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Chelerythrine and genistein inhibit the endothelin-1-induced increase in myofilament Ca^{2+} sensitivity in rabbit ventricular myocytes

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Abstract

We performed experiments to elucidate the cellular mechanism for the biphasic inotropic response to endothelin-1 of single rabbit ventricular myocytes loaded with a fluorescent dye, acetoxymethylester of indo-1. Endothelin-1 at 10 nM elicited a biphasic inotropic effect: a transient decrease in cell shortening and Ca^{2+} transients followed by an increase in cell shortening without significant elevation of peak Ca^{2+} transients. The selective endothelin ET_A receptor antagonist FR139317 (2(*R*)-[2(*R*)-[2(*S*)-[(1-hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)propionyl]amino-3-(2-pyridyl)propionic acid) at 1 μM abolished the biphasic effect of endothelin-1 on cell shortening and Ca^{2+} transients. The selective protein kinase C inhibitor chelerythrine at 1 μM and the tyrosine kinase inhibitor genistein at 5 μM inhibited the endothelin-1-induced increase in cell shortening without significantly affecting Ca^{2+} transients and the transient decrease in cell shortening and Ca^{2+} transients. The present results indicate that both protein kinase C and tyrosine kinase may contribute to the increase in myofilament Ca^{2+} sensitivity induced by endothelin-1, whereas the decrease in Ca^{2+} transients induced by endothelin-1 may be mediated by a signalling pathway different from that involved in the increase in cardiac contractility in rabbit ventricular myocytes.

Keywords: Endothelin-1; Cell shortening; Ca^{2+} transient; Chelerythrine; Genistein; Ventricular myocyte, rabbit

1. Introduction

Endothelin-1 is a 21-amino acid vasoconstrictor peptide originally isolated from culture medium of porcine aortic endothelial cells (Yanagisawa et al., 1988). It is released by endothelial, vascular smooth muscle and cardiac cells under various pathophysiological conditions such as congestive heart failure and cardiac ischemia, and plays an important role in the regulation of cardiovascular function in various cardiovascular disorders (Wei et al., 1994; Sakai et al., 1996).

In cardiac muscle of most mammalian species, including rabbits, rats, guinea pigs, ferrets and humans, endothelin-1 has a positive inotropic effect of different magnitude (Moravec et al., 1989; Shah et al., 1989; Takanashi and Endoh, 1992). The signal transduction pathway subsequent to endothelin receptor activation is considered to be coupled to several subcellular mechanisms, including facilitation of various types of ion channels and ion transport systems, that result in an increase in intracellular Ca^{2+} mobilization and/or an increase in the sensitivity of myofilament to Ca^{2+} ions in the final step of cardiac contraction (Krämer et al., 1991; Yang et al., 1999; Wang et al., 2000). The positive inotropic effect of endothelin-1 is postulated to be strongly related to the receptor-mediated activation of phospholipase Cβ and the resultant acceleration of hydrolysis of phosphoinositide, which leads to the production of inositol 1,4,5-trisphosphate and diacylglycerol (Berridge, 1993; Hansen et al., 1995; Meyer et al., 1996).

The cardiovascular regulation induced by endothelin-1 and its signal transduction pathway show a wide range of species-dependent variation. In rabbit ventricular myocardium, endothelin-1 elicits a transient negative inotropic effect followed by a pronounced and sustained positive inotropic effect, whereas it does not have any inotropic effect on dog right ventricular trabeculae (Takanashi and Endoh, 1991; 1992). Although many studies have been carried out to investigate the mechanism underlying the positive inotropic effect of endothelin-1,

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subcellular mechanisms are still unclear and controversial. In addition, little is known about the mechanism for the negative inotropic effect of endothelin-1.

The present study was undertaken to elucidate the mechanism involved in the regulation of contraction induced by endothelin-1 in mammalian ventricular myocardium. For this purpose, we examined the influence of the selective endothelin ET_A receptor antagonist FR139317, the selective protein kinase C inhibitor chelerythrine and the tyrosine kinase inhibitor genistein on the endothelin-1-induced modulation of cell shortening and Ca^{2+} transients in adult rabbit ventricular myocytes loaded with a fluorescent dye, acetoxymethylester of indo-1 (indo-1/AM).

2. Methods

2.1. Isolation of rabbit ventricular myocytes

Rabbit ventricular cardiomyocytes were isolated by means of a procedure that has been described previously (Fujita and Endoh, 1996). Briefly, adult male Japanese White rabbits (1.8–2.2 kg) were anesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.v.). The heart was excised and perfused by the Langendorff method with Tyrode solution that contained HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulphonic acid]) for about 1 min at 37 °C to wash blood out of the heart. HEPES–Tyrode solution contained (in mM) NaCl, 136.5; KCl, 5.4; $MgCl_2$, 0.53; $CaCl_2$, 1.8; NaH_2PO_4, 0.33; glucose, 5.0; HEPES, 5.0 (pH 7.4) and was continuously bubbled with 100% O_2. Then, the heart was perfused with nominally Ca^{2+}-free HEPES–Tyrode solution for 5 min, followed by perfusion with a solution containing 0.6 mg ml^{-1} collagenase (type II, Worthington Biochemical, Freehold, NJ, USA) and 0.1 mg ml^{-1} protease (type XIV; Sigma, St. Louis, MO, USA) for 16–20 min. Finally, the heart was washed with HEPES–Tyrode solution containing 0.2 mM $CaCl_2$ and ventricles were cut into small pieces. The cell suspension was rinsed several times with a gradual increase in the Ca^{2+} concentration up to 1.8 mM. The myocytes displaying rod-shaped, well-defined striations and no spontaneous contractions were used for the experiments.

2.2. Loading of myocytes with indo-1/AM

Myocytes were loaded with indo-1/AM by incubating them in 5 μM indo-1 solution for about 3 min at room temperature. The loading solution consisted of 10 μl of 1 μM indo-1/AM, 40 μl dimethylsulfoxide, 90 μl fetal bovine serum, 10 μl of 20% (w w^{-1} in dimethylsulfoxide) pluronic F-127 and 1 ml HEPES–Tyrode solution. The loading solution described above was sonicated for 3 min and 1 ml of cell suspension was added to it. After loading, the cells were centrifuged at about $5 \times g$ for 1 min. The supernatant was discarded and the pellet was resuspended in HEPES–Tyrode solution and stored at room temperature (~24 °C) until they were used for the experiments.

2.3. Measurements of cell shortening and Ca^{2+} transients

Myocytes were dispersed in a chamber that contained Krebs–Henseleit bicarbonate buffer and placed on the stage of an inverted microscope (Diaphot TMD 300; Nikon, Tokyo, Japan). They were allowed to settle down to attach loosely to the bottom of the chamber for 10 min. Then perfusion was started with Krebs–Henseleit bicarbonate buffer at a rate of 1 ml min^{-1} at room temperature (~24 °C) and the cells were stimulated electrically by square-wave pulses with voltage about 30–40% above the threshold at a frequency of 0.5 Hz. The Krebs–Henseleit bicarbonate buffer contained (in mM) NaCl, 116.4; KCl, 5.4; $MgSO_4$, 0.8; $CaCl_2$, 1.8; NaH_2PO_4, 1.0; $NaHCO_3$, 23.8 and glucose, 5.0 (pH 7.4) and had been equilibrated with 95% O_2 and 5% CO_2.

