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**Endothelin-1 decreases  $[Ca^{2+}]_i$  via  $Na^+/Ca^{2+}$  exchanger in CHO cells stably expressing endothelin  $ET_A$  receptor**

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## Abstract

Endothelin ET<sub>A</sub> receptor couples to G<sub>q/11</sub> protein that transduces a variety of receptor signals to modulate diverse cellular responses including Ca<sup>2+</sup> mobilization. Stimulation of endothelin ET<sub>A</sub> receptor with endothelin-1 is generally believed to induce an increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) via G<sub>q/11</sub> protein. Here we provide the first convincing evidence that endothelin-1 elicited G<sub>q/11</sub> protein-dependent and -independent 'decrease' in [Ca<sup>2+</sup>]<sub>i</sub> via Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) in Chinese hamster ovary (CHO) cells stably expressing human endothelin ET<sub>A</sub> receptor. In the cells treated with 1 μM thapsigargin, an inhibitor of endoplasmic Ca<sup>2+</sup> pump, that induces an increase in [Ca<sup>2+</sup>]<sub>i</sub> via capacitative Ca<sup>2+</sup> entry, endothelin-1 induced a decrease in [Ca<sup>2+</sup>]<sub>i</sub> which was partially inhibited by YM-254890, a specific inhibitor of G<sub>q/11</sub>, indicating that G<sub>q/11</sub>-dependent and -independent pathways are involved in the decrease. The endothelin-1-induced decrease in [Ca<sup>2+</sup>]<sub>i</sub> was markedly suppressed by 3',4'-dichlorobenzamil hydrochloride, a potent NCX inhibitor, and also by a replacement of extracellular Na<sup>+</sup> with Li<sup>+</sup>, which was not transported by NCX, indicating a major role of NCX operating in the forward mode in the endothelin-1-induced decrease in [Ca<sup>2+</sup>]<sub>i</sub>. Molecular approach with RT-PCR demonstrated the expression of mRNA for NCX1, NCX2 and NCX3. These results suggest that stimulation of endothelin ET<sub>A</sub> receptor with endothelin-1 activates the forward mode NCX through G<sub>q/11</sub>-dependent and -independent mechanisms: the NCX exports Ca<sup>2+</sup> out of the cell depending on Na<sup>+</sup> gradient across the cell membrane, resulting in the decrease in [Ca<sup>2+</sup>]<sub>i</sub>.

**Key words:** Endothelin-1; endothelin receptor; G<sub>q/11</sub> protein; Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; intracellular free Ca<sup>2+</sup> concentration.

## 1. Introduction

Endothelin ET<sub>A</sub> receptor is a well studied member of the seven membrane spanning receptors that exert their intracellular effects through G protein activation (Kawanabe et al., 2002a, b; Miwa et al., 2005). Stimulation of endothelin ET<sub>A</sub> receptor with endothelin-1 leads to G<sub>q/11</sub> protein-mediated activation of phospholipase C (PLC), resulting in the production of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) that binds to the IP<sub>3</sub> receptor in the endoplasmic reticulum (ER) membrane to initiate Ca<sup>2+</sup> release into the cytosol (Kawanabe et al., 2002a; Miwa et al., 2005). This signal transduction pathway is associated with diverse subcellular mechanisms underlying an increase in intracellular free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) induced by endothelin-1 such as activation of Ca<sup>2+</sup>-permeable nonselective cation channel and store-operated Ca<sup>2+</sup> channel (Miwa et al., 2005).

Recently, several studies demonstrated that endothelin-1 activated Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE), which increases intracellular Na<sup>+</sup> concentration ([Na<sup>+</sup>]<sub>i</sub>), leading to activation of Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (NCX) operating in the reverse mode to increase [Ca<sup>2+</sup>]<sub>i</sub> (Aiello et al., 2005; Fujita and Endoh, 1999; Yang et al., 1999). In addition, it has been reported that, after endothelin-1 stimulation, the NCX protein was phosphorylated by protein kinase C (PKC) which is a downstream signaling molecule activated by endothelin ET<sub>A</sub> receptor (Iwamoto et al., 1996). A study with whole-cell patch clamp technique also showed that endothelin-1 induced a direct PKC-dependent increase in NCX-mediated outward ionic current which is reverse mode Na<sup>+</sup>/Ca<sup>2+</sup> exchange (Zhang et al., 2001). These results suggested that endothelin-1-induced Ca<sup>2+</sup> influx is mediated by both mechanisms, the increase in [Na<sup>+</sup>]<sub>i</sub> via NHE, which drives the NCX in the reverse mode, and a direct [Na<sup>+</sup>]<sub>i</sub>-independent activation of the NCX.

The NCX plays an important physiological role in maintaining Ca<sup>2+</sup> homeostasis in many mammalian tissues (for review see, Blaustein and Lederer, 1999). The NCX can

trigger the electrogenic exchange of one  $\text{Ca}^{2+}$  for three  $\text{Na}^+$  either into or out of the cells, depending on the prevailing electrochemical driving force on the exchanger. In other words, excessive increase in  $[\text{Ca}^{2+}]_i$  can lead to activation of NCX operating in the forward mode to transport  $\text{Ca}^{2+}$  out of cells. Therefore, we hypothesized that under high  $[\text{Ca}^{2+}]_i$  conditions, endothelin-1 induces the decrease but not increase in  $[\text{Ca}^{2+}]_i$  via the forward mode NCX driven by endothelin  $\text{ET}_A$  receptor activation, if endothelin-1 could directly activate NCX as described above.

