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ENDOTHELIN-SENSITIVE INTRACELLULAR Ca^{2+} STORE OVERLAPS WITH CAFFEINE-SENSITIVE ONE IN RAT AORTIC SMOOTH MUSCLE CELLS IN PRIMARY CULTURE

Hisashi Kai, Hideo Kanaide and Motoomi Nakamura

Research Institute of Angiocardiology, Division of Molecular Cardiology and Cardiovascular Clinic, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

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SUMMARY: We made use of quin2 microfluorometry to determine the effects of endothelin (ET) on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in rat aortic smooth muscle cells in primary culture. In Ca^{2+}-containing medium, ET induced a rapid and sustained elevation of $[Ca^{2+}]_i$. In the latter component, in particular, the elevation of $[Ca^{2+}]_i$ was inhibited by diltiazem. In Ca^{2+}-free medium, ET induced a rapid and transient $[Ca^{2+}]_i$ elevation, which was not inhibited by diltiazem. When the caffeine-sensitive intracellular Ca^{2+} store was practically depleted by repeated treatment with caffeine in Ca^{2+}-free media, ET did not elevate $[Ca^{2+}]_i$. Thus, it was suggested that ET induces $[Ca^{2+}]_i$ elevation not only by extracellular Ca^{2+}-dependent mechanisms but also by releasing Ca^{2+} from the intracellular store, and that the ET-sensitive Ca^{2+} store may overlap with the caffeine-sensitive one, in cultured vascular smooth muscle cells.

The potent vasoconstrictor peptide endothelin (ET) seems to function as an endogenous modulator of the voltage-dependent Ca^{2+} channel (1). Using rat aortic vascular smooth muscle cells in primary culture (VSMCs), we found that ET induces an increase in the rate of ^{45}Ca efflux, even in the absence of extracellular Ca^{2+}, and we suggested that ET functions in a manner so as to mobilize Ca^{2+} from the intracellular store (2). In the present study, we made use of quin2 microfluorometry and VSMCs to investigate the effects of ET on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and on the release of Ca^{2+} from the intracellular store.

Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium concentrations; VSMCs, vascular smooth muscle cells; PSS, physiological salt solution; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid; ET, endothelin.

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MATERIALS AND METHODS

Synthetic porcine endothelin was purchased from Peptide Institute (Japan), and quin2/AM was purchased from Dotite (Japan). Diltiazem hydrochloride, a Ca^{2+} antagonist, kindly donated by Tanabe Pharmac. Co. (Japan).

Cell Culture and quin2 loading of cells. VSMCs were cultured from rat aorta, as described (3). Only primary cultures were used for all experiments. Just before reaching confluence, VSMCs on the chamber slide were loaded with quin2 as acetoxymethyl ether (quin2/AM) (4), by incubating in growth medium containing 50 μM quin2/AM for 60 min at 37°C. Optic measurements were performed in physiological salt solution (PSS) at 25°C (5,6). The millimolar composition of normal PSS (pH 7.4 at 25°C) was: NaCl 135; KCl 5; $CaCl_2$ 1; $MgCl_2$ 1; glucose 5.5; HEPES 10. High K^+ PSS was prepared by replacing NaCl with KCl, isosmotically. Ca^{2+}-free PSS contained 2 mM EGTA instead of $CaCl_2$.

Quin2 Microfluorometry. The details of quin2 microfluorometry have been described elsewhere (5,6). Briefly, the fluorescence intensity in a small spot (< 1 μm^2) in the cytosol 3 μm apart from nucleus was measured using a fluorescence microscope (Model Standard 18, Zeiss) equipped with a water immersion objective system (Plan-Neofluor 63, Zeiss), an appropriate combination of filters (Zeiss and Toshiba) and a pinhole diaphragm (Zeiss) in the light axis. For optical measurements, each cell was exposed to the excitation light, only once, for no longer than 2 sec to avoid the photobleaching effect on quin2. The estimate of $[Ca^{2+}]_i$ was carried out as described (6,7).

RESULTS

Effects of ET on $[Ca^{2+}]_i$ in VSMCs in the presence and absence of the extracellular Ca^{2+}.