Fluorescence of indo-1 was excited with light from a xenon lamp (150 W) at a wavelength of 355 nm, reflected by a 380-nm long-pass dichroic mirror, and detected by a fluorescence spectrophotometer (CAM-230; Japan Spectroscopic, Tokyo, Japan). Excitation light was applied to myocytes intermittently through a neutral density filter to minimize the photobleaching of indo-1. The emitted fluorescence was collected by an objective lens (CF Fluor DL40, Nikon) and then separated by a 580-nm long-pass dichroic mirror (Omega Optical, Brattleboro, VT, USA). The fluorescence light was subsequently split with a 425-nm dichroic mirror to permit simultaneous measurements of light at both 405 and 500 nm wavelengths through band-pass filters.

The emission field was restricted to a single cell with the aid of an adjustable window. The fluorescence ratio (405/500 nm) was used as an indicator of $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). Cells were simultaneously illuminated with red light (wavelength above 620 nm) through the normal bright-field illumination optics of the microscope and the bright-field image of a myocyte was collected by means of an objective lens and first separated by a 580-nm long-pass dichroic mirror. A bright-field cell image was projected onto a photodiode array of the edge detector (C6294-01, Hamamatsu Photonics K.K., Hamamatsu, Japan) with 5 ms temporal resolution and the cell length was monitored simultaneously with indo-1 fluorescence.

2.4. Experimental protocols

After an equilibration period of 40 min, myocytes were perfused with the solution containing the agent examined. When the response of myocytes to the agent applied reached a stable level, indo-1 fluorescence was measured

and then the perfusion was switched to the solution that contained an additional agent. The cell length was continuously measured throughout the experiments, while the fluorescence of indo-1 was monitored intermittently to reduce quenching. The cell length and Ca^{2+} transients were simultaneously recorded at baseline and in the presence of the agent examined when the response reached a maximal or steady level. Endothelin-1 was applied only once to each preparation since the effect of endothelin-1 was not reversible by washout. Selective inhibitors of the endothelin ET_A receptor, protein kinase C and tyrosine kinase, were allowed to act for 10 min prior to and during the administration of endothelin-1.

2.5. Data recording and analysis

Data on cell length and fluorescence of indo-1 were stored and displayed by means of a computer (Power Macintosh 8100/100AV, Apple Computer, Cupertino, CA, USA) equipped with an A/D converter (MP-100A, BIOPAC Systems, Santa Barbara, CA, USA) at 200 Hz and analyzed after low-pass filtering (cutoff frequency of 20 Hz). The data used for statistical analysis were obtained by signal-averaging of five successive tracings of cell shortening and Ca^{2+} transients. In analysis of data, systolic cell shortening and indo-1 fluorescence ratio prior to first application of the agent examined in individual experiments were regarded as basal values in each myocyte and were assigned a value of 100%, and all the data are expressed as a percentage of the basal values.

2.6. Drugs

The drugs and reagents used were endothelin-1 (Peptide Institute, Osaka; Japan), chelerythrine chloride, genistein, isoprenaline hydrochloride and protease type XIV (Sigma); indo-1/AM (Dojindo Chemical, Kumamoto, Japan); collagenase type II (Worthington Biochemical); FR139317 (2(*R*)-[2(*R*)-[2(*S*)-[(1-hexahydro-1*H*-azepinyl)]carbonyl]-amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indolyl)propionyl]amino-3-(2-pyridyl)propionic acid; Fujisawa Pharmaceutical, Tsukuba, Japan). Other reagents used were of the highest grade in purity that were commercially available.

2.7. Statistical analysis

Data are expressed as means ± S.E.M. For analysis of multiple measurements obtained from a single preparation, we used one-way analysis of variance (ANOVA) for repeated measures with Dunnett's test. Significance of differences between two mean values was estimated by Student's *t*-test. Differences were considered to be significant when the P value was less than 0.05.