To test this hypothesis, the present study examined the effects of endothelin-1 on the increase in  $[\text{Ca}^{2+}]_i$  generated by thapsigargin, a sarcoplasmic and endoplasmic  $\text{Ca}^{2+}$ -ATPase inhibitor, in Chinese hamster ovary (CHO) cells stably expressing human endothelin  $\text{ET}_A$  receptor. The mechanisms underlying the  $\text{Ca}^{2+}$  mobilization activated by endothelin-1 were also studied using YM-254890, a novel and specific  $\text{G}_{q/11}$  protein inhibitor (Takasaki *et al.*, 2004; Taniguchi *et al.*, 2003), and 3',4'-dichlorobenzamil hydrochloride (3',4'-DCB), a potent forward and reverse mode NCX inhibitor (Teubl *et al.*, 1999).

## 2. Materials and methods

### 2.1. Materials

YM-254890 (Fig. 1, molecular weight = 959, Taniguchi *et al.*, 2003) was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). Other chemicals were obtained commercially from the following sources: synthetic human endothelin-1 from Peptide Institute (Osaka, Japan); 3',4'-dichlorobenzamil hydrochloride (3',4'-DCB), probenecid and thapsigargin from Sigma-Aldrich Co. (St. Louis, Mo., U.S.A.); U-73122 (1-[6-((17 $\beta$ -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione) from Calbiochem (San Diego, Calif., U.S.A.); fura-2/acetoxymethyl ester (fura-2/AM),

fluo-3/acetoxymethyl ester (fluo-3/AM) and Pluronic F-127 from Dojindo Laboratories (Kumamoto, Japan).

## 2.2. Cell culture

CHO cells were cultured in Ham's F-12 medium supplemented with 10% fetal calf serum and penicillin-streptomycin at 37°C in humidified air with 5% CO<sub>2</sub>.

## 2.3. Stable expression of human endothelin ET<sub>A</sub> receptor in CHO cells

To generate CHO cells stably expressing endothelin ET<sub>A</sub> receptor, human endothelin ET<sub>A</sub> receptor gene was introduced into CHO cells by retroviral gene transfer. Briefly, retroviruses were produced by triple transfection of HEK293T cells with retroviral constructs along with gag-pol and vesicular stomatitis virus G glycoprotein expression constructs (Yee *et al.*, 1994). The supernatants containing virus were collected 24 h after transfection and added to CHO cells. The CHO cells were then centrifuged at 900 × g for 45 min at room temperature followed by incubation for 6 h at 37°C in 5% CO<sub>2</sub>-95% air. Then, the supernatants were replaced with fresh culture media. Endothelin ET<sub>A</sub> receptor-positive cells were selected for growth in medium containing 5 µg/ml puromycin for a week.

## 2.4. Measurement of [Ca<sup>2+</sup>]<sub>i</sub>

CHO cells grown to confluence in 10-cm dishes were incubated in the culture medium with 4 µM fura-2/AM or 10 µM fluo-3/AM admixed with 2.5 mM probenecid and 0.04% Pluronic F-127, a detergent, at 37°C for 45 min under reduced light. Cells were then collected and washed once with Ca<sup>2+</sup>-free Krebs-HEPES solution (in mM:

140 NaCl, 3 KCl, 1 MgCl<sub>2</sub>·6H<sub>2</sub>O, 11 D-(+)-glucose, 10 HEPES; adjusted to pH 7.3 with LiOH). The resulting cell pellets were resuspended in Ca<sup>2+</sup>-free Krebs-HEPES solution at 4 × 10<sup>5</sup> cells/ml and kept on ice under reduced light until Ca<sup>2+</sup> measurement. In some experiments, cells were resuspended in low Na<sup>+</sup> Krebs-HEPES solutions (0, 10 and 70 mM Na<sup>+</sup>) prepared by replacing NaCl with equimolar LiCl. Before each experiment, CaCl<sub>2</sub> was added to 0.5-ml aliquot of the cell suspension at the final concentration of 1 mM.

Changes of [Ca<sup>2+</sup>]<sub>i</sub> in cells were measured at 25°C using CAF-110 spectrophotometer (JASCO, Tokyo, Japan) with the following wavelengths: fura-2 excitation = 340 and 380 nm, emission = 500 nm; fluo-3 excitation = 490 nm, emission = 540 nm. At the end of each experiment, Triton X-100 was added to the cells at the final concentration of 0.2% to determine the maximal fluorescence intensity (F<sub>max</sub>) and/or ratio (R<sub>340/380</sub><sub>max</sub>). This was followed by an addition of EGTA at the final concentration of 30 mM to determine the minimal fluorescence intensity (F<sub>min</sub>) and/or ratio (R<sub>340/380</sub><sub>min</sub>). [Ca<sup>2+</sup>]<sub>i</sub> was calculated as previously described (Grynkiewicz *et al.*, 1985; Minta *et al.*, 1989).

In the experiments with 3',4'-DCB, [Ca<sup>2+</sup>]<sub>i</sub> was measured using fluo-3 instead of fura-2, because 3',4'-DCB over 10 μM used in the present study was found to interfere with fluorescence signals of fura-2 (data not shown).

## 2.5. Detection of mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

mRNA was extracted and purified using the mRNA purification kit (GE Healthcare UK Ltd., Buckinghamshire, U.K.) following the instructions of the manufacturer. cDNA was synthesized by using both Omniscript<sup>TM</sup> RT Kit (QIAGEN, Tokyo, Japan) for reverse transcription (RT) and HotStarTaq<sup>TM</sup> Master Mix Kit (QIAGEN, Tokyo, Japan) for polymerase chain reaction (PCR). Briefly, cDNA was synthesized by RT of

2.0  $\mu$ g of each mRNA using oligo (dT)<sub>15</sub> (Promega, Madison, U.S.A.) as a primer, and the RT reaction mixture was incubated at 37°C for 60 min, then at 95°C for 5 min before being placed on ice. A negative control without reverse transcriptase was run in parallel to verify that amplification did not proceed from residual genomic DNA.

PCR amplification was carried out on cDNA equivalent to 100 ng of starting mRNA, using specific oligonucleotide primers for NCX1 (forward, 5'-AATGGAGAGACCACCAAGAC-3' and reverse, 5'-CCTTCCCAGACCTCCAC-3'), NCX2 (forward, 5'-GAGATCACCATCACCAAGGC-3' and reverse, 5'-ATGAGATAAAGCCAGACATAGGC-3') and NCX3 (forward, 5'-TCTCACCTCTGCCTTCCTCCATTT-3' and reverse, 5'-TCAGGTTGGAGACAGTTTCATTCCA-3'), synthesized at Hokkaido System Science Co., Ltd. (Sapporo, Japan). cDNA was heated for 15 min at 95°C, then amplified by 35 cycles (95°C for 30 s, 55°C for 30 s, 72°C for 30 s) followed by 5 min of extension at 72°C. The PCR products were confirmed by a single band on electrophoresis with 2.0% ethidium bromide stained agarose gels. Because the sequences for NCX1, NCX2 and NCX3 of Chinese hamster were unknown, the primer sets were designed using conserved sequences for NCX mRNA among human, rat and mouse. To confirm the sequences of these PCR products, the direct sequencing was carried out. Briefly, the PCR products of NCX1, NCX2 and NCX3 were purified using QIAquick<sup>TM</sup> PCR Purification Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. The purified PCR products were labeled using BigDye<sup>TM</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Calif., U.S.A.) and the aforementioned primers. DNA sequencing was performed on the ABI PRISM<sup>TM</sup>3100 Genetic Analyzer (Applied Biosystem, Calif., U.S.A.).

## 2.6. Data Analysis



The percentage of decrease in  $[Ca^{2+}]_i$  induced by endothelin-1 was calculated by considering the  $[Ca^{2+}]_i$  level obtained just before administration of endothelin-1 as 0% decrease, and the basal  $[Ca^{2+}]_i$  level before application of thapsigargin as 100% decrease. Data are presented as means  $\pm$  S.E.M. where  $n$  refers to the number of experiments. The significance of the difference between mean values was evaluated with GraphPad PRISM™ (version 3.00) by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A  $P$  value less than 0.05 was considered to indicate significant differences.

### 3. Results

#### 3.1. Dual effects of endothelin-1 in the regulation of $[Ca^{2+}]_i$

Figures 2A and 3A show representative traces for the opposite effects of 0.3 nM endothelin-1 on  $[Ca^{2+}]_i$  in CHO cells stably expressing endothelin  $ET_A$  receptor. In untreated cells, endothelin-1 induced transient and subsequent sustained increases in  $[Ca^{2+}]_i$  (Fig. 2A, left trace), which were submaximal responses. Both phases were completely abolished by pretreatment with 0.3  $\mu$ M YM-254890, a specific and potent  $G_{q/11}$  protein inhibitor (Fig. 2A, right trace). In addition, U-73122, an inhibitor of PLC, at a concentration of 3  $\mu$ M depressed the transient and sustained  $[Ca^{2+}]_i$  increases in response to 0.3 nM endothelin-1 (Fig. 2B, C). The result is in accord with our previous report (Sugawara *et al.*, 1996). As shown in Fig. 3A, the treatment of CHO cells with 1  $\mu$ M thapsigargin, a sarcoplasmic and endoplasmic  $Ca^{2+}$ -ATPase inhibitor, elicited transient and sustained increases in  $[Ca^{2+}]_i$ . Surprisingly, endothelin-1 at 0.3 nM caused a significant reduction of  $[Ca^{2+}]_i$  ( $32.2 \pm 2.8\%$  of thapsigargin-induced sustained increase in  $[Ca^{2+}]_i$ ,  $n = 5$ ; Fig. 3B), after the sustained increase in  $[Ca^{2+}]_i$  induced by thapsigargin reached a plateau level (Fig. 3A, left trace). The decrease in

