Molecular Mechanisms for the Activation of Voltage-Independent Ca\textsuperscript{2+} Channels by Endothelin-1 in Chinese Hamster Ovary Cells Stably Expressing Human Endothelin\textsubscript{A} Receptors

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ABSTRACT

We demonstrated that in Chinese hamster ovary cells stably expressing human recombinant endothelin\textsubscript{A} receptors (CHO-ET\textsubscript{AR}), endothelin-1 (ET-1) activates two types of Ca\textsuperscript{2+} -permeable nonselective cation channels (designated NSCC-1 and NSCC-2) and a store-operated Ca\textsuperscript{2+} channel (SOCC), which can be distinguished by Ca\textsuperscript{2+} channel blockers such as 1-\{\(\beta\)[-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl\}-1H-imidazole hydrochloride (SK\&F 96365) and (R,S)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-\(N,N\)-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate (LOE 908). We also reported that CHO-ET\textsubscript{AR} couples with G\textsubscript{12} in addition to G\textsubscript{q} and G\textsubscript{s}. The purpose of the present study was to identify the G proteins involved in the activation of these Ca\textsuperscript{2+} channels by ET-1, using mutated ET\textsubscript{AR}S with coupling to either G\textsubscript{q} or G\textsubscript{s}. We also reported that CHO-ET\textsubscript{AR} pretreated with U73122, an inhibitor of phospholipase C (PLC), ET-1 activated only NSCC-1. Dibutyryl cAMP alone did not activate any Ca\textsuperscript{2+} channels in the resting and ET-1–stimulated CHO-SerET\textsubscript{AR}. Microinjection of G\textsubscript{12}G228A abolished the activation of NSCC-1 and NSCC-2 in CHO-ET\textsubscript{AR} and that of NSCC-1 in CHO-SerET\textsubscript{AR}. These results indicate that ET\textsubscript{AR} activates three types of Ca\textsuperscript{2+} channels via different G protein-related pathways. NSCC-1 is activated via a G\textsubscript{12}-dependent pathway, NSCC-2 via G\textsubscript{q}/PLC- and G\textsubscript{12}-dependent pathways, and SOCC via a G\textsubscript{q}/PLC-dependent pathway.

Endothelin-1 (ET-1), a 21–amino acid peptide, is one of the most potent endogenous vasoconstricting agents (Yanagisawa et al., 1988). Subsequent studies have described its multiple and wide-ranging biological activities, including modulation of neurotransmission (Koseki et al., 1989) and stimulation of cell proliferation (Komuro et al., 1988; Shichiri et al., 1991). Recent reports have demonstrated that extracellular Ca\textsuperscript{2+} influx through voltage-independent Ca\textsuperscript{2+} channels (VICCs) plays a critical role for ET-1–induced contraction of rat aorta (Zhang et al., 1999) and ET-1–induced proliferation of vascular smooth muscle cells (VSMCs) (Kawanabe et al., 2002a). Thus, it is important to elucidate activation mechanisms of VICCs by ET-1. Biological actions of ET-1 are mediated by two distinct receptor subtypes: endothelin\textsubscript{A} and endothelin\textsubscript{B} receptors (ET\textsubscript{AR}S and ET\textsubscript{BR}S, respectively), which belong to a family of G protein–coupled receptors (Arai et al., 1990; Sakurai et al., 1990). Therefore, in the present study, we focused on investigating which G protein subtypes were involved in the activation of each Ca\textsuperscript{2+} channel by ET-1.

