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Characterization of Ca^{2+} Channels and G Proteins Involved in Arachidonic Acid Release by Endothelin-1/Endothelin$_A$ Receptor

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ABSTRACT

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) in Chinese hamster ovary cells expressing endothelin$_A$ receptors (CHO-ET$_A$R). These channels can be distinguished by their sensitivity to Ca^{2+} channel blockers 1-(β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl)-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). NSCC-1 is sensitive to LOE 908 and resistant to SK&F 96365; NSCC-2 is sensitive to both blockers, and SOCC is resistant to LOE 908 and sensitive to SK&F 96365. In this study, we examined the mechanism of ET-1–induced arachidonic acid (AA) release. Both SK&F 96365 and LOE 908 inhibited ET-1–induced AA release with the IC_{50} values correlated to those of ET-1–induced Ca^{2+} influx. Moreover, combined treatment with these blockers abolished ET-1–induced AA release. Wortmannin and LY294002, inhibitors of phosphoinositide 3-kinase (PI3K), partially inhibited ET-1–induced AA release. LOE 908, but not SK&F 96365, inhibited ET-1–induced AA release in wortmannin-treated CHO-ET$_A$R. ET-1 also induced AA release in CHO cells expressing ET$_A$R truncated at the carboxyl terminal downstream of Cys385 (CHO-ET$_A$RΔ385) or an unpalmitoylated ($Cys^{383}Cys^{385–388}$→$Ser^{383}Ser^{385–388}$) ET$_A$R (CHO-SerET$_A$R), each of which is coupled with G_q or G_s/G_{12}, respectively. In CHO-SerET$_A$R, a dominant-negative mutant of G_{12} inhibited AA release. SK&F 96365 inhibited ET-1–induced AA release in CHO-ET$_A$RΔ385, whereas LOE 908 inhibited it in CHO-SerET$_A$R. These results indicate the following: 1) ET-1–induced AA release depends on Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC in CHO-ET$_A$R; 2) G_q and G_{12} mediate AA release through ET$_A$R in CHO cells; and 3) PI3K is involved in ET-1–induced AA release, which depends on NSCC-2 and SOCC.

The release of arachidonic acid (AA) from the membrane lipids is catalyzed by phospholipase A_2 (PLA_2) in mammalian cells (Dennis, 1997). Hormones and growth factors including endothelin-1 (ET-1) stringently regulate PLA_2 activity (Dennis, 1997; Leslie, 1997; Trevisi et al., 2002). AA is converted into other biologically active metabolites such as leukotrienes, lipoxins, prostaglandins, and thromboxanes by different enzymes. These metabolites seem to play significant roles in several important processes, including vascular contraction and cell growth (Gong et al., 1995; Anderson et al., 1997). Previous reports indicate that the key enzyme responsible for agonist-induced AA release is cytosolic PLA_2 (cPLA$_2$) (Lin et al., 1992; Roshak et al., 1994). ET-1 also induces AA release through cPLA$_2$ activation (Trevisi et al., 2002). cPLA$_2$ is a cytosolic 85-kDa Ca^{2+}-dependent PLA_2 and is activated by both an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and Ser-505 phosphorylation by mitogen-activated protein kinase or protein kinase C (Leslie, 1997). Extracellular Ca^{2+} influx plays critical roles in the ET-1–induced AA release (Stanimirovic et al., 1994; Wu-Wong et al., 1996). However, it remains unclear what types of Ca^{2+} channels are

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ABBREVIATIONS: AA, arachidonic acid; $AACOCF_3$, arachydonyl trifluoromethyl ketone; CHO, Chinese hamster ovary; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CHO-ET$_A$R, Chinese hamster ovary cells expressing endothelin$_A$ receptors; CHO-ET$_A$RΔ385, Chinese hamster ovary cells that express human endothelin$_A$ receptor truncated at the carboxyl-terminal downstream of Cys385; CHO-SerET$_A$R, Chinese hamster ovary cells that express an unpalmitoylated ($Cys^{383}Cys^{385–388}$→$Ser^{383}Ser^{385–388}$) human endothelin$_A$ receptor; cPLA$_2$, cytosolic phospholipase A_2; ET-1, endothelin-1; G_{12}G228A, dominant-negative mutant of G_{12}; NSCC, nonselective cation channel; PI3K, phosphoinositide 3-kinase; PLA_2, phospholipase A_2; SOCC, store-operated Ca^{2+} channel; VICC, voltage-independent Ca^{2+} channel; SK&F 96365, 1-(β-[3-(4-methoxyphenyl) propoxy]-4-methoxyphenethyl)-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; LY294002, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; BQ123, cyclo(D-Trp-D-Asp-Pro-D-Val-Leu-)Na^+; BQ788, 2-6-dimethylpiperidinecarbonyl-γ-methyl-Leu-N_{in}-[methoxycarbonyl]-D-Trp-D-Nle.