$[Ca^{2+}]_i$  induced by endothelin-1 was partly but significantly inhibited by 1  $\mu$ M YM-254890 ( $17.4 \pm 2.6\%$ ,  $n = 5$ ; Fig. 3B). However, a higher concentration (10  $\mu$ M) of YM-254890 did not show further inhibition of  $[Ca^{2+}]_i$  reduction ( $16.2 \pm 2.5\%$ ,  $n = 5$ ; Fig. 3B), indicating that the inhibitory effect of YM-254890 was maximal at 1  $\mu$ M. Similarly, U-73122 inhibited the endothelin-1-induced  $[Ca^{2+}]_i$  decrease (Fig. 3C). These results suggested that the decrease in  $[Ca^{2+}]_i$  induced by endothelin-1 was mediated through  $G_{q/11}$ -dependent and -independent pathways.

### 3.2. Functional role of NCX in the decrease in $[Ca^{2+}]_i$ induced by endothelin-1

To clarify the mechanisms involving the decrease in  $[Ca^{2+}]_i$  induced by endothelin-1, the effects of 3',4'-dichlorobenzamil hydrochloride (3',4'-DCB), a forward and reverse mode NCX inhibitor (Teubl *et al.*, 1999), were investigated using a  $Ca^{2+}$  indicator fluo-3 instead of fura-2 (see Materials and Methods). As shown in Fig. 3D, 3',4'-DCB at concentrations over 30  $\mu$ M depressed the decrease in  $[Ca^{2+}]_i$  induced by endothelin-1 in a concentration-dependent manner (control,  $41.3 \pm 2.3\%$ ; 10  $\mu$ M 3',4'-DCB,  $41.8 \pm 3.0\%$ ; 30  $\mu$ M,  $29.6 \pm 4.1\%$ ; 100  $\mu$ M,  $6.5 \pm 2.2\%$ ;  $n = 5$  for each). In addition, the YM-254890-insensitive ( $G_{q/11}$ -independent) decrease was markedly inhibited by 100  $\mu$ M 3',4'-DCB (in the presence of 1  $\mu$ M YM-254890,  $23.2 \pm 0.9\%$ ; the combination of 1  $\mu$ M YM-254890 and 100  $\mu$ M 3',4'-DCB,  $2.4 \pm 0.6\%$ ;  $n = 6$ ). These results indicate that the forward mode NCX transporting  $Ca^{2+}$  out of cells plays an important role in both  $G_{q/11}$ -dependent and -independent decreases in  $[Ca^{2+}]_i$  triggered by endothelin-1.

To further confirm the possible involvement of NCX operating in the forward mode, where its driving force is generated by the concentration gradient of  $Na^+$  across the membrane, we attempted to examine the decreasing concentrations of extracellular  $Na^+$  on the activity of NCX associated with the endothelin-1-induced decrease in  $[Ca^{2+}]_i$ .

For this purpose, we reduced the extracellular  $\text{Na}^+$  concentration by replacing  $\text{Na}^+$  with  $\text{Li}^+$ , which was not transported by NCX (Blaustein and Lederer, 1999). As expected, the endothelin-1-induced decrease in  $[\text{Ca}^{2+}]_i$  became smaller depending on the decreasing concentrations of extracellular  $\text{Na}^+$  (Fig. 3E). Taken together, these results suggest that stimulation of endothelin  $\text{ET}_A$  receptor with endothelin-1 activates the forward mode NCX to drive  $\text{Ca}^{2+}$  efflux and  $\text{Na}^+$  influx in  $\text{G}_{q/11}$ -dependent and -independent manner, resulting in the decrease in  $[\text{Ca}^{2+}]_i$ .

### 3.3. Expression of mRNA for NCX1, NCX2 and NCX3 in CHO cells

To validate the pharmacological and physiological evidence for the presence of functional NCX in CHO cells stably expressing endothelin  $\text{ET}_A$  receptor, RT-PCR experiments were carried out. As shown in Fig. 4, PCR products were detected by agarose gel electrophoresis at positions of approximately 350, 330 and 390 bp, which correspond to the expected size for PCR products of NCX1 (350 bp), NCX2 (329 bp) and NCX3 (386 bp), respectively. The absence of PCR products without reverse transcription provided evidence that there is no non-specific amplification and these bands are derived from mRNA but not contaminating genomic DNA (data not shown). Finally, the PCR products were purified, sequenced and identified as NCX1, NCX2 and NCX3. The degree of sequence homology for NCX1, NCX2 and NCX3 between Chinese hamster and the three species (human, rat and mouse) was approximately 90%.

## 4. Discussion

In the present study, we have provided the first evidence that stimulation of endothelin  $\text{ET}_A$  receptor with endothelin-1 induces  $\text{G}_{q/11}$  protein-dependent and -independent decreases in  $[\text{Ca}^{2+}]_i$  via NCX operating in the forward mode in CHO cells

stably expressing human endothelin ET<sub>A</sub> receptor. In addition, we were able to detect all three types of NCX (NCX1, NCX2 and NCX3) by using RT-PCR.