Transfection and functional expression of wild-type or mutant ET\textsubscript{AR} cDNAs into the same cell type provides a model

ABBREVIATIONS: ET-1, endothelin-1; VICC, voltage-independent Ca\textsuperscript{2+} channel; VSMC, vascular smooth muscle cell; ET\textsubscript{AR}, endothelin\textsubscript{A} receptor; ET\textsubscript{BR}, endothelin\textsubscript{B} receptor; CHO, Chinese hamster ovary; NSCC, nonselective cation channel; SOCC, store-operated Ca\textsuperscript{2+} channel; PLC, phospholipase C; SK\&F 96365, 1-\{\(\beta\)[-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl\}-1H-imidazole hydrochloride; LOE 908, (R,S)-(3,4-dihydro-6,7-dimethoxy-isochinolin-1-yl)-2-phenyl-\(N,N\)-di[2-(2,3,4-trimethoxyphenyl)ethyl]acetamid mesylate; AM, acetoxymethyl ester; U73122, 1-\{[(17\beta-3-methoxyoxostra-1,3,5(10)\}-tri-en-17-\(\beta\)-l-amino\}hexyl\}-1H-pyrole-2,5-dione; IP, inositol phosphate; DAG, diacylglycerol; G\textsubscript{12}, dominant-negative mutant of G\textsubscript{12}; CHO-SerET\textsubscript{AR}, Chinese hamster ovary cells that express an unpalmitoylated (Cys\textsubscript{383}Cys\textsubscript{385}–388) human endothelin\textsubscript{A} receptor; CHO-ET\textsubscript{AR}R3385, Chinese hamster ovary cells that express human endothelin\textsubscript{A} receptor truncated at the carboxyl-terminal downstream of Cys\textsubscript{385}; CHO-ET\textsubscript{AR}, Chinese hamster ovary cells stably expressing human endothelin\textsubscript{A} receptor.
system for study of the precise characteristics of signal transduction by a single receptor subtype. We used Chinese hamster ovary (CHO) cells stably expressing wild-type or mutant ETαRs in this study. We have recently shown that a sustained increase in intracellular free Ca2+ concentration ([Ca2+]i) caused by ET-1 results from Ca2+ entry through three types of VICCs into CHO cells stably expressing wild-type ETαR (CHO-ETαR): two types of Ca2+-permeable non-selective cation channels (designated NSCC-1 and NSCC-2) and a store-operated Ca2+ channel (SOCC) (Kawanabe et al., 2001). In particular, these channels can be distinguished using Ca2+ channel blockers such as SK&F 96365 and LOE 908. Thus, NSCC-1 is sensitive to LOE 908 and resistant to SK&F 96365, NSCC-2 is sensitive to both LOE 908 and SK&F 96365, and the SOCC is resistant to LOE 908 and sensitive to SK&F 96365 (Kawanabe et al., 2001). The VICCs activated by ET-1 in CHO-ETαR are pharmacologically identical to those in VSMCs (Kawanabe et al., 2002a). Therefore, CHO-ETαR may be a good model for studying the mechanism of activation of VICCs. ETαRs are functionally coupled with Gα and G12 in CHO cells (Kawanabe et al., 2002b). Activation of Gα and G12 causes stimulation of phospholipase C (PLC) and adenylyl cyclase, respectively (Aramori and Nakaniishi, 1992). In addition, ETαRs also couple with G12 via its C terminus to induce actin stress fiber formation in CHO cells (Kawanabe et al., 2002b). In the present study, we used a dominant-negative mutant of G12 and two types of mutated ETαRs designated ETαR385 and SerETαR to clarify the involvement of Gα, G12, and Gα for Ca2+ channel activation by ET-1. ETαR385 lacks a C terminus downstream of Cys385 and couples only with Gα in CHO cells (Kawanabe et al., 2002b). SerETαR is unpalmitoylated because of substitution of all the cysteine residues to serine (Cys385Ser388→Ser385Ser388) and coupling with Gα and G12 in CHO cells (Kawanabe et al., 2002b).

Experimental Procedures

Cell Culture. We used CHO-ETαR, CHO-ETαR385, and CHO-SerETαR, which were constructed as described previously (Kawanabe et al., 2002b). The Kα (picomolar) and Bmax (pmol/mg of protein) values for CHO-ETαR, CHO-ETαR385, and CHO-SerETαR were 52.8 ± 2.4 and 1.08 ± 0.16, 49.5 ± 4.3 and 1.12 ± 0.08, and 70.2 ± 4.4 and 1.04 ± 0.14, respectively. CHO cells were maintained in Ham’s F12 medium supplemented with 10% fetal calf serum. Microinjection of G12G228A, constructed as described previously (Kawanabe et al., 2002b), was performed using a Zeiss microinjection system (Carl Zeiss). Plasmid (100 ng/μl) was used for microinjection into the cell nuclei.