ET induced an increase in the cytosolic fluorescence intensity, $[Ca^{2+}]_i$, of quin2-loaded VSMCs, both in the presence and absence of extracellular Ca^{2+}. In normal PSS containing 1 mM Ca^{2+} and 5 mM K^+, ET induced a rapid and dose-dependent (10^{-9} -10^{-7} M) elevation of $[Ca^{2+}]_i$, within 30 sec, and this elevated level remained unchanged for at least 20 min of observation (Fig.1A). The estimated levels of $[Ca^{2+}]_i$ observed just before and 5 min after application with 10^{-7} M ET in normal PSS were 109 $\pm$ 6 nM and 225 $\pm$ 21 nM (n=5), respectively. The extent of increase in $[Ca^{2+}]_i$ was comparable to that observed when VSMCs were exposed to 16 mM K^+ PSS, and it was much smaller than observed with 100 mM K^+ PSS or with 10^{-5} M norepinephrine (the peak level of $[Ca^{2+}]_i$; 543 $\pm$ 82 nM and 368 $\pm$ 63 nM (n=5), respectively). The effect of ET was little influenced by wash-out with normal PSS, and the level of $[Ca^{2+}]_i$ at 20 min after wash-out was higher than that observed prior to application of ET (data not shown).

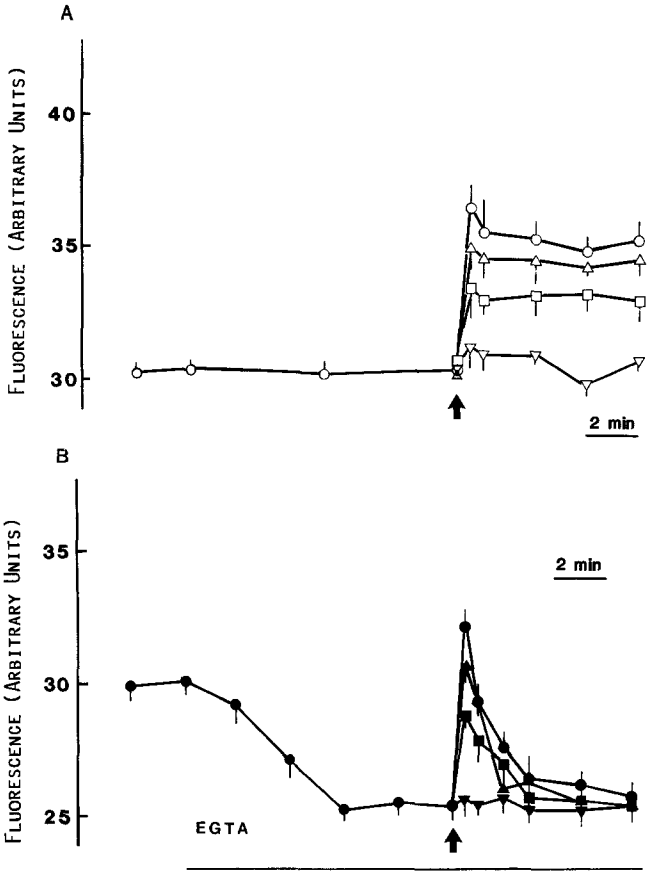

Figure 1. (A) Time courses of the fluorescence changes of cytosolic spots observed when 10^{-10} M (▽), 10^{-9} M (□), 10^{-8} M (△) and 10^{-7} M (○) ET were applied to VSMCs in normal PSS, as indicated by arrow. (B) Time courses of the fluorescence changes of cytosolic spots when 10^{-10} M (▼), 10^{-9} M (■), 10^{-8} M (▲) and 10^{-7} M (●) ET were applied to VSMCs in Ca^{2+}-free 2 mM EGTA PSS, as indicated by arrow.
Data are mean ± S.D. of 5 experiments, and the number of cells counted in each experiment was 8.

When VSMCs were exposed to Ca^{2+}-free PSS, $[Ca^{2+}]_i$ rapidly decreased to reach a lower steady level, within 6 min. The subsequent application with ET induced a rapid and transient elevation of $[Ca^{2+}]_i$ with a peak level seen at 30 sec and a gradual return to the pre-exposed level, within 3 min, in a dose-dependent manner. 10^{-7} M was the minimum concentration required to induce the maximum response (Fig.1B). $[Ca^{2+}]_i$ levels of the lower steady state and the peak of the transient elevation induced by 10^{-7} M ET in Ca^{2+}-free PSS were estimated to be 55 ± 4 nM and 135 ± 11 nM (n=5), respectively. Therefore, ET seems to induce a release of Ca^{2+} from intracellular Ca^{2+} store, in VSMCs.