#### *4.1. Involvement of G<sub>q/11</sub> protein-dependent and -independent pathways in endothelin ET<sub>A</sub> receptor signaling*

To characterize endothelin ET<sub>A</sub> receptor signaling, we examined the effects of YM-254890, a novel and specific G<sub>q/11</sub> protein inhibitor, on endothelin-1-mediated Ca<sup>2+</sup> mobilization. Initial studies confirmed that YM-254890 blocked Ca<sup>2+</sup> mobilization mediated by several G<sub>q/11</sub>-coupled receptors but not by G<sub>i</sub>- or G<sub>α15</sub>-coupled receptors (Takasaki *et al.*, 2004). In the present study, in CHO cells expressing human endothelin ET<sub>A</sub> receptor, YM-254890 (0.3 μM) completely inhibited endothelin-1-induced transient and sustained increases in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 2A). Similar results were obtained for a PLC inhibitor, U-73122. These results indicate the activation of G<sub>q/11</sub> and its downstream signaling pathways by endothelin-1. Interestingly, in CHO cells pretreated with thapsigargin which induces an increase in [Ca<sup>2+</sup>]<sub>i</sub> mediated by capacitative Ca<sup>2+</sup> entry, endothelin-1 induced a marked decrease in [Ca<sup>2+</sup>]<sub>i</sub>, as shown in Fig. 3A. The decrease consisted of two components, which were sensitive and insensitive to YM-254890 at pharmacologically relevant concentrations. This result indicates that the endothelin-1-induced decrease in [Ca<sup>2+</sup>]<sub>i</sub> is mediated via both G<sub>q/11</sub>-dependent and -independent pathways. We have recently shown that endothelin ET<sub>A</sub> receptor is functionally coupled with G<sub>s</sub> and G<sub>12</sub> in addition to G<sub>q/11</sub> in CHO cells (Kawanabe *et al.*, 2002a; Takagi *et al.*, 1995). G<sub>s</sub> protein is a possible candidate for G<sub>q/11</sub>-independent mechanisms involving the endothelin-1-induced [Ca<sup>2+</sup>]<sub>i</sub> decrease, because the activation of β-adrenoceptors, a member of G<sub>s</sub> protein-coupled receptors, is reported to induce the reduction of [Ca<sup>2+</sup>]<sub>i</sub> via NCX in the porcine coronary arterial smooth muscle (Yamanaka *et al.*, 2003). However, G<sub>s</sub>-adenylyl cyclase

pathway(s) might be ruled out, since an inhibitor of adenylyl cyclase (2',5'-dideoxyadenosine, 50  $\mu$ M) had no effect on the endothelin-1-induced decrease in  $[Ca^{2+}]_i$  (Horinouchi *et al.*, 2007, unpublished data). Therefore,  $G_{12}$  and/or other unidentified G protein(s) may be responsible for  $G_{q/11}$ -independent pathway(s).

#### *4.2. Identification of molecules involved in the endothelin-1-induced decrease in $[Ca^{2+}]_i$*

Although the mechanisms for endothelin-1-induced increase in  $[Ca^{2+}]_i$  have so far been reported repeatedly, there was no report for the endothelin-1-induced decrease in  $[Ca^{2+}]_i$ . We therefore attempted to clarify possible mechanisms underlying this response. For this purpose, we focused on the physiological role of NCX that can transport  $Ca^{2+}$  either into or out of cells depending on transmembrane ion gradient and membrane potential, since stimulation of  $G_{q/11}$  protein-coupled receptors with endothelin-1, phenylephrine and angiotensin II activated cardiac NCX operating in the forward mode (Ballard and Schaffer, 1996; Philipson and Nicoll, 2000). Endothelin-1-induced decrease in  $[Ca^{2+}]_i$  was, as expected, significantly depressed by 3',4'-DCB, a forward and reverse mode NCX inhibitor (Teubl *et al.*, 1999), in a concentration-dependent manner. In addition, the response to endothelin-1 was also inhibited by the reduction of extracellular  $Na^+$  concentration which is expected to decrease NCX driving force in the forward mode. These results strongly indicate that the forward mode NCX transporting  $Ca^{2+}$  out of cells plays a significant role in  $G_{q/11}$ -dependent and -independent  $Ca^{2+}$  export activated by endothelin-1. However, the removal of  $Na^+$  from the medium to block ion transport via NCX did not completely prevent endothelin-1-induced decrease in  $[Ca^{2+}]_i$ . This raises the possibility that endothelin-1-induced  $[Ca^{2+}]_i$  decrease is mediated through NCX-dependent and -independent pathways. Unlike the NCX-dependent pathway, the NCX-independent mechanism involving  $Ca^{2+}$  export is still unclear.