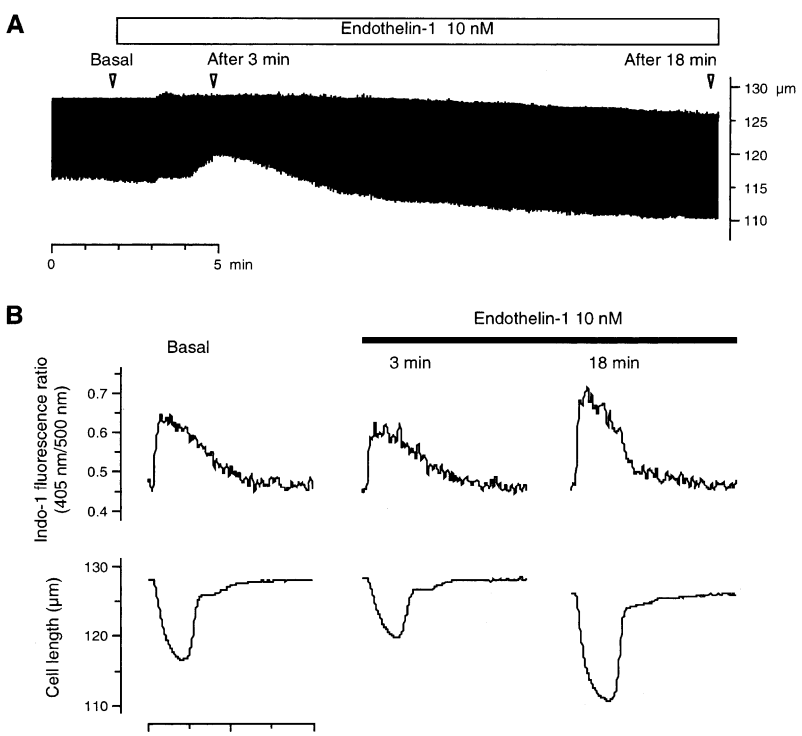

Fig. 1. Effects of endothelin-1 on cell shortening and indo-1 ratio in rabbit ventricular myocytes loaded with indo-1/AM. **A**: actual tracings of the biphasic effect of endothelin-1 at 10 nM on cell shortening recorded continuously. **B**: actual tracings of the effects of endothelin-1 at 3 and 18 min after the administration of 10 nM on indo-1 fluorescence ratio (upper panel) and cell length (lower panel) recorded at open triangles in **A**. Basal: baseline Ca^{2+} transient and cell shortening prior to administration of endothelin-1.

3. Results

3.1. Effects of endothelin-1 at 10 nM on cell shortening and indo-1

We first examined the characteristics of the effects of endothelin-1 at 10 nM on the Ca^{2+} transients and cell shortening. Endothelin-1 at 10 nM induced a biphasic effect on cell shortening in a rabbit ventricular myocyte: an initial transient decrease in cell shortening was followed by a long-lasting increase. The maximal decrease and the sustained increase in cell shortening were induced 3–5 and 15 min after the administration of endothelin-1, respectively (Fig. 1A). Actual tracings and summarized data on the effects of endothelin-1 are shown in Figs. 1B and 2, respectively. Endothelin-1 at 10 nM induced a significant decrease in cell shortening ($71.6 \pm 4.5\%$ of the baseline level, $n = 6$), which was associated with a significant decrease in the indo-1 ratio ($85.2 \pm 3.3\%$ of the baseline level, $n = 6$). The sustained increase in cell shortening ($132.2 \pm 9.2\%$, $n = 6$) was not associated with a significant increase in the indo-1 ratio ($121.5 \pm 14.7\%$, $n = 6$).

3.2. Influence of the endothelin ET_A receptor antagonist FR139317 on the effects of endothelin-1

The endothelin ET_A receptor antagonist FR139317 at 1 μM was allowed to act 10 min prior to and during application of endothelin-1. FR139317 did not affect the baseline level of cell shortening or the indo-1 ratio, but it abolished the biphasic action of endothelin-1 at 10 nM on cell shortening and indo-1 ratio (Fig. 3).