Effect of diltiazem on ET-induced Ca^{2+} transients.

As we reported (6), diltiazem produced no significant change in the level of $[Ca^{2+}]_i$ in VSMCs, both in normal PSS and Ca^{2+}-free PSS. As shown in Fig.2A and C, in normal PSS, the pretreatment with diltiazem for 15 min dose-dependently inhibited the ET-induced Ca^{2+} transients, in particular the sustained component of the $[Ca^{2+}]_i$ elevation. In the absence of extracellular Ca^{2+}, the peak level and the time course of ET-induced Ca^{2+} transients were affected by pretreatment with diltiazem (Fig.2B and C).

Characteristics of the ET-sensitive intracellular Ca^{2+} store.

Caffeine and norepinephrine will release Ca^{2+} from intracellular stores in VSMCs in primary culture (6,7). To determine the characteristics

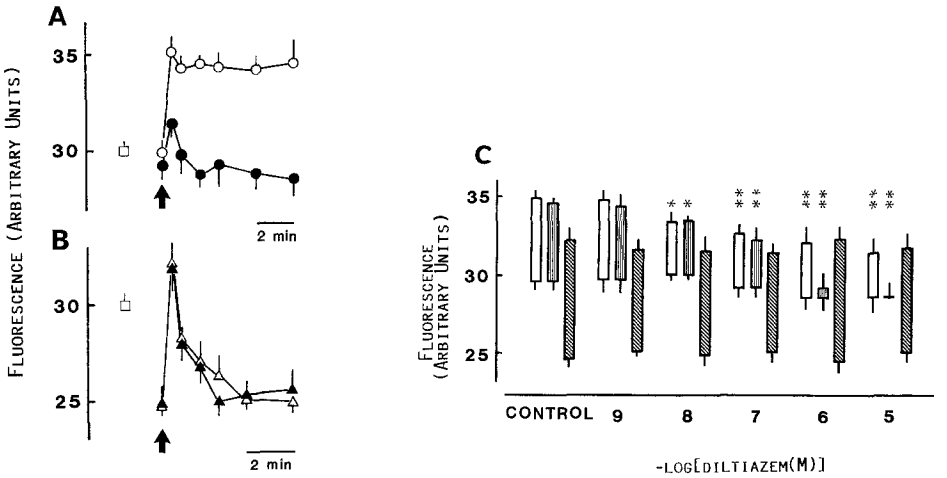

Figure 2. Effects of diltiazem on ET-induced Ca^{2+} transients. (**A**) The time courses of the fluorescence intensities of cytosolic spots when 10^{-7} M ET was applied to VSMCs, as indicated by arrow, with (●) or without (○) pretreatment with 10^{-5} M diltiazem for 15 min in normal PSS. The fluorescence level of VSMCs in normal PSS (□). Data are means ± S.D. of 4 experiments. Eight cells were used for each experiment. (**B**) The time courses of the fluorescence intensities of cytosolic spots when 10^{-7} M ET was applied to VSMCs in Ca^{2+}-free PSS, with (▲) or without (△), 15 min pretreatment with 10^{-5} M diltiazem. Cells were exposed to ET 10 min after incubation in Ca^{2+}-free PSS, as indicated by arrow. The fluorescence level of VSMCs in normal PSS (□). Data are means ± S.D. of 4 experiments. Eight cells were used for each experiment. (**C**) Dose-dependent inhibition effects of diltiazem on ET-induced Ca^{2+} transients. After pretreatment with the indicated concentration of diltiazem for 15 min, 10^{-7} M ET was applied to VSMCs in normal PSS and in Ca^{2+}-free PSS, as shown in (A) and (B), respectively. The bottom of each column indicates mean value just before application of ET. The top of the column indicates the values at 0.5 and 5 min after application with ET in normal PSS (□and▥, respectively) and the value at 0.5 min after application with ET in Ca^{2+}-free media (▨). Vertical line indicates S.D. of 4 experiments. * and ** denote statistical difference from the values of control at $p<0.05$ and $p<0.01$, respectively.