#### 4.3. Molecular evidence for the presence of NCX in CHO cells

Previous molecular studies have identified NCX1, NCX2 and NCX3 that are encoded by distinct genes in mammalian cells (Li *et al.*, 1994; Nicoll *et al.*, 1990; Nicoll *et al.*, 1996). To determine whether either the NCX member is present in CHO cells, we performed RT-PCR using specific primers for each of these NCX genes. The RT-PCR studies demonstrated that all three members of NCX were expressed in CHO cells stably expressing endothelin ET<sub>A</sub> receptor. That is, the sequence of PCR products was highly homologous (approximately 90%) to that of the corresponding NCX member reported in human, rat and mouse, and hence, the mRNAs containing these sequences were identified as NCX1, NCX2 and NCX3. This is the first report of mRNA expression and function of NCX in CHO cells. However, it remains to be determined which of the NCX members plays a major role after stimulation of endothelin ET<sub>A</sub> receptor, because NCX1, NCX2 and NCX3 cannot be functionally differentiated (Iwamoto and Shigekawa, 1998; Linck *et al.*, 1998). Therefore, the pharmacological and molecular identity should be determined by using RNA interference method for specific NCX gene silencing in future.

In summary, the present study demonstrated that the functionally tight coupling of endothelin ET<sub>A</sub> receptor with NCX induced the decrease in [Ca<sup>2+</sup>]<sub>i</sub> in CHO cells. This association would play a regulatory role in Ca<sup>2+</sup> homeostasis.

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## Legends for figures

Fig. 1. Chemical structure of YM-254890.

Fig. 2. Characterization of endothelin-1-induced increase in intracellular free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) in CHO cells stably expressing human endothelin  $\text{ET}_A$  receptor. (A) Endothelin-1 induced transient and sustained increases in  $[\text{Ca}^{2+}]_i$  (left trace). Both phases were completely abolished by YM-254890 (0.3  $\mu\text{M}$ ), a specific  $\text{G}_{q/11}$  protein inhibitor (right trace). The traces shown are representative of 5 similar individual experiments. (B and C) Effects of YM-254890 and U-73122, a PLC inhibitor, on the transient and sustained increases in  $[\text{Ca}^{2+}]_i$  induced by endothelin-1. The transient and sustained  $[\text{Ca}^{2+}]_i$  increases were inhibited by the pretreatment with YM-254890 and U-73122. Thus the  $\text{Ca}^{2+}$  responses to endothelin-1 are mediated by  $\text{G}_{q/11}/\text{PLC}$  pathway. The ordinate is the change in  $[\text{Ca}^{2+}]_i$  measured using a  $\text{Ca}^{2+}$  indicator fura-2. Results are presented as means  $\pm$  S.E.M of the results obtained from 4 - 6 separate experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

Fig. 3. Endothelin-1 can induce a decrease in  $[\text{Ca}^{2+}]_i$  in CHO cells stably expressing human endothelin  $\text{ET}_A$  receptor. (A) Thapsigargin (1  $\mu\text{M}$ ) also evoked transient and sustained increases in  $[\text{Ca}^{2+}]_i$ . Stimulation of  $\text{ET}_A$  receptors with endothelin-1 inhibited the sustained phase generated by thapsigargin (left trace). Endothelin-1-induced decrease in  $[\text{Ca}^{2+}]_i$  was partly inhibited by the pretreatment with the high concentration (1  $\mu\text{M}$ ) of YM-254890 (right trace). The ordinate is the change in  $[\text{Ca}^{2+}]_i$  measured using a  $\text{Ca}^{2+}$  indicator fura-2. The traces shown are representative of 5 similar individual experiments. (B and C) YM-254890 and U-73122 could partially inhibit endothelin-1-induced decrease in  $[\text{Ca}^{2+}]_i$ . Thus the  $\text{Ca}^{2+}$  response to endothelin-1 is mediated by  $\text{G}_{q/11}/\text{PLC}$ -dependent and -independent pathways. (D) 3',4'-DCB, a potent  $\text{Na}^+/\text{Ca}^{2+}$  exchanger inhibitor, significantly suppressed endothelin-1-induced decrease in  $[\text{Ca}^{2+}]_i$ . (E) A reduction of extracellular  $\text{Na}^+$  concentration

by replacement of  $\text{Na}^+$  with  $\text{Li}^+$  in bathing medium resulted in marked inhibition of endothelin-1-induced decrease in  $[\text{Ca}^{2+}]_i$ . The  $[\text{Ca}^{2+}]_i$  level of sustained phase generated by thapsigargin before administration of endothelin-1 and the basal  $[\text{Ca}^{2+}]_i$  level before administration of thapsigargin were set at 0% and 100%, respectively. Results are presented as means  $\pm$  S.E.M of the results obtained from 4 - 6 separate experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

Fig. 4. Detection by RT-PCR of NCX1, NCX2 and NCX3 mRNA in CHO cells stably expressing human endothelin  $\text{ET}_A$  receptor. RT-PCR experiments were carried out using mRNA prepared from 3 individual cell populations. Expected size of PCR products for NCX1, NCX2 and NCX3 were 350, 329 and 386 bp, respectively. Left lane shows 100 bp DNA ladder (Promega).

Figure 1.