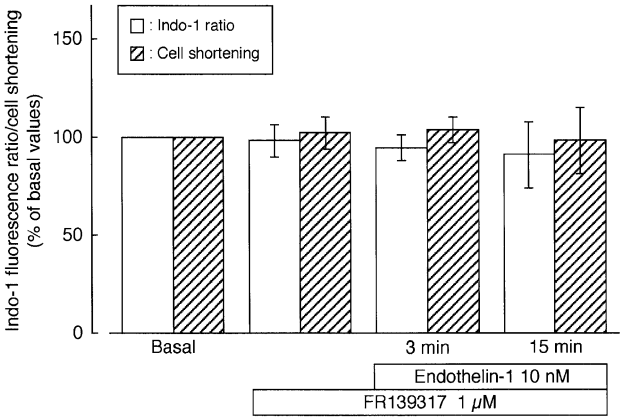

Fig. 3. Influence of FR139317 on the effects of endothelin-1 at 10 nM on indo-1 fluorescence ratio and cell shortening in rabbit single ventricular myocytes loaded with indo-1/AM ($n = 5$, each). FR139317 was applied 10 min prior to and during administration of endothelin-1.

3.3. Influence of the protein kinase C inhibitor chelerythrine on the effects of endothelin-1 on cell shortening and indo-1 ratio

The role of protein kinase C in the contractile regulation induced by endothelin-1 was studied by means of the protein kinase C inhibitor chelerythrine. Chelerythrine at 1 μM affected neither the baseline cell shortening nor the indo-1 ratio. In the presence of 1 μM chelerythrine, endothelin-1 induced a significant decrease in cell shortening and indo-1 ratio, whereas the increase in cell shortening was almost completely inhibited (Fig. 4). The effects of isoprenaline were not affected by chelerythrine 1 μM ($n = 4$, data not shown).

3.4. Influence of the tyrosine kinase inhibitor genistein on the effects of endothelin-1 on cell shortening and indo-1 ratio

Fig. 5 shows the effect of 10 nM endothelin-1 in the presence of the tyrosine kinase inhibitor genistein. Genistein at 5 μM had no effect on baseline cell shortening or on the indo-1 ratio. In the presence of 5 μM genistein, endothelin-1 induced a significant decrease in cell shorten-

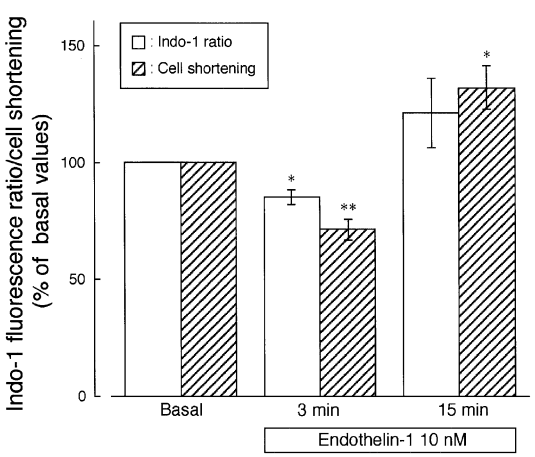

Fig. 2. Summarized data for the effects of endothelin-1 at 10 nM on indo-1 fluorescence ratio and cell length in rabbit single ventricular myocytes loaded with indo-1/AM ($n = 6$). Basal: baseline Ca^{2+} transient and cell shortening prior to administration of endothelin-1. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. respective baseline values.