of the ET-sensitive intracellular store, the following experiments were performed. The effects of the duration of incubation in Ca^{2+}-free solution on Ca^{2+} transients, as induced by the first application with 10^{-7} M ET, in comparison with those with 10^{-2} M caffeine and 10^{-5} M norepinephrine are shown in Fig.3. When VSMCs were exposed to ET after 10 - 60 min incubation in Ca^{2+}-free PSS, the peak levels of ET-induced Ca^{2+} transients were not affected by duration of the incubation period. As already noted (7), the longer the duration of incubation in Ca^{2+}-free PSS the lesser was the extent of transient elevations of $[Ca^{2+}]_i$ induced by norepinephrine. The caffeine-induced Ca^{2+} transients were little affected by the duration of exposure to Ca^{2+}-free media, as in the case of ET.

As shown in Fig.4A, when VSMCs were treated repetitively with 10^{-2} M caffeine in Ca^{2+}-free PSS for 2 min at 2 min intervals, a series of Ca^{2+} transients were observed and the peak levels of the responses progressively decreased with each application. The fifth application of caffeine elicited no cellular response. After pretreatment with caffeine in Ca^{2+}-free media, the transient elevation of $[Ca^{2+}]_i$ induced by the subsequent application with ET was reduced. The greater the number of treatments with caffeine the lesser was the extent of $[Ca^{2+}]_i$ elevation induced by ET (Fig.4A a-c). Under conditions where the caffeine-sensitive intracellular Ca^{2+} store was practically depleted by five times of treatment with caffeine in Ca^{2+}-free

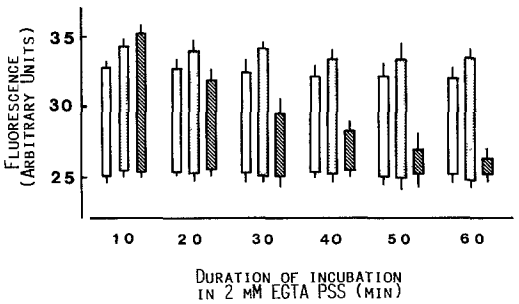

Figure 3. Effects of the duration of incubation in Ca^{2+}-free PSS on the levels in response to the first applications with 10^{-7} M ET (□), 10^{-2} M caffeine (▨) and 10^{-5} M norepinephrine (▧). The bottom of the column indicates mean value just before application of the agent, and top of the column indicates the peak value of the Ca^{2+} transients observed after application with ET, caffeine and norepinephrine, respectively, in Ca^{2+}-free PSS. Bars indicate S.D. of 4 experiments.