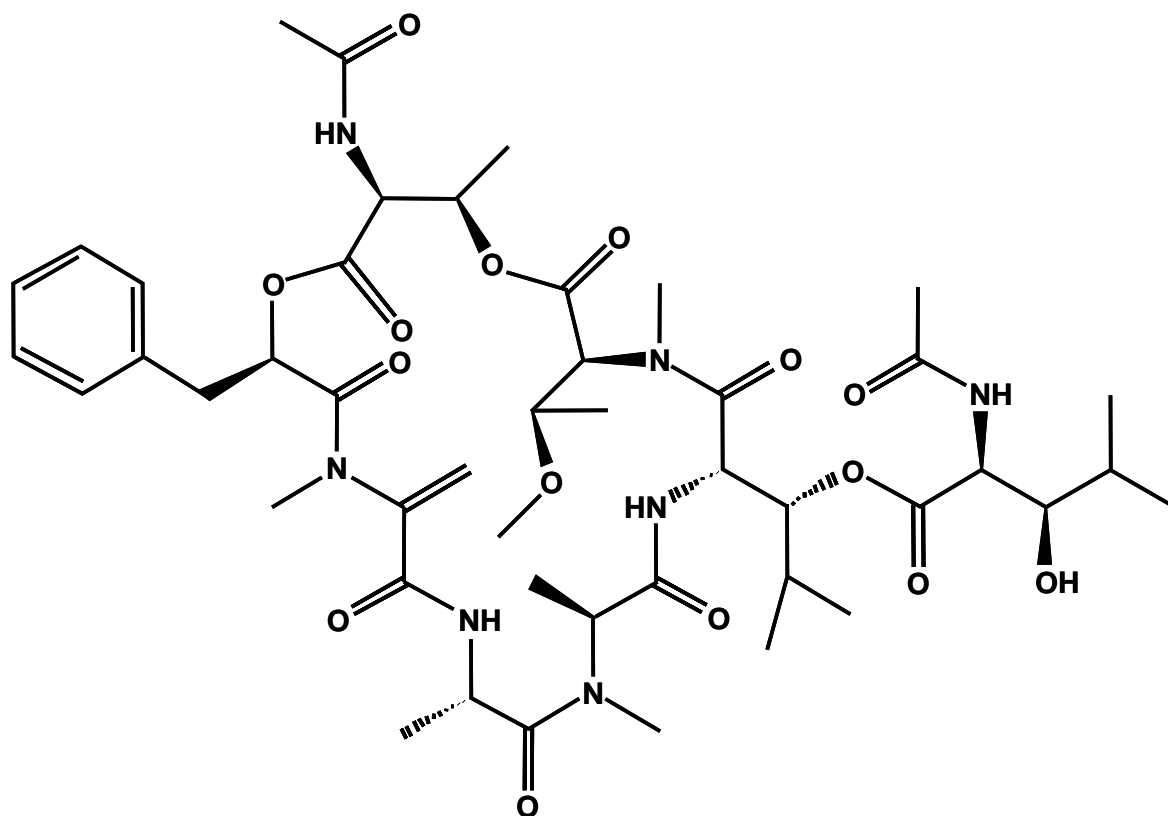
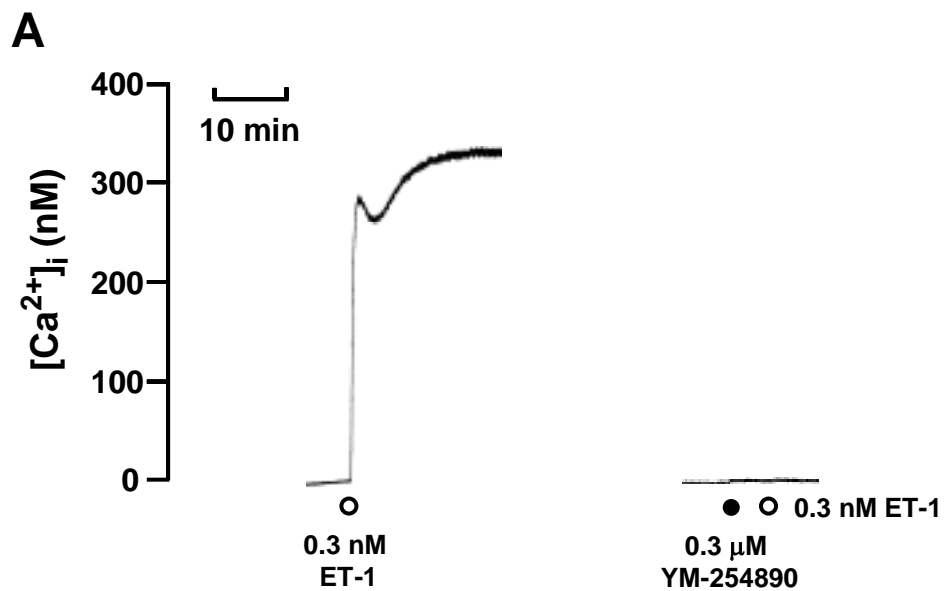
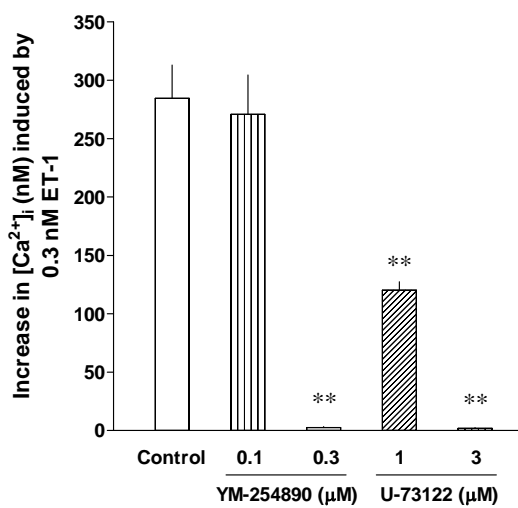


Figure 2.



**B. Transient**



**C. Sustained**

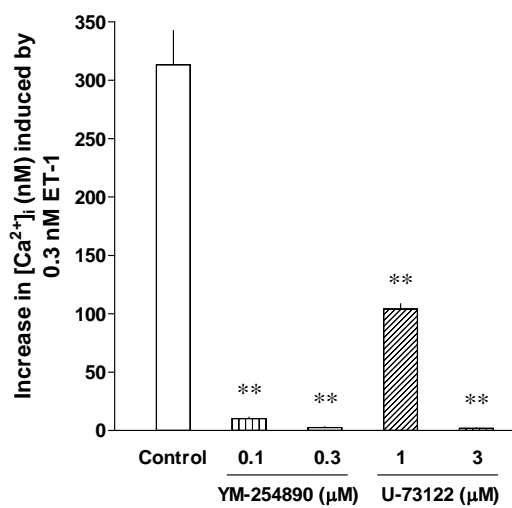


Figure 3.

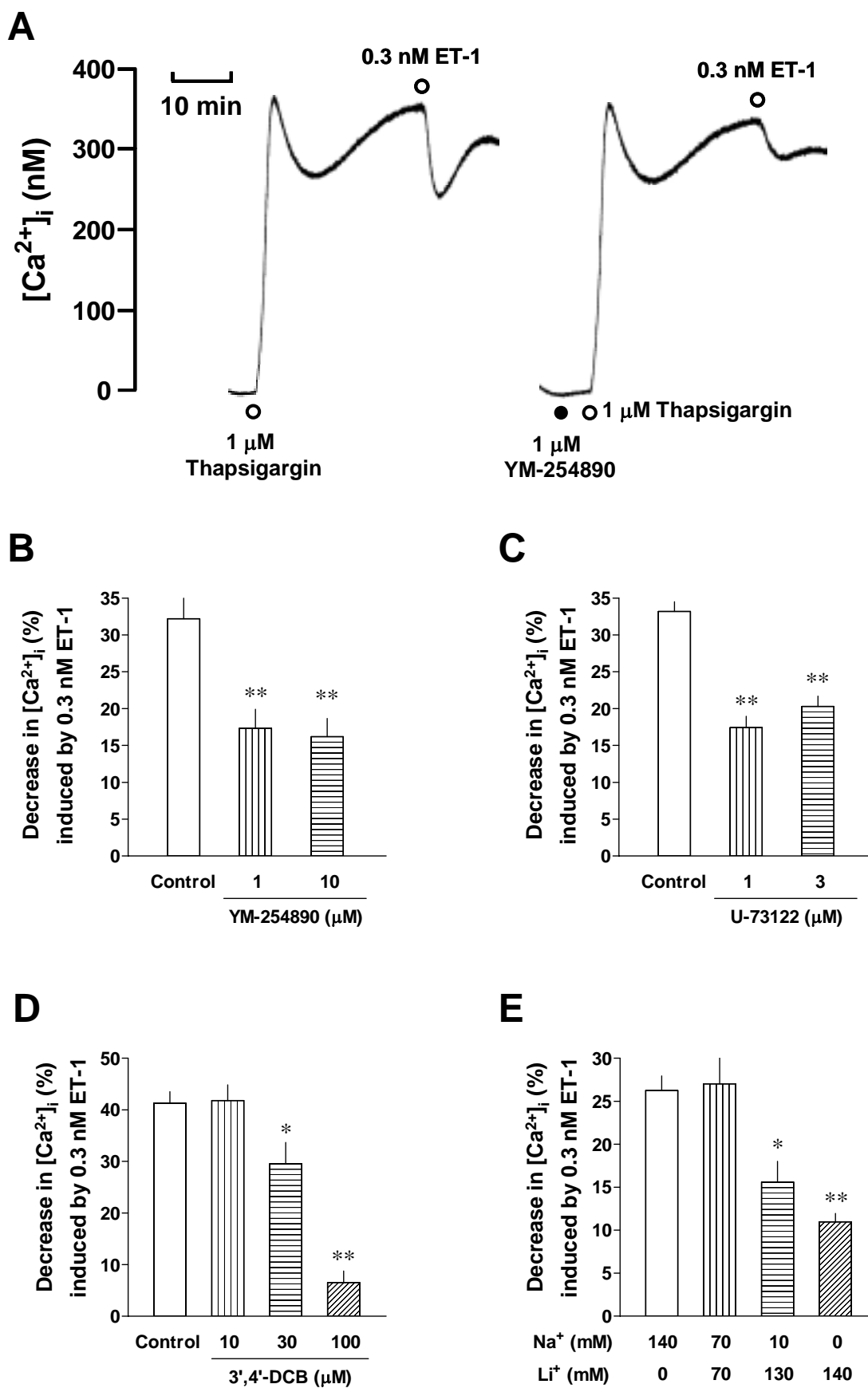


Figure 4.