Materials. Boehringer Ingelheim GmbH (Ingelheim, Germany) kindly provided LOE 908. Other chemicals were obtained commercially from the following sources: ET-1 from Peptide Institute (Osaka, Japan); SK&F 96365 from Biomol Research Laboratories (Plymouth Meeting, PA); fluo-3/AM from Doinj Laboratories (Kumamoto, Japan); and U73122 from Funakoshi (Tokyo, Japan).

Statistical Analysis. All results were expressed as mean ± S.E.M.

Results

Basic Properties of the ET-1–Induced Increase in [Ca2+]i, in CHO-ETαR, CHO-ETαR385, and CHO-SerETαR. ET-1 induced a biphasic increase in [Ca2+]i in CHO-ETαR, consisting of an initial transient phase and a subsequent sustained phase (Fig. 1A). Both the transient and sustained increase in [Ca2+]i were dependent on the concentrations of ET-1 with EC50 values of approximately 1 nM, and they reached the maximal value at concentrations ≥10 nM (Fig. 1, D and E).

In CHO-ETαR385, which is coupled with Gα alone, ET-1 also induced a biphasic increase in [Ca2+]i (Fig. 1B). ET-1 caused a transient peak and subsequent sustained increase in [Ca2+]i (Fig. 2B). The magnitude of the transient increase in [Ca2+]i, in CHO-ETαR385 was essentially similar to that in CHO-ETαR (Fig. 1D). On the other hand, the magnitude of the sustained increase in [Ca2+]i, in CHO-ETαR385 was lower than that in CHO-ETαR (Fig. 1E). Moreover, an ET-1 concentration ≥10 nM induced a sustained increase in [Ca2+]i in CHO-ETαR385, whereas ET-1 induced an increase at only 0.1 nM in CHO-ETαR (Fig. 1E).

In CHO-SerETαR, which is coupled with Gα and G12, the pattern of the ET-1–induced increase in [Ca2+]i, was different from that in CHO-ETαR and CHO-ETαR385. That is, ET-1 failed to induce an initial transient increase in [Ca2+]i, and it induced only a sustained increase in [Ca2+]i (Fig. 1C). The magnitude of the sustained increase in [Ca2+]i, in CHO-SerETαR was lower than that in CHO-ETαR (Fig. 1E).

Pharmacological Identification of Ca2+ Channels Activated by ET-1 in CHO-ETαR, CHO-ETαR385, and CHO-SerETαR. As described previously (Kawanabe et al., 2001), in CHO-ETαR, the ET-1–induced sustained increase in [Ca2+]i, was partially suppressed by the maximally effective concentration (10 μM) of either SK&F 96365 or LOE 908, and it was abolished by combined treatment with both block-
ers (Fig. 2, A and D). In CHO-ET₅RΔ385, the ET-1–induced sustained increase in [Ca²⁺]ᵢ was completely inhibited by 10 μM SK&F 96365, whereas LOE 908 at concentrations up to 10 μM had no effects (Fig. 2, B and E). In CHO-SerET₅R, the ET-1–induced sustained increase in [Ca²⁺]ᵢ was completely inhibited by 10 μM LOE 908, whereas SK&F 96365 at concentrations up to 10 μM had no effects (Fig. 2, C and F).

Effects of Inhibition of PLC on the Species of ET-1–Activated Ca²⁺ Channels in CHO-ET₅R. In CHO-SerET₅R, coupling between the receptor and G₉ is missing, and hence, PLC as an effector of G₉ cannot be activated upon stimulation of the receptor. To mimic the stimulation in CHO-SerET₅R and confirm that PLC actually acts as an effector for activation of Ca²⁺ channels, we used U73122, a PLC blocker. Previous reports demonstrated that 5 to 10 μM U73122 inhibits PLC activation completely (Okamoto et al., 1995; Kanki et al., 2001). ET-1 stimulated [³H]inositol phosphates (IPs) formation in CHO-ET₅R (Kawanabe et al., 2001). On the other hand, ET-1 failed to induce [³H]IPs formation in CHO-ET₅R treated with 5 μM U73122 (data not shown). ET-1 at 10 nM induced only the sustained increase in [Ca²⁺]ᵢ in CHO-ET₅R treated with 5 μM U73122 (Fig. 3, A and B). The magnitude of the sustained increase in [Ca²⁺]ᵢ was approximately 20% of that in the absence of U73122. This sustained increase in [Ca²⁺]ᵢ was completely inhibited by 10 μM LOE 908, whereas SK&F 96365 at concentrations up to 10 μM had no effects (Fig. 3, A and B).

Fig. 1. Original tracings illustrating the effects of ET-1 on the increase in [Ca²⁺]ᵢ in CHO-ET₅R (A), CHO-ET₅RΔ385 (B), and CHO-SerET₅R (C). The cells were loaded with fluo-3 and stimulated with 10 nM ET-1 at the time indicated by horizontal bars. Effects of various concentrations of ET-1 on the transient increase in [Ca²⁺]ᵢ (D) and the sustained increase in [Ca²⁺]ᵢ (E) in CHO-ET₅R, CHO-ET₅RΔ385, and CHO-SerET₅R. Each point represents the mean ± S.E.M. of five experiments.

Fig. 2. Original tracings illustrating the effects of maximally effective concentration of LOE 908 and SK&F 96365 on the ET-1–induced sustained increase in [Ca²⁺]ᵢ in CHO-ET₅R (A), CHO-ET₅RΔ385 (B), and CHO-SerET₅R (C). The cells were loaded with fluo-3 and stimulated with 10 nM ET-1 at the time indicated by horizontal bars. After [Ca²⁺]ᵢ reached a steady-state, 10 μM LOE 908 or 10 μM SK&F 96365 was sequentially added, as indicated by horizontal bars. Effects of maximally effective concentration of LOE 908, SK&F 96365, and their combination on the ET-1–induced sustained increase in [Ca²⁺]ᵢ in CHO-ET₅R (D), CHO-ET₅RΔ385 (E), and CHO-SerET₅R (F). The experimental protocols were described under Materials and Methods, and the values of [Ca²⁺]ᵢ after the addition of 10 μM LOE 908 and/or 10 μM SK&F 96365 were determined. Each point represents the mean ± S.E.M. of five experiments.
Effects of Dibutyryl cAMP on the Resting [Ca\(^{2+}\)] and the ET-1–Induced Increase in [Ca\(^{2+}\)] in CHO-SerETAR. Because CHO-SerETAR is coupled with G\(_s\) and G\(_{12}\) (Kawanabe et al., 2002b), it is unknown which of the G proteins is involved in the activation of Ca\(^{2+}\) channels. To clarify whether G\(_s\) was involved in Ca\(^{2+}\) channel activation, we examined the effects of dibutyryl cAMP on the resting [Ca\(^{2+}\)] and the ET-1–induced increase in [Ca\(^{2+}\)]. A previous report demonstrated that 1 mM dibutyryl cAMP activates protein kinase A in CHO cells (Lee and Fraser, 1993). Dibutyryl cAMP at 1 mM failed to evoke an increase in [Ca\(^{2+}\)] in CHO-SerETAR (Fig. 3C). Moreover, the ET-1–induced sustained increase in [Ca\(^{2+}\)] was not affected by 1 mM dibutyryl cAMP in CHO-SerETAR (Fig. 3D). Dibutyryl cAMP also failed to affect the resting [Ca\(^{2+}\)] and the ET-1–induced increase in [Ca\(^{2+}\)] in CHO-ETAR (data not shown).

**Effects of G\(_{12}\) on the ET-1–Induced Sustained Increase in [Ca\(^{2+}\)] in CHO-ETAR or CHO-SerETAR.** To confirm that G\(_{12}\) is involved in the activation of Ca\(^{2+}\) channels, we investigated the effects of G\(_{12}\),G228A on the ET-1–induced increase in [Ca\(^{2+}\)], in CHO-ETAR and CHO-SerETAR. In this experiment, G\(_{12}\),G228A was microinjected into CHO-ETAR and CHO-SerETAR, and the ET-1–induced increase in [Ca\(^{2+}\)] in these cells was analyzed using microfluorimetry.

In CHO-ETAR microinjected with G\(_{12}\),G228A, ET-1 evoked transient and subsequently sustained increase in [Ca\(^{2+}\)]. The magnitude of the sustained increase in [Ca\(^{2+}\)] was approximately 40% of that in CHO-ETAR microinjected with an expression vector alone (data not shown). The sustained increase in [Ca\(^{2+}\)] was inhibited by 10 \(\mu\)M SK&F 96365, whereas it remained unaffected by 10 \(\mu\)M LOE 908 (Fig. 4A). ET-1 failed to induce a sustained increase in [Ca\(^{2+}\)] in CHO-SerETAR microinjected with G\(_{12}\),G228A (Fig. 4B).

**Discussion**

As reported previously (Kawanabe et al., 2001), the ET-1–induced sustained increase in [Ca\(^{2+}\)] in CHO-ETAR results from extracellular Ca\(^{2+}\) influx through three types of VICCs: NSCC-1, NSCC-2, and SOCC. Pharmacological identification of these Ca\(^{2+}\) channels and calculation for contribution of Ca\(^{2+}\) influx through each channel are explained schematically in Fig. 5. We have pharmacologically defined these channels in CHO cells expressing human recombinant ET-R and VSMCs expressing endogenous ET-R and have found that the same Ca\(^{2+}\) channels are activated in these cells (Kawanabe et al., 2001, 2002a).

In CHO-ETARΔ385, the only Ca\(^{2+}\) channels activated by ET-1 are SOCCs, judging from the sensitivity of the ET-1–induced sustained increase in [Ca\(^{2+}\)] to SK&F 96365 and LOE 908 (Fig. 2, B and E). Because CHO-ETARΔ385 is coupled with G\(_s\) but not with G\(_{12}\) (Kawanabe et al., 2002b), this result indicates that G\(_s\) is required (sufficient) for activation of SOCC.

Furthermore, activation of SOCC in CHO-ETAR is lost after treatment with U73122 (Fig. 3, A and B), indicating that PLC acts as an effector downstream of G\(_s\). These results, taken together, show that SOCC is activated via a G\(_s\)/PLC-dependent pathway.

Conversely, the results obtained from CHO-ETARΔ385 suggest that the activation of NSCC-1 and NSCC-2 requires G proteins other than G\(_s\). Because CHO-ETAR is coupled with G\(_s\) and G\(_{12}\) in addition to G\(_q\) (Aramori and Nakanishi, 1992; Kawanabe et al., 2002b), activation of NSCC-1 and NSCC-2 might be mediated by either G\(_s\) or G\(_{12}\). To address this point, we used CHO-SerETAR, which couples with G\(_s\) and G\(_{12}\) but not with G\(_q\) (Kawanabe et al., 2002b). In CHO-SerETAR, ET-1 activated NSCC-1 but not NSCC-2, because of the pharmacology of the sustained increase in [Ca\(^{2+}\)], (sensitive to LOE 908 and resistant to SK&F 96365) (Fig. 3, C and D). These results indicate that either G\(_s\) or G\(_{12}\) is required for activation of NSCC-1, whereas either G\(_s\) or G\(_{12}\) or both are not sufficient for activation of NSCC-2. Regarding a
mechanism for activation of NSCC-1, dibutyryl cAMP alone was without effect in the resting [Ca\textsuperscript{2+}]i, and the ET-1-induced sustained increase in [Ca\textsuperscript{2+}]i in CHO-SerET\textsubscript{AR} (Fig. 3), excluding the possibility that activation of NSCC-1 is mediated by cAMP, a product of G\textsubscript{i} adenylate cyclase-dependent pathway. Moreover, disruption of signaling through endogenous G\textsubscript{12}, by its dominant-negative mutant (G\textsubscript{12}G228A) abrogated activation of NSCC-1 in CHO-SerET\textsubscript{AR} as well as CHO-ET\textsubscript{AR} (Fig. 4), indicating that activation of NSCC-1 is mediated by G\textsubscript{12}. Taken together, these results strongly demonstrate that NSCC-1 is activated via a G\textsubscript{12}-dependent pathway.