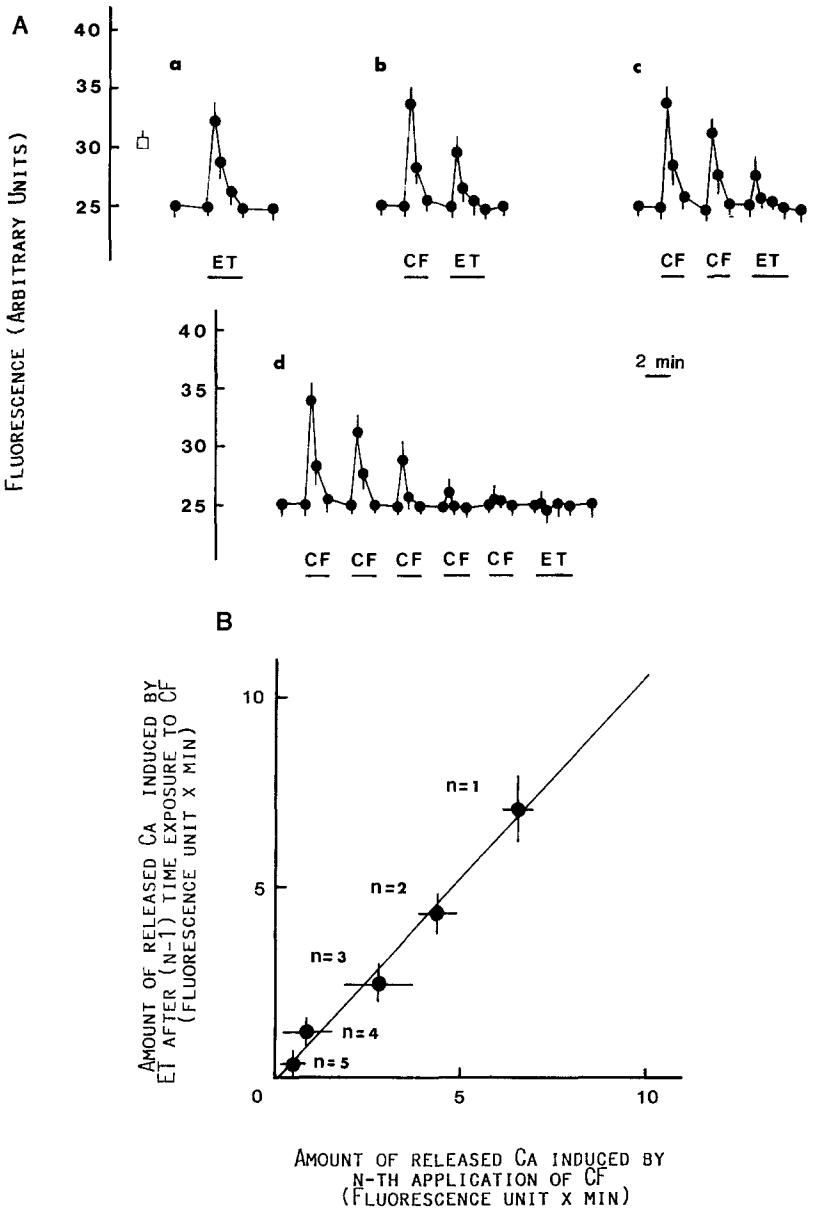

Figure 4. Effects of the repetitive caffeine treatments on ET-induced Ca^{2+} transients in Ca^{2+}-free PSS. (A) Representative samples of experiments; After 10 min incubation in Ca^{2+}-free PSS, the procedures were initiated. a, VSMCs were not exposed to caffeine b, VSMCs were exposed to 10^{-2} M caffeine (CF) once for 2 min. c and d, VSMCs were exposed to caffeine for 2 min twice and 5 times, respectively, each with 2 min interval. The fluorescence level of VSMCs in normal PSS (□). (B) The Comparison between ET- and caffeine-induced Ca^{2+} transients in Ca^{2+}-free media. The mean values of 5 experiments carried out as in A. Bars are S.D. Abscissa; amount of the Ca^{2+} released by the n-th application with 10^{-2} M caffeine. Ordinate; amount of Ca^{2+} released by 10^{-7} ET after (n-1) time applications with 10^{-2} M caffeine. The line was obtained by linear regression analysis. A positive slope with 1.09 with a correlation coefficient of 0.98.

media, the subsequent application of 10^{-7} M ET produced no significant elevation of $[Ca^{2+}]_i$ (Fig.4A d). To estimate the amount of Ca^{2+} released with each treatment, the area bound by the fluorescence trace and the steady state baseline in Ca^{2+}-free PSS was measured using a computerized manipulator (Houston Instrument, U.S.A.). The area was considered to indicate relative amounts of Ca^{2+} released from the intracellular store (5). As shown in Fig.4B, the amount of Ca^{2+} released, as induced by ET after (n-1) times applications ($1 \leq n \leq 5$) of caffeine, was equal to that induced by the n-th application of caffeine. The line obtained by linear regression analysis showed a positive slope of 1.09 with a correlation coefficient of 0.98.

DISCUSSION

ET may be an endogenous voltage-dependent Ca^{2+} channel agonist because of the properties of action and the structural homologies with neurotoxins which affect ion channels (1,8). The most recent evidence suggests that extracellular Ca^{2+}-independent mechanisms are involved in the action of ET on vascular smooth muscles (2,9). In the present study, ET induced a sustained elevation of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}, and a transient elevation of $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, in VSMCs.

Since the sustained component of ET-induced Ca^{2+} transients in Ca^{2+}-containing media was markedly inhibited by the Ca^{2+} antagonist, diltiazem, and was not observed in Ca^{2+}-free media, the component was postulated to be mediated by an extracellular Ca^{2+}-dependent mechanism such as Ca^{2+} influx. However, the maximum increase in $[Ca^{2+}]_i$ induced by ET was slight, as compared with that observed when VSMCs were exposed to 100 mM K^+ PSS or 10^{-5} M norepinephrine, in normal PSS. This may explain why a significant increase in ^{45}Ca accumulation into VSMCs by ET was not observed in the previous study (2).

Since the ET-sensitive intracellular Ca^{2+} store was resistant to $[Ca^{2+}]_i$ depletion in Ca^{2+}-free media, as in case of the caffeine-sensitive

one, the ET-sensitive store was distinguishable from the norepinephrine-sensitive one, in which Ca^{2+} was readily depleted by the decrease in $[Ca^{2+}]_i$ in Ca^{2+}-free media, in VSMCs in primary culture (7). When the caffeine-sensitive Ca^{2+} store was practically depleted by repeated treatments with caffeine, the subsequent application of ET induced no increase in $[Ca^{2+}]_i$. Thus, it was suggested that the ET-sensitive store overlaps with the caffeine-sensitive one, in VSMCs.

Although a high affinity binding site of ET was noted on the plasma membrane (10,11), ET had no significant effect on accumulation of total inositol phosphates(10), thereby indicating that transmembrane signal conduction through inositol phosphates turnover may not be involved in the ET-induced release of Ca^{2+} from the intracellular store. We reported that, in the absence of extracellular Ca^{2+}, high extracellular K^+-induced depolarization mobilizes the stored Ca^{2+} from the caffeine-sensitive store in VSMCs (5). It seems possible that a similar mechanism is involved in the process of ET-induced Ca^{2+} release. It was also reported that some of the ET penetrated through the plasma membrane into the cytoplasm (10). Thus, the pathway of signal transduction of ET into VSMCs to release intracellularly stored Ca^{2+} remains to be determined. Although ET has been reported to be one of the most potent vasoconstrictors known (1), the present study also shows that the maximum $[Ca^{2+}]_i$ elevation induced by ET was moderate. Thus, in addition to the Ca^{2+}-mediated contraction, mechanisms independent of Ca^{2+}-mediated ones are presumably involved in the ET-induced contraction of vascular smooth muscles.

In conclusion, we obtained the evidence that ET induces a $[Ca^{2+}]_i$ elevation not only through extracellular Ca^{2+}-dependent mechanisms but also by the direct release of Ca^{2+} from the intracellular store. The store may well overlap with caffeine-sensitive one, in VSMCs in primary culture.

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REFERENCES

1. Yanagisawa M., Kurihara H., Kimura S., Tomobe Y., Kobayashi M., Mitsui M., Yazaki Y., Goto K. and Masaki T. (1988) Nature 332, 411-415.
2. Miasiro N., Yamamoto H., Kanaide H. and Nakamura M. (1988) Biochem. Biophys. Res. Comm. 156, 312-317.
3. Yamamoto H., Kanaide H. and Nakamura M. (1983) Br. J. Exp. Pathol. 64, 156-165.
4. Tsien R.Y., Pozzan T. and Rink T.J. (1982) J. Cell Biol. 94, 325-334.
5. Kobayashi S., Kanaide H. and Nakamura M. (1986) J. Biol. Chem. 261, 15709-15713.
6. Kanaide H., Kobayashi S., Nishimura J., Hasegawa M., Shogakiuchi Y., Matsumoto T. and Nakamura M. (1988) Circ. Res. 63, 16-26.
7. Kanaide H., Shogakiuchi Y. and Nakamura M. (1987) FEBS Lett. 214, 130-134.
8. Ishikawa T., Yanagisawa M., Kimura S., Goto K. and Masaki T. (1988) Am. J. Physiol. 255 (Heart. Circ. Physiol. 24), H970-H973.
9. Auguest M., Delaflotte S., Chabrier P-E., Pirotzky E., Clostre F. and Braquest P. (1988) Biochem. Biophys. Res. Comm. 156, 186-192.
10. Hirata Y., Yoshimi S., Takata S., Watanabe T.X., Kumagai S., Nakajima K. and Sakakibara S. (1988) Biochem. Biophys. Res. Comm. 154, 868-875.
11. Hirata Y., Yoshimi S., Takata S., Yanagisawa M. and Masaki T. (1988) FEBS Lett. 239, 13-17.