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involved in ET-1–induced AA release. These uncertainties are mainly caused by the lack of specific Ca^{2+}-channel blockers. We have recently shown that a sustained increase in $[Ca^{2+}]_i$ caused by ET-1 results from Ca^{2+} entry through three types of voltage-independent Ca^{2+} channel (VICC) into CHO cells expressing 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. 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). Thus, SK&F 96365 and LOE 908 may be useful for identifying which Ca^{2+} channels are involved in ET-1–induced AA release in CHO-ET_AR. Moreover, phosphoinositide 3-kinase (PI3K) was reported to be involved in the angiotensin II-induced $cPLA_2$ activation and AA release in vascular smooth muscle cells (Silfani and Freeman, 2002). PI3K plays essential roles in the activation of NSCC-2 and SOCC by ET-1 in CHO-ET_AR (Kawanabe et al., 2002a). Therefore, we examined the effects of PI3K on ET-1–induced AA release in CHO-ET_AR.

Biological actions of ET-1 are mediated by two distinct receptor subtypes, ET_AR and ET_BR, that belong to a family of G protein-coupled receptors (Arai et al., 1990; Sakurai et al., 1990). ET_AR are functionally coupled with G_q, G_s, and G_{12} in CHO cells (Aramori and Nakanishi, 1992; Kawanabe et al., 2002c). Therefore, in the present study, we investigated which G protein subtypes were involved in ET-1–induced AA release. For this purpose, we used a dominant-negative mutant of G_{12} (G_{12}G228A) and two types of mutated ET_AR designated $ET_AR\Delta385$ and $SerET_AR$ to clarify the involvement of G_q, G_s, and G_{12} in ET-1–induced AA release. $ET_AR\Delta385$ lacks a C terminus downstream of Cys^{385} and couples only with G_q in CHO cells (Kawanabe et al., 2002c). $SerET_AR$ is unpalmitoylated because of substitution of all of the cysteine-to-serine residues ($Cys^{383}Cys^{385–388}$→$Ser^{383}Ser^{385–388}$) and couples with G_s and G_{12} in CHO cells (Kawanabe et al., 2002c). Moreover, ET-1 activates SOCC in CHO-$ET_AR\Delta385$ and NSCC-1 in CHO-$SerET_AR$ (Kawanabe et al., 2002b).

Materials and Methods

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,c). CHO cells were maintained in Ham's F-12 medium supplemented with 10% fetal calf serum under a humidified 5% CO_2/95% air atmosphere.

[^{3}H]Arachidonic Acid Release. The level of [^{3}H]arachidonic acid release was determined as described previously (Perez et al., 1993). Briefly, cells in 100-mm dishes were incubated overnight with [^{3}H]arachidonic acid (final concentration, 1 μCi/ml). After washing, ET-1 was added for 5 min. The medium was then removed, acidified with 100 μl of 1 N formic acid, and extracted with 3 ml of chloroform. The extracts were evaporated to dryness, resuspended in 50 μl of chloroform, and applied to silica gel plates for thin-layer chromatography (Merck, Darmstadt, Germany). The plates were developed in heptane/diethyl ether/acetic acid/water (v/v, 75:25:4). The distance of movement was visualized with iodine vapor. The location of arachidonic acid was verified with the use of a purified arachidonic acid (PerkinElmer Life Sciences, Boston, MA). The plate was scraped, and the radioactivity was counted with use of a liquid scintillation counter.

Transfection of G_{12}G228A. We used G_{12}G228A, which was constructed as described previously (Kawanabe et al., 2002b,c). For transient expression, cells were transfected with plasmid (100 ng/μl) encoding for G_{12}G228A by the MBS Mammalian Transfection Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. After 24 h of incubation, we used these cells for measurement of [^{3}H]arachidonic acid release.

Drugs. LOE 908 was kindly provided by Boehringer Ingelheim GmbH (Ingelheim, Germany). All other chemicals were of reagent grade and were obtained commercially.

Statistical Analysis. All results were expressed as mean ± S.E.M. The data were subjected to a two-way analysis of variance. When a significant F value was encountered, the Newman-Keuls multiple range test was used to test for significant differences between treatment groups. A probability level of $P < 0.05$ was considered statistically significant.

Results

Effects of ET-1 on AA Release in CHO-ET_AR. ET-1 induced AA release in a concentration-dependent manner with an EC_{50} value of approximately 1 nM, and maximal effects were observed at concentrations ≥10 nM (Fig. 1A). In the absence of extracellular Ca^{2+}, the magnitudes of ET-1–induced AA release were near the basal level (Fig. 1B). ET-1–induced AA release was abolished by BQ123, a specific antagonist of ET_AR, but it was unaffected by BQ788, a specific antagonist of ET_BR (Fig. 1B). Moreover, ET-1–induced AA release was inhibited by arachydonyl trifluoromethyl ketone ($AACOCF_3$), a selective inhibitor of $cPLA_2$.

Effects of SK&F 96365 and LOE 908 on ET-1–Induced AA Release in CHO-ET_AR. SK&F 96365 inhibited ET-1–induced AA release in a concentration-dependent manner with IC_{50} values of approximately 1 μM (Fig. 2A). Maximal inhibition was observed at concentrations ≥10 μM. The extent of maximal inhibