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Molecular Mechanisms for the Activation of Voltage-Independent Ca^{2+} Channels by Endothelin-1 in Chinese Hamster Ovary Cells Stably Expressing Human $Endothelin_A$ Receptors

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ABSTRACT

We demonstrated recently that in Chinese hamster ovary cells stably expressing human recombinant $endothelin_A$ receptors ($CHO\text{-}ET_AR$), endothelin-1 (ET-1) activates two types of Ca^{2+}-permeable nonselective cation channels (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC), which can be distinguished by Ca^{2+} channel blockers such as 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1*H*-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 $CHO\text{-}ET_AR$ couples with G_{12} in addition to G_q and G_s. The purpose of the present study was to identify the G proteins involved in the activation of these Ca^{2+} channels by ET-1, using mutated ET_ARs with coupling to either G_q or G_s/G_{12} (designated $ET_AR\Delta385$ and $SerET_AR$, respectively) and a dominant-negative mutant of G_{12} ($G_{12}G228A$). $ET_AR\Delta385$ is truncated immediately downstream of Cys^{385} in the C terminus as palmitoylation sites, whereas $SerET_AR$ is unpalmitoylated because of substitution of all the cysteine residues to serine ($Cys^{383}Cys^{385–388} \rightarrow Ser^{383}Ser^{385–388}$). In $CHO\text{-}ET_AR\Delta385$, stimulation with ET-1 activated only SOCC. In $CHO\text{-}SerET_AR$ or $CHO\text{-}ET_AR$ pretreated with U73122, an inhibitor of phospholipase C (PLC), ET-1 activated only NSCC-1. Dibutyryl cAMP alone did not activate any Ca^{2+} channels in the resting and ET-1–stimulated $CHO\text{-}SerET_AR$. Microinjection of $G_{12}G228A$ abolished the activation of NSCC-1 and NSCC-2 in $CHO\text{-}ET_AR$ and that of NSCC-1 in $CHO\text{-}SerET_AR$. These results indicate that ET_AR activates three types of Ca^{2+} channels via different G protein-related pathways. NSCC-1 is activated via a G_{12}-dependent pathway, NSCC-2 via G_q/PLC- and G_{12}-dependent pathways, and SOCC via a G_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^{2+} influx through voltage-independent Ca^{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: $endothelin_A$ and $endothelin_B$ receptors (ET_ARs and ET_BRs, 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^{2+} channel by ET-1.

Transfection and functional expression of wild-type or mutant ET_AR cDNAs into the same cell type provides a model

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ABBREVIATIONS: ET-1, endothelin-1; VICC, voltage-independent Ca^{2+} channel; VSMC, vascular smooth muscle cell; ET_AR, $endothelin_A$ receptor; ET_BR, $endothelin_B$ receptor; CHO, Chinese hamster ovary; NSCC, nonselective cation channel; SOCC, store-operated Ca^{2+} channel; PLC, phospholipase C; SK&F 96365, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenylethyl}-1*H*-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-(6-{[17β-3-methoxyoestra-1,3,5(10)-trien-17-yl]amino}hexyl)-1*H*-pyrrole-2,5-dione; IP, inositol phosphate; DAG, diacylglyceride; $G_{12}G228A$, dominant-negative mutant of G_{12}; $CHO\text{-}SerET_AR$, Chinese hamster ovary cells that express an unpalmitoylated ($Cys^{383}Cys^{385–388} \rightarrow Ser^{383}Ser^{385–388}$) human $endothelin_A$ receptor; $CHO\text{-}ET_AR\Delta385$, Chinese hamster ovary cells that express human $endothelin_A$ receptor truncated at the carboxyl-terminal downstream of Cys385; $CHO\text{-}ET_AR$, Chinese hamster ovary cells stably expressing human $endothelin_A$ 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 ET_ARs in this study. We have recently shown that a sustained increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) caused by ET-1 results from Ca^{2+} entry through three types of VICCs into CHO cells stably expressing wild-type ET_AR (CHO-ET_AR): two types of Ca^{2+}-permeable nonselective cation channels (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC) (Kawanabe et al., 2001). In particular, these channels can be distinguished using Ca^{2+} 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-ET_AR are pharmacologically identical to those in VSMCs (Kawanabe et al., 2002a). Therefore, CHO-ET_AR may be a good model for studying the mechanism of activation of VICCs. ET_ARs are functionally coupled with G_q and G_s in CHO cells (Aramori and Nakanishi, 1992). Activation of G_q and G_s causes stimulation of phospholipase C (PLC) and adenylyl cyclase, respectively (Aramori and Nakanishi, 1992). In addition, ET_ARs also couple with G_{12} 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 G_{12} and two types of mutated ET_ARs designated $ET_AR\Delta385$ and $SerET_AR$ to clarify the involvement of G_q, G_s, and G_{12} for Ca^{2+} channel activation by ET-1. $ET_AR\Delta385$ lacks a C terminus downstream of Cys^{385} and couples only with G_q in CHO cells (Kawanabe et al., 2002b). $SerET_AR$ is unpalmitoylated because of substitution of all the cysteine residues to serine ($Cys^{383}Cys^{385–388} \rightarrow Ser^{383}Ser^{385–388}$) and couples with G_s and G_{12} in CHO cells (Kawanabe et al., 2002b).

Experimental Procedures

Cell Culture. We used CHO-ET_AR, CHO-$ET_AR\Delta385$, and CHO-$SerET_AR$, which were constructed as described previously (Kawanabe et al., 2002b). The K_d (picomolar) and B_{max} (picomoles per milligram of protein) values for CHO-ET_AR, CHO-$ET_AR\Delta385$, and CHO-$SerET_AR$ 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 under a humidified 5% CO_2/95% air atmosphere.

Measurement of Intracellular Free Ca^{2+} Concentration. $[Ca^{2+}]_i$ was measured using the fluorescent probe fluo-3. The measurement of fluorescence by a CAF 110 spectrophotometer (Jasco, Tokyo, Japan) was performed as described previously (Kawanabe et al., 2001).

Microfluorimetry of fluo-3 was performed as described previously (Zhang et al., 1999). Briefly, the cells seeded on 35-mm glass-bottomed plastic dishes (MatTek, Ashland, MA), which were marked with a cross to facilitate the localization of injected cells, were loaded with fluo-3 by incubating them with Ca^{2+}-free Krebs-HEPES solution containing 10 μM fluo-3/AM for 30 min at 37°C under a reduced light. Ca^{2+}-free Krebs-HEPES solution contained 140 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 11 mM glucose, and 10 mM HEPES, pH 7.4, adjusted with NaOH. After washing with Krebs-HEPES solution (2.2 mM $CaCl_2$ was added to Ca^{2+}-free Krebs-HEPES solution), they were kept in fresh Krebs-HEPES solution at 37°C for at least 30 min. Fluo-3 microfluorimetry was performed at 25°C by use of an Atto-fluor Ratio-Vision real-time digital fluorescence analyzer (Atto Instruments, Rockville, MD) equipped with an Axiovert-100 inverted epifluorescent microscope (Carl Zeiss Inc., Thornwood, NY). A 100-W mercury lamp served as the source of excitation. For measurement of $[Ca^{2+}]_i$, fluo-3 was excited at 450 to 490 nm, and fluorescence was detected at 515 to 565 nm. At the end of the experiment, ionomycin and subsequently EGTA were added at final concentrations of 10 μM and 10 mM, respectively, to obtain the fluorescence intensity maximum (F_{max}) and the fluorescence intensity minimum (F_{min}). $[Ca^{2+}]_i$ was determined from the equilibrium equation $[Ca^{2+}]_i = K_d(F - F_{min}) / (F_{max} - F)$, where F was the experimental value of fluorescence and K_d was defined as 0.4 μM (Minta et al., 1989).

Microinjection. For microinjection, cells were seeded onto glass coverslips coated with fibronectin (Iwaki Glass, Chiba, Japan), which were marked with a cross to facilitate the localization of injected cells, and were incubated overnight in Ham's F12 medium containing 1% fetal calf serum. Microinjection of G_{12}G228A, 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 Dojin 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 $[Ca^{2+}]_i$ in CHO-ET_AR, CHO-$ET_AR\Delta385$, and CHO-$SerET_AR$. ET-1 induced a biphasic increase in $[Ca^{2+}]_i$ in CHO-ET_AR, consisting of an initial transient phase and a subsequent sustained phase (Fig. 1A). Both the transient and sustained increase in $[Ca^{2+}]_i$ were dependent on the concentrations of ET-1 with EC_{50} values of approximately 1 nM, and they reached the maximal value at concentrations ≥10 nM (Fig. 1, D and E).

In CHO-$ET_AR\Delta385$, which is coupled with G_q alone, ET-1 also induced a biphasic increase in $[Ca^{2+}]_i$ (Fig. 1B). ET-1 caused a transient peak and subsequent sustained increase in $[Ca^{2+}]_i$ (Fig. 2B). The magnitude of the transient increase in $[Ca^{2+}]_i$ in CHO-$ET_AR\Delta385$ was essentially similar to that in CHO-ET_AR (Fig. 1D). On the other hand, the magnitude of the sustained increase in $[Ca^{2+}]_i$ in CHO-$ET_AR\Delta385$ was lower than that in CHO-ET_AR (Fig. 1E). Moreover, an ET-1 concentration ≥ 10 nM induced a sustained increase in $[Ca^{2+}]_i$ in CHO-$ET_AR\Delta385$, whereas ET-1 induced an increase at only 0.1 nM in CHO-ET_AR (Fig. 1E).

In CHO-$SerET_AR$, which is coupled with G_s and G_{12}, the pattern of the ET-1–induced increase in $[Ca^{2+}]_i$ was different from that in CHO-ET_AR and CHO-$ET_AR\Delta385$. That is, ET-1 failed to induce an initial transient increase in $[Ca^{2+}]_i$, and it induced only a sustained increase in $[Ca^{2+}]_i$ (Fig. 1C). The magnitude of the sustained increase in $[Ca^{2+}]_i$ in CHO-$SerET_AR$ was lower than that in CHO-ET_AR (Fig. 1E).

Pharmacological Identification of Ca^{2+} Channels Activated by ET-1 in CHO-ET_AR, CHO-$ET_AR\Delta385$, and CHO-$SerET_AR$. As described previously (Kawanabe et al., 2001), in CHO-ET_AR, the ET-1–induced sustained increase in $[Ca^{2+}]_i$ 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-$ET_AR\Delta385$, the ET-1–induced sustained increase in $[Ca^{2+}]_i$ 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-SerET_AR, the ET-1–induced sustained increase in $[Ca^{2+}]_i$ 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 Ca^{2+} Channels in CHO-ET_AR. In CHO-SerET_AR, coupling between the receptor and G_q is missing, and hence, PLC as an effector of G_q cannot be activated upon stimulation of the receptor. To mimic the stimulation in CHO-SerET_AR and confirm that PLC actually acts as an effector for activation of Ca^{2+} 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 [^{3}H]inositol phosphates (IPs) formation in CHO-ET_AR (Kawanabe et al., 2001). On the other hand, ET-1 failed to induce [^{3}H]IPs formation in CHO-ET_AR treated with 5 μM U73122 (data not shown). ET-1 at 10 nM induced only the sustained increase in $[Ca^{2+}]_i$ in CHO-ET_AR treated with 5 μM U73122 (Fig. 3, A and B). The magnitude of the sustained increase in $[Ca^{2+}]_i$ was approximately 20% of that in the absence of U73122. This sustained increase in $[Ca^{2+}]_i$ 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).

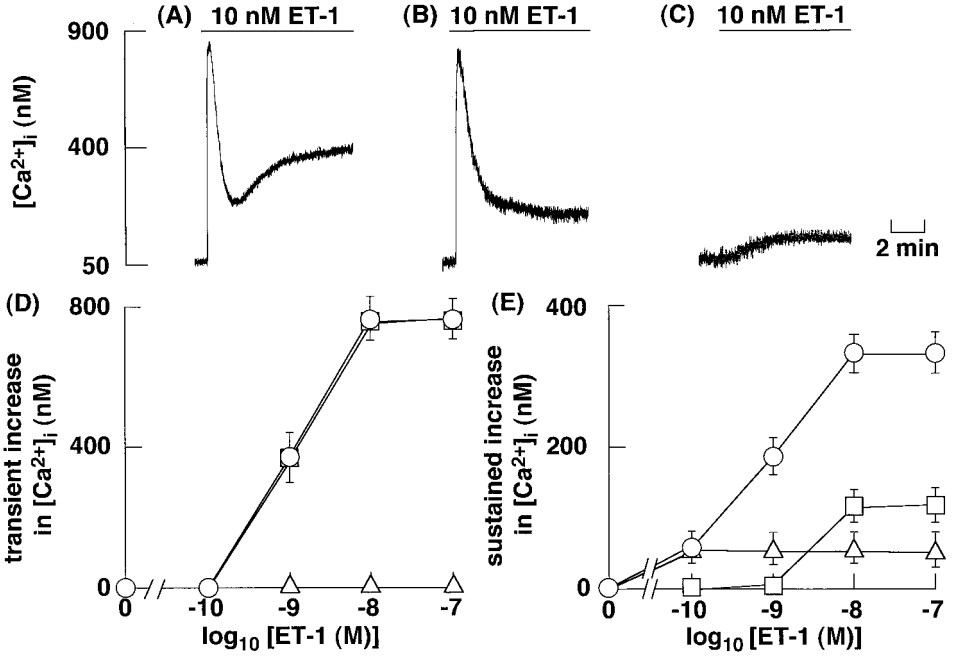

Fig. 1. Original tracings illustrating the effects of ET-1 on the increase in $[Ca^{2+}]_i$ in CHO-ET_AR (A), CHO-$ET_AR\Delta385$ (B), and CHO-SerET_AR (C). The cells were loaded with fluo-3 and stimulated with 10 nM ET-1 at the time indicated by horizontal bars. Effects of various concentrations of ET-1 on the transient increase in $[Ca^{2+}]_i$ (D) and the sustained increase in $[Ca^{2+}]_i$ (E) in CHO-ET_AR, CHO-$ET_AR\Delta385$, and CHO-SerET_AR. ○, CHO-ET_AR; □, CHO-$ET_AR\Delta385$, and △, CHO-SerET_AR. Each point represents the mean ± S.E.M. of five experiments.

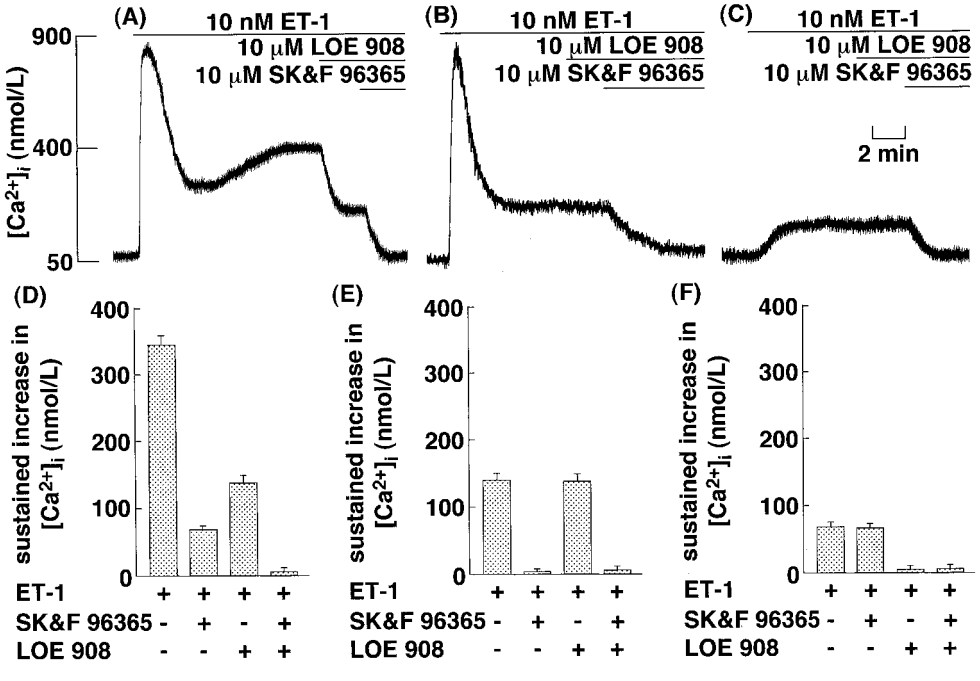

Fig. 2. Original tracings illustrating the effects of maximally effective concentration of LOE 908 and SK&F 96365 on the ET-1–induced sustained increase in $[Ca^{2+}]_i$ in CHO-ET_AR (A), CHO-$ET_AR\Delta385$ (B), and CHO-SerET_AR (C). The cells were loaded with fluo-3 and stimulated with 10 nM ET-1 at the time indicated by horizontal bars. After $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ in CHO-ET_AR (D), CHO-$ET_AR\Delta385$ (E), and CHO-SerET_AR (F). The experimental protocols were described under *Materials and Methods,* and the values of $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ and the ET-1–Induced Increase in $[Ca^{2+}]_i$ in CHO-SerET$_A$R. Because CHO-SerET$_A$R 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 $[Ca^{2+}]_i$ and the ET-1–induced increase in $[Ca^{2+}]_i$. 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 $[Ca^{2+}]_i$ in CHO-SerET$_A$R (Fig. 3C). Moreover, the ET-1–induced sustained increase in $[Ca^{2+}]_i$ was not affected by 1 mM dibutyryl cAMP in CHO-SerET$_A$R (Fig. 3D). Dibutyryl cAMP also failed to affect the resting $[Ca^{2+}]_i$ and the ET-1–induced increase in $[Ca^{2+}]_i$ in CHO-ET$_A$R (data not shown).

Effects of G_{12} on the ET-1–Induced Sustained Increase in $[Ca^{2+}]_i$ in CHO-ET$_A$R or CHO-SerET$_A$R. 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 $[Ca^{2+}]_i$ in CHO-ET$_A$R and CHO-SerET$_A$R. In this experiment, G_{12}G228A was microinjected into CHO-ET$_A$R and CHO-SerET$_A$R, and the ET-1–induced increase in $[Ca^{2+}]_i$ in these cells was analyzed using microfluorimetry.

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

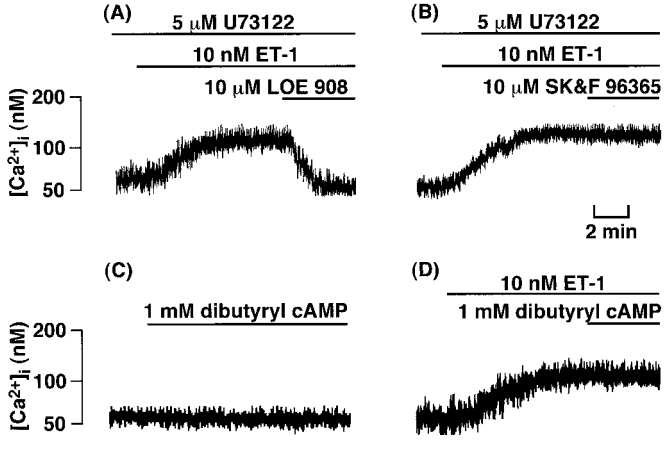

Fig. 3. Original tracings illustrating the effects of maximally effective concentration of LOE 908 (A) and SK&F 96365 (B) on the ET-1–induced sustained increase in $[Ca^{2+}]_i$ in CHO-ET$_A$R pretreated with U73122. The cells loaded with fluo-3 were incubated with 5 μM U73122 for 10 min before stimulation with 10 nM ET-1. After $[Ca^{2+}]_i$ reached a steady state, 10 μM LOE 908 or 10 μM SK&F 96365 was added at the time indicated by horizontal bars. Original tracings illustrating the effects of dibutyryl cAMP on the resting $[Ca^{2+}]_i$ (C) and the ET-1–induced sustained increase in $[Ca^{2+}]_i$ (D) in CHO-SerET$_A$R. C, the cells loaded with fluo-3 were stimulated by 1 mM dibutyryl cAMP at the time indicated by the horizontal bar. D, the cells loaded with fluo-3 were stimulated by 10 nM ET-1. After $[Ca^{2+}]_i$ reached a steady state, 1 mM dibutyryl cAMP was added at the time indicated by the horizontal bar.

Discussion

As reported previously (Kawanabe et al., 2001), the ET-1–induced sustained increase in $[Ca^{2+}]_i$ in CHO-ET$_A$R 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$_A$R and VSMCs expressing endogenous ET$_A$R and have found that the same Ca^{2+} channels are activated in these cells (Kawanabe et al., 2001, 2002a).

In CHO-ET$_A$RΔ385, the only Ca^{2+} channels activated by ET-1 are SOCCs, judging from the sensitivity of the ET-1–induced sustained increase in $[Ca^{2+}]_i$ to SK&F 96365 and LOE 908 (Fig. 2, B and E). Because CHO-ET$_A$RΔ385 is coupled with G_q but not with G_s or G_{12} (Kawanabe et al., 2002b), this result indicates that G_q is required (sufficient) for activation of SOCC. Furthermore, activation of SOCC in CHO-ET$_A$R is lost after treatment with U73122 (Fig. 3, A and B), indicating that PLC acts as an effector downstream of G_q. These results, taken together, show that SOCC is activated via a G_q/PLC-dependent pathway.

Conversely, the results obtained from CHO-ET$_A$RΔ385 suggest that the activation of NSCC-1 and NSCC-2 requires G proteins other than G_q. Because CHO-ET$_A$R 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-SerET$_A$R, which couples with G_s and G_{12} but not with G_q (Kawanabe et al., 2002b). In CHO-SerET$_A$R, ET-1 activated NSCC-1 but not NSCC-2, because of the pharmacology of the sustained increase in $[Ca^{2+}]_i$ (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, G_{12}, or both are not sufficient for activation of NSCC-2. Regarding a

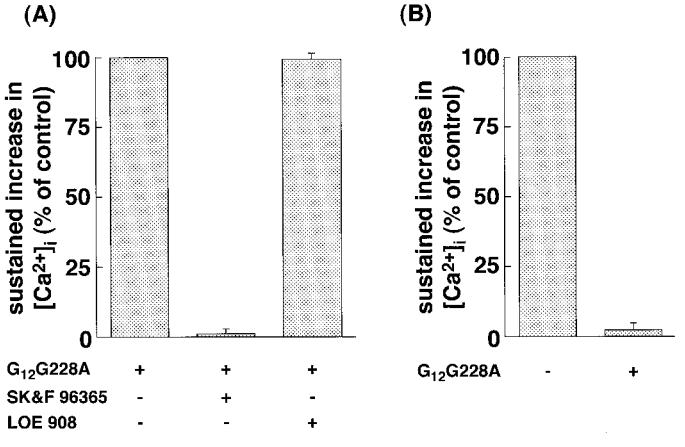

Fig. 4. A, effects of a maximally effective concentration of SK&F 96365 (10 μM) or LOE 908 (10 μM) on ET-1–induced sustained increase in $[Ca^{2+}]_i$ in CHO-ET$_A$R microinjected with G_{12}G228A. The sustained increase in $[Ca^{2+}]_i$ in the presence of SK&F 96365 or LOE 908 is represented as a percentage of values in its absence. B, effects of G_{12}G228A on ET-1–induced sustained increase in $[Ca^{2+}]_i$ in CHO-SerET$_A$R. The cells loaded with fluo-3 were stimulated by 10 nM ET-1. The sustained increase in $[Ca^{2+}]_i$ in the presence of G_{12}G228A is represented as a percentage of values in its absence. Data are presented as the mean ± S.E.M. of three experiments.

mechanism for activation of NSCC-1, dibutyryl cAMP alone was without effect in the resting $[Ca^{2+}]_i$ and the ET-1–induced sustained increase in $[Ca^{2+}]_i$ in CHO-SerET$_A$R (Fig. 3), excluding the possibility that activation of NSCC-1 is mediated by cAMP, a product of G_s/adenylate cyclase-dependent pathway. Moreover, disruption of signaling through endogenous G_{12} by its dominant-negative mutant (G_{12}G228A) abrogated activation of NSCC-1 in CHO-SerET$_A$R as well as CHO-ET$_A$R (Fig. 4), indicating that activation of NSCC-1 is mediated by G_{12}. Taken together, these results strongly demonstrate that NSCC-1 is activated via a G_{12}-dependent pathway.