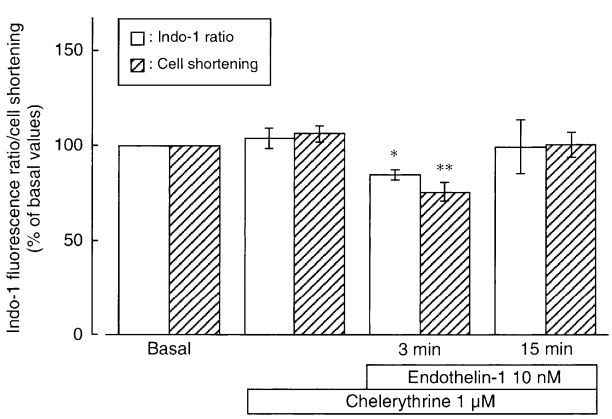

Fig. 4. Influence of chelerythrine on the effects of endothelin-1 at 10 nM on indo-1 fluorescence ratio and cell shortening in rabbit single ventricular myocytes loaded with indo-1/AM ($n = 4$, each). Chelerythrine was applied 10 min prior to and during administration of endothelin-1. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. respective baseline values.

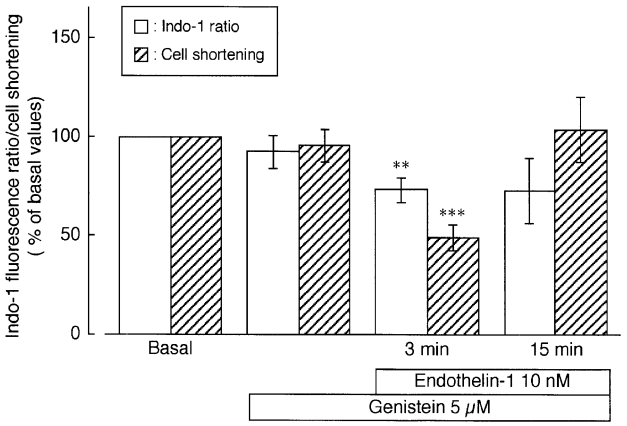

Fig. 5. Influence of genistein on the effects of endothelin-1 at 10 nM on indo-1 fluorescence ratio and cell shortening in rabbit single ventricular myocytes loaded with indo-1/AM ($n = 5$, each). Genistein was applied 10 min prior to and during administration of endothelin-1. $^{**}P < 0.01$; $^{***}P < 0.001$ vs. respective baseline values.

ing and indo-1 ratio, whereas the increase in cell shortening induced by endothelin-1 was markedly decreased. The effects of isoprenaline were not influenced by genistein 5 μM ($n = 3$, data not shown).

4. Discussion

Endothelin-1 at 10 nM elicited a sustained increase subsequent to a transient decrease in cell shortening, both of which were mediated by endothelin ET_A receptors that were susceptible to the selective endothelin ET_A antagonist FR139317 in rabbit ventricular myocytes. We carried out recently a detailed analysis of the concentration and time dependence of the effects of endothelin-1 in rabbit ventricular myocytes, and found that endothelin-1 elicited a pronounced inhibitory effect at higher concentrations (Talukder et al., in press). It should be noted that the concentration of endothelin-1 used in the current study is very high since the maximum response is achieved by endothelin-1 at 1 nM in rabbit ventricular myocytes (Yang et al., 1999; Talukder et al., in press). We selected this concentration to elucidate whether the same or a different signalling process mediates the dual effect of endothelin-1. We hypothesized that if the excessive activation of protein kinase C induced by a high concentration of endothelin-1 is responsible for the dual effect, the inhibition of protein kinase C may enhance the endothelin-1-induced increase in cell shortening. The current study, however, implies that the dual effect of endothelin-1 is mediated by different signalling pathways.

