, Ca²⁺ store contents were estimated by rapid application of 20 mM caffeine, and fractional Ca²⁺ release (FCR) from the stores was calculated as the ratio of the amplitude of electrically evoked CaTs to the amplitude of the caffeine-induced CaTs (see Materials and Methods for further details). Fig. 9A illustrates an original recording of cytoplasmic (black) and nuclear (red) CaTs before (left) and after (right) ET application. Each time, two electrically stimulated CaTs followed by a caffeine-induced CaT are shown first in the absence and then in the presence of ET. ET elicited an increase in the cytoplasmic CaT (+44%) and an even more pronounced increase in the nuclear CaT (+97%). Application of caffeine, however, revealed that neither the SR nor perinuclear Ca²⁺ store content was elevated by ET. Fractional Ca²⁺ release from the SR increased from 0.53 to 0.92 and fractional Ca²⁺ release from perinuclear stores increased from 0.38 to 0.86. Average data from seven myocytes confirm these observations (Fig. 9B). The Ca²⁺ contents of both stores were unchanged, whereas fractional Ca²⁺ release was significantly augmented by ET (SR: from 0.65±0.05 to 0.78±0.07; perinuclear: from 0.52±0.06 to 0.75±0.08; n=7, both P<0.05). The ET-induced increase in FCR (ΔFCR) from perinuclear stores (0.23±0.06) was significantly larger than the increase in FCR from the SR (0.13±0.04; n=7, P<0.05).

For comparison, analogous experiments were conducted with ISO (10 nM, n=7). ISO increased cytoplasmic and nuclear CaTs by the same extent (~50%; data not shown). The increase in cytoplasmic CaTs was associated with a 13±4% elevation of SR Ca²⁺ content (Fig. 9B). Furthermore, ISO increased FCR both from SR and perinuclear regions by the same amount (SR: 0.22±0.07; perinuclear: 0.21±0.06, Fig. 9B). Thus, ISO induced alterations in SR and perinuclear Ca²⁺ handling that were distinctly different from the ET-induced alterations.

Discussion

The physiological and pathophysiological relevance of Ins(1,4,5)P₃ signalling in cardiac myocytes has long been enigmatic. Recently, some elegant studies have led to novel insights into the putative roles of Ins(1,4,5)P₃ in cardiac myocytes. For example, ET-induced Ins(1,4,5)P₃ signalling generates arrhythmogenic alterations in Ca²⁺ signalling in atrial and ventricular myocytes (Bootman et al., 2007; Li et al., 2005; Mackenzie et al., 2002; Proven et al., 2006; Zima and Blatter, 2004). In addition, most recent work has implicated Ca²⁺ release through Ins(1,4,5)P₃ receptors from the nuclear envelope into the nucleoplasm in Ca²⁺-dependent activation of transcription and hypertrophy in adult cardiac myocytes, a concept referred to as excitation-transcription coupling (Bare et al., 2005; Wu et al., 2006). The present study, however, is the first to demonstrate directly active modulation of nuclear CaTs during normal excitation-contraction coupling in cardiac myocytes by Ins(1,4,5)P₃-dependent Ca²⁺ release from perinuclear stores. Our major new findings are: (1) ET increases cytoplasmic and nuclear CaTs in beating adult atrial myocytes through activation of ETα receptors coupling to PLC-Ins(1,4,5)P₃ signalling. (2) The increase in nuclear CaTs is larger than the increase in cytoplasmic CaTs. At low concentrations, ET causes selective increases in nuclear CaTs. (3) The enhancement of nuclear CaTs is specific for ET-induced Ins(1,4,5)P₃ signalling and elicited by increased fractional Ca²⁺ release from perinuclear stores presumably mediated by Ca²⁺ release through Ins(1,4,5)P₃ receptors in the nuclear envelope. These results thus provide a sound framework for the cellular and subcellular mechanisms underlying a compartmentalised increase in nuclear [Ca²⁺] in cardiac myocytes evoked by ET.

Mechanisms underlying the ET-induced increases in cytoplasmic and nuclear CaTs

According to our results, ET elicited increases in both cytoplasmic and nuclear CaTs through activation of ETα receptor coupling to PLC-Ins(1,4,5)P₃ signalling. Interestingly, inhibition of the PLC-Ins(1,4,5)P₃ pathway completely suppressed the ET effects on cytoplasmic and nuclear CaTs. This is somewhat surprising, given
that ET activates various signalling cascades (Sugden, 2003), and suggests that PLC-Ins(1,4,5)P₃ signalling is the major pathway utilised by atrial myocytes to elevate cytoplasmic and nuclear CaTs. Our findings are in line with a recent study in which an ET-induced increase in cytoplasmic and nuclear [Ins(1,4,5)P₃] in cardiac myocytes was demonstrated directly by means of novel FRET-based Ins(1,4,5)P₃ biosensors (Remus et al., 2006). Furthermore, previous studies have shown the expression of Ins(1,4,5)P₃ receptors in the cytoplasm as well as in the nuclear envelope of cardiac myocytes (Bare et al., 2005; Lipp et al., 2000; Mackenzie et al., 2002; Yamada et al., 2001). Thus, it is likely that ET increased the [Ins(1,4,5)P₃] in the cytoplasm and nucleus and augmented CaTs in the two compartments through Ca²⁺ release from Ins(1,4,5)P₃ receptors located in the SR and the nuclear envelope, respectively. Ins(1,4,5)P₃-dependent Ca²⁺ release from perinuclear stores into the nucleoplasm has been observed before in rat neonatal myocytes (Ibarra et al., 2004; Luo et al., 2007), in cat atrial myocytes (Zima et al., 2007) and in nuclei isolated from rat skeletal myotubes (Cardenas et al., 2005) or rat heart (Zima et al., 2007). The most elegant and direct evidence for independent Ins(1,4,5)P₃-mediated nuclear Ca²⁺ signalling comes from a recent study on permeabilised atrial myocytes and isolated cardiac nuclei (Zima et al., 2007). In that study, it was shown that Ins(1,4,5)P₃ and the potent Ins(1,4,5)P₃ receptor agonist adenophostin were able to release Ca²⁺ from the nuclear envelope directly into the nucleoplasm, suggesting that nuclear Ins(1,4,5)P₃ receptors are located predominantly on the inner membrane of the nuclear envelope (Zima et al., 2007). A further study provided evidence for the involvement of the ET-PLC-Ins(1,4,5)P₃ pathway in localised nuclear Ca²⁺ signalling in cultured rabbit and mouse ventricular myocytes (Wu et al., 2006). An Ins(1,4,5)P₃-mediated increase in nuclear [Ca²⁺], however, could not be detected (Wu et al., 2006). Thus, in conjunction with most recent work on rat atrial myocytes [see fig. 3 in Bootman et al. (Bootman et al., 2007)], the present study is the first to demonstrate directly an Ins(1,4,5)P₃-mediated [Ca²⁺] increase in the nucleus of adult beating cardiac myocytes. The results suggest that localised Ins(1,4,5)P₃-mediated increases in nuclear [Ca²⁺] might occur in intact cardiac myocytes during normal excitation-contraction coupling.

The ET-induced alterations in the kinetics of cytoplasmic and nuclear CaTs are also consistent with Ins(1,4,5)P₃-dependent Ca²⁺ release being the underlying mechanism. As shown by Zima and Blatter, the duration of Ins(1,4,5)P₃-mediated Ca²⁺ release events in atrial myocytes is approximately three times longer than Ry receptor-2-mediated Ca²⁺ release events (Zima and Blatter, 2004). This might explain the increase in the time-to-peak of the cytoplasmic CaT following ET exposure. Because of the much slower kinetics of nuclear CaTs, such an effect might be obscured and not detectable in the nucleus. Moreover, Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is coupled to Ins(1,4,5)P₃ receptors (Bare et al., 2005) and is responsible for the frequency-dependent acceleration of CaT decay (DeSantiago et al., 2002), suggesting that the ET-induced acceleration of cytoplasmic and nuclear CaT decay was mediated by Ins(1,4,5)P₃-dependent stimulation of CaMKII activity.

ET increases nuclear CaTs selectively Low concentrations of ET elicited selective increases in nuclear CaTs, meaning that increases in nuclear CaTs occurred in the absence of increases in cytoplasmic CaTs. This finding has two important implications: first, it demonstrates that ET can elicit a compartmentalised increase in nuclear [Ca²⁺]; and second, ET might alter nuclear Ca²⁺ signalling and, therefore, presumably also Ca²⁺-dependent gene expression without causing relevant changes in cytoplasmic Ca²⁺ signalling. The question remains how ET-induced Ins(1,4,5)P₃ signalling can alter the nuclear [Ca²⁺] without altering cytoplasmic Ca²⁺ signalling. One possible explanation for this apparent paradox is that nuclear Ins(1,4,5)P₃ receptors might exhibit either an increased density or an increased sensitivity to Ins(1,4,5)P₃. This means that, at the same concentration of Ins(1,4,5)P₃, nuclear Ins(1,4,5)P₃ receptors might be activated, whereas cytoplasmic Ins(1,4,5)P₃ receptors are not. As Ins(1,4,5)P₃ receptors are modulated by a variety of factors (Bultynck et al., 2003), differences in Ins(1,4,5)P₃ sensitivity between cytoplasmic and nuclear Ins(1,4,5)P₃ receptors could be caused by subcellular differences in the concentrations or activities of these modulatory factors. Alternatively, the local [Ins(1,4,5)P₃] near nuclear Ins(1,4,5)P₃ receptors might be higher than in the vicinity of cytoplasmic Ins(1,4,5)P₃ receptors. A preferential increase of perinuclear [Ins(1,4,5)P₃] could be caused by activation of nuclear PLC through nuclear ET receptors (Bkaily et al., 2003; Boivin et al., 2003). This explanation, however, appears less likely for two reasons: first, it is unclear how extracellular ET could activate intracellular ET receptors and, second, ET-induced elevation of the [Ins(1,4,5)P₃] in cardiomycytes occurs first in the cytoplasm and with a delay in the nucleus (Remus et al., 2006), suggesting that Ins(1,4,5)P₃ is generated in the cytoplasm before diffusing into the nucleus.

The nuclear CaT consists of two components Another important finding of the current study is that the nuclear CaT exhibits distinct kinetics and that it consists of two components, a passive and an active component. The passive component is probably mediated by cytoplasmic Ca²⁺ diffusing through nuclear pores to increase nuclear [Ca²⁺]. Several lines of evidence support this notion. First, nuclear pore complexes are readily permeable to Ca²⁺ ions. Second, the nuclear CaT lags behind the cytoplasmic CaT. This delay is most likely caused by slow diffusion of cytoplasmic Ca²⁺ through the nuclear pore complexes. In addition, the effective diffusion of Ca²⁺ within the nucleus is significantly slower than in the cytoplasm (Soeller et al., 2003), and this could contribute to the slower kinetics of the nuclear CaT. Third, in the absence of Ins(1,4,5)P₃ production, any increases in cytoplasmic CaTs are followed by increases in nuclear CaTs of identical magnitude. During ET treatment, however, an additional active component comes into play. This active component is reflected by the ET-induced extra increase in nuclear CaTs. It is inhibited by U-73122, 2-APB and xestospongin C and, therefore, is mediated by PLC-Ins(1,4,5)P₃ signalling. Determination of the Ca²⁺ load of perinuclear stores revealed that, during ET exposure, the Ca²⁺ store content was unchanged. Fractional release from perinuclear stores, however, was increased greatly by ET. Taken together, these data imply that ET increases nuclear CaTs by increasing fractional Ca²⁺ release from the nuclear envelope through recruitment of Ins(1,4,5)P₃ receptor-mediated Ca²⁺ release.