As for a mechanism for activation of NSCC-2, the channel was not activated in CHO-ET$_A$R pretreated with U73122 or in CHO-ET$_A$R microinjected with G_{12}G228A (Figs. 3 and 4). These results indicate that both a G_q/PLC-dependent pathway and a G_{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$_A$R lacking coupling with either G_q or G_{12}, i.e., SerET$_A$R and ET$_A$RΔ385.

Signaling mechanisms downstream of G proteins are presently unknown. In the case of G_q, it is very likely that PLC is activated downstream of G_q, considering that CHO-ET$_A$R treated with U73122 (an inhibitor of PLC) mimicked CHO-SerET$_A$R (which lost the ability to couple with G_q) in terms of temporal pattern and magnitude of the ET-1–induced changes in $[Ca^{2+}]_i$ 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^{2+} store to release Ca^{2+} and subsequently deplete the store (Berridge, 1993). Thus, the store depletion could be a trigger for activation of the Ca^{2+} channels on the plasma membrane called capacitative Ca^{2+} channel (Thasreup et al., 1990). In fact, SOCC is activated in CHO-ET$_A$R and VSMCs, after depletion of the Ca^{2+} store by treatment with thapsigargin (an inhibitor of Ca^{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 $[Ca^{2+}]_i$ and IP accumulation did not induce SOCC activation (Kawanabe et al., 2001). Judging from these data, we concluded that the transient increase in $[Ca^{2+}]_i$ (depletion of Ca^{2+} from intracellular Ca^{2+} store) essentially follows the characteristics of SOCC. On the other hand, another second messenger, DAG, can directly activate Ca^{2+} channels such as transient receptor potential (Hofmann et al., 1999). Thus, DAG might be an effector downstream of G_q. In the case of G_{12}, several kinds of proteins are reported to be its effectors (Seasholtz et al., 1999). We have recently shown that stimulation of CHO-ET$_A$R with ET-1 induces actin stress fiber formation through G_{12}, and that downstream of G_{12}, a small GTP-binding protein Rho and ρ-associated 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_{12}. Additional study is needed to identify the effectors downstream of G_q and G_{12} for activation of NSCC-1, NSCC-2, and SOCC.

In summary, stimulation of ET$_A$R with ET-1 activates NSCC-1, NSCC-2, and SOCC in CHO-ET$_A$R. NSCC-1 is activated via a G_{12}-dependent pathway, NSCC-2 is activated via G_q- and G_{12}-dependent pathways, and SOCC is activated via a G_q-dependent pathway.

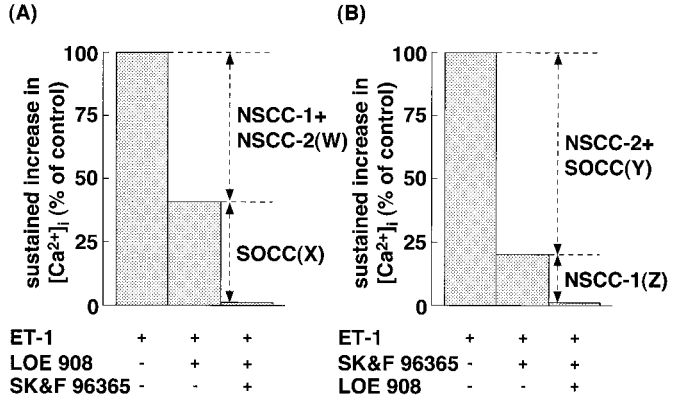

Fig. 5. A and B, pharmacological identification of three types of voltage-independent Ca^{2+} channels activated by 10 nM ET-1 and calculation for contribution of Ca^{2+} influx through each channel in CHO-ET$_A$R. The ET-1–induced sustained increase in $[Ca^{2+}]_i$ in the presence of 10 μM LOE 908 and/or 10 μM SK&F 96365 are represented as a percentage of values in its absence. The contributions of SOCC and NSCC-1 are represented as X and Z, respectively. The contribution of NSCC-2 is represented as W-Z or Y-X.

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