4.1. Increase in cell shortening induced by endothelin-1

The increase in cell shortening induced by endothelin-1 at 10 nM was associated with an alteration of Ca^{2+} transients that was not significantly different from the basal level (Fig. 2), an indication that the effect of endothelin-1 is partially due to an increase in myofilament Ca^{2+} sensitivity. Since chelerythrine inhibited the increase in cell shortening induced by endothelin-1 (Fig. 4), activation of protein kinase C may play a crucial role in the effect of endothelin-1. In rabbit ventricular myocytes, we have shown that the selective inhibition of the Na^+/H^+ exchanger by HOE642 (4-isopropyl-3-methyl-sulphonyl-benzoyl guanidine methanesulphonate) and KB-R9032 (*N*-(4-isopropyl-2,2-dimethyl-3-oxo-3,4-dihydro-2*H*-benzo-[1,4]oxazine-6-carbonyl) guanidine methanesulphonate) or of the Na^+/Ca^{2+} exchanger by KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulphonate), respectively, is able to suppress selectively the endothelin-1-induced increase in cell shortening and Ca^{2+} transients (Yang et al., 1999; Wang et al., 2000). These findings together imply that the signalling pathway activated by endothelin-1 that leads to activation of protein kinase C and to activation of the Na^+/H^+ exchanger may be important for the increase in contractility induced by endothelin-1 in rabbit ventricular myocytes. The resultant intracellular alkalinization may increase myofilament Ca^{2+} sensitivity (Krämer et al., 1991), and a small increase in Ca^{2+} mobilization (though the increase did not reach a significant level in the current study) may be elicited through the Na^+/Ca^{2+} exchanger, which elevates $[Ca^{2+}]_i$ in exchange for $[Na^+]_i$ resulting from activation of Na^+/H^+ exchanger (Yang et al., 1999).

A novel finding of the current study is that the increase in cell shortening induced by endothelin-1 is also susceptible to the tyrosine kinase inhibitor genistein (Fig. 5). Endothelin-1 stimulates the hydrolysis of phosphoinositides in rabbit ventricular myocardium (Takanashi and Endoh, 1992). While it has recently been shown that the angiotensin II-induced generation of inositol phosphate is mediated through the tyrosine kinase pathway in cardiomyocytes (Goutsouliak and Rabkin, 1997), it has not been shown that such a pathway is also shared by the process triggered by endothelin receptor activation. The signalling process through which tyrosine kinase contributes to the increase in cell shortening induced by endothelin-1, therefore, awaits further study.

4.2. Decrease in cell shortening induced by endothelin-1

Endothelin-1 at 10 nM induced a transient decrease in cell shortening in rabbit ventricular myocytes. The endothelin-1-induced decrease in contractility is considered to be elicited more prominently by endothelin-1 at higher concentrations since an increase in contractility predominates at lower concentrations: endothelin-1 elicited a pronounced concentration-dependent increase in contractility over the concentration range 0.3 pM–1 nM in rabbit ventricular myocytes (Fujita and Endoh, 1996; Yang et al.,

1999). The transient negative inotropic effect of endothelin-1 was observed also in isolated rabbit papillary muscle (Takanashi and Endoh, 1992). The decrease in cell shortening was associated with a definite decrease in the amplitude of Ca^{2+} transients (Fig. 2). The findings for chelerythrine and genistein indicate that neither activation of protein kinase C nor activation of tyrosine kinase is involved in the induction of the decrease in contractility induced by endothelin-1. The endothelin-1-induced decrease in cell shortening may be partly due to the inhibitory effect of endothelin-1 on L-type Ca^{2+} currents in rabbit ventricular myocytes (Watanabe and Endoh, 1999). The inhibitory action of endothelin-1 on L-type Ca^{2+} currents is more pronounced in the presence of β-adrenoceptor stimulation, which is partly mediated by G_i proteins that are susceptible to pertussis toxin (Watanabe and Endoh, 1999). The signalling process that is responsible for the inhibitory effect of endothelin-1 on the baseline level of L-type Ca^{2+} currents or cell shortening is not clear at the moment and is difficult to analyze because of the relatively small alterations in the parameters measured.

In conclusion, different mechanisms, which are mediated by endothelin receptors in rabbit ventricular myocytes, are responsible for the biphasic inotropic effect of endothelin-1. The activation of protein kinase C and tyrosine kinase may partially contribute to the increase in contractility induced by endothelin-1. The signalling mechanism underlying the inhibitory effect of endothelin-1 is unrelated to the process involved in the endothelin-1-induced increase in contractility.

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