As a mechanism for activation of NSCC-2, the channel was not activated in CHO-ET\textsubscript{AR} pretreated with U73122 or in CHO-ET\textsubscript{AR} microinjected with G\textsubscript{12}G228A (Figs. 3 and 4). These results indicate that both a G\textsubscript{q}/PLC-dependent pathway and a G\textsubscript{12}-dependent pathway are required for activation of NSCC-2. In accordance with this conclusion, NSCC-2 was not activated after the stimulation of ET\textsubscript{AR} lacking coupling with either G\textsubscript{q} or G\textsubscript{12}, i.e., SerET\textsubscript{AR} and ET\textsubscript{AR}Δ385.

Signaling mechanisms downstream of G proteins are presently unknown. In the case of G\textsubscript{q}, it is very likely that PLC is activated downstream of G\textsubscript{q}, considering that CHO-ET\textsubscript{AR} treated with U73122 (an inhibitor of PLC) mimicked CHO-SerET\textsubscript{AR} (which lost the ability to couple with G\textsubscript{q}) in terms of temporal pattern and magnitude of the ET-1–induced changes in [Ca\textsuperscript{2+}]i and species of activated channels. Stimulation of PLC leads to increased formation of inositol-1,4,5-trisphosphate and diacylglycerol (DAG). Inositol-1,4,5-trisphosphate acts on its receptor on sarcoplasmic reticulum as an intracellular Ca\textsuperscript{2+} store to release Ca\textsuperscript{2+} and subsequently deplete the store (Berridge, 1993). Thus, the store depletion could be a trigger for activation of the Ca\textsuperscript{2+} channel on the plasma membrane called capacitative Ca\textsuperscript{2+} channel (Thasreup et al., 1980). In fact, SOCC is activated in CHO-ET\textsubscript{AR} and VSMCs, after depletion of the Ca\textsuperscript{2+} store by treatment with thapsigargin (an inhibitor of Ca\textsuperscript{2+}-pump ATPase on the membrane of sarcoplasmic reticulum) (Kawanabe et al., 2001, 2002a). The concentrations of ET-1 that failed to induce a transient increase in [Ca\textsuperscript{2+}]i, and IP accumulation did not induce SOCC activation (Kawanabe et al., 2001). Judging from these data, we concluded that the transient increase in [Ca\textsuperscript{2+}]i (depletion of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} store) essentially follows the characteristics of SOCC. On the other hand, another second messenger, DAG, can directly activate Ca\textsuperscript{2+} channels such as transient receptor potential (Hofmann et al., 1999). Thus, DAG might be an effector downstream of G\textsubscript{q}. In the case of G\textsubscript{12}, several kinds of proteins are reported to be its effectors (Seasholtz et al., 1999). We have recently shown that stimulation of CHO-ET\textsubscript{AR} with ET-1 induces actin stress fiber formation through G\textsubscript{12}, and that downstream of G\textsubscript{12}, a small GTP-binding protein Rho and p-associate coiled-coil–forming protein kinase is activated (Kawanabe et al., 2002b). Thus Rho/Rho-associated coiled-coil–forming protein kinase might be a signal downstream of G\textsubscript{12}. Additional study is needed to identify the effectors downstream of G\textsubscript{q} and G\textsubscript{12}, for activation of NSCC-1, NSCC-2, and SOCC.

In summary, stimulation of ET\textsubscript{AR} with ET-1 activates NSCC-1, NSCC-2, and SOCC in CHO-ET\textsubscript{AR}. NSCC-1 is activated via a G\textsubscript{12}-dependent pathway, NSCC-2 is activated via G\textsubscript{q} and G\textsubscript{12}-dependent pathways, and SOCC is activated via a G\textsubscript{q}-dependent pathway.

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