Physiological and pathophysiological implications The ET receptor density and plasma levels are elevated in cardiovascular diseases, including human heart failure (Pieske et al., 1999). Nuclear Ca²⁺ is a crucial factor for the regulation of gene expression. The ET-induced increases in nuclear [Ca²⁺]
characterised in the present study, therefore, are expected to alter cardiac gene expression, consistent with the fact that ET exerts hypertrophic actions in the heart. There is clear experimental evidence for the functional relevance of Ins(1,4,5)P3-dependent Ca2⁺ release for gene expression in striated muscle. In isolated nuclei of skeletal myotubes, Ins(1,4,5)P3-dependent Ca2⁺ release into the nucleoplasm causes phosphorylation of the transcription factor cAMP response element binding protein (Cardenas et al., 2005). Moreover, in cardiac myocytes, Ins(1,4,5)P3 receptors and CaMKII colocalise in the nucleus (Bare et al., 2005), and Ins(1,4,5)P3-dependent Ca2⁺ release into the nucleoplasm has been implicated in CaMKII-mediated phosphorylation of histone deacetylases and de-repression of gene expression (Wu et al., 2006). Remarkably, our finding that low concentrations of ET cause selective increases in nuclear CaTs emphasises that the nuclear [Ca2⁺] cannot be regulated independently from the cytoplasmic [Ca2⁺] in beating cardiac myocytes during normal excitation-contraction coupling. Furthermore, it suggests that the hypertrophic effects of ET might occur in the low concentration range found in cardiac tissue and precede the onset of the inotropic and arrhythmogenic effects of ET caused by alterations in cytoplasmic [Ca2⁺].

**Materials and Methods**

**Atrial myocytes**

Atrial myocytes from adult rabbit hearts were isolated by a standard collagenase-based Langendorff perfusion protocol as described previously for ventricular myocytes (Schillinger et al., 2000). Isolated myocytes were plated on glass-bottomed culture dishes and allowed to attach to the glass bottom for >45 minutes. The cell isolation procedure was in accordance with national and international animal care guidelines.

**Confocal Ca2⁺ imaging**

Fast, two-dimensional (2D) confocal [Ca2⁺] imaging was performed as described previously (Kockskamper et al., 2001) using a confocal imaging system (Visitech International, UK) consisting of an inerted microscope (Nikon) equipped with a ×40 oil-immersion objective lens (N.A. 1.3), a Nikon dual disc-based confocal unit and an ICCD camera with a temporal resolution of 120 Hz. Myocytes were loaded with Fluo-4 by a 20-25 minute incubation in Tyrode’s solution containing 8 μM Fluo-4/AM. Fifteen minutes were allowed for de-esterification. Myocytes were placed on the stage of the microscope, superfused with Tyrode’s solution by means of a gravity-driven superfusion pipette and field-stimulated at 0.7 Hz. Fluo-4 was excited by 488 nm light from an argon-ion laser. The fluorescence emission was collected at wavelengths >505 nm. Changes of the [Ca2⁺] were expressed as changes of background-corrected normalised fluorescence, F/F0, where F denotes Fluo-4 fluorescence and F0 resting fluorescence at the beginning of an experiment.

Line-scan imaging of atrial CaTs was performed using a confocal microscope (Zeiss LSM 5 Pascal) equipped with a ×40 oil-immersion objective lens (N.A. 1.3). A zoom factor of six was used in all recordings, yielding a spatial resolution of 0.07 μm. The scan time was 0.77 ms/scan per line. Myocytes were treated as described above for 2D Ca2⁺ imaging (20-25 minutes loading with 8 μM Fluo-4/AM; 0.7 Hz stimulation). Fluo-4 was excited by the 488 nm line of an argon-ion laser and fluorescence was collected at >515 nm.

**Staining of the nucleus**

The nucleus was stained by the nucleic acid dye Syto-16 (Molecular Probes) and NaOH. Endothelin-1 (ET), BQ-123 (ETa antagonist), 1-[6-((17β-3-methoxyestra-1,3,5(10)atrien-17β)-amino]hexyl]-1H-pyrrrole-2,5-dione (U-73122; PLC inhibitor), 2-aminoethoxy-diphenylborate (2-APB; Ins(1,4,5)P3 receptor blocker), xestospongin C (Ins(1,4,5)P3 receptor blocker), caffeine and isoproterenol (ISO) were from Calbiochem or Sigma.

**Statistics**

The data are presented as mean ± s.e.m. Differences between data sets were evaluated by ANOVA or Student’s t-test and considered significant when P<0.05.

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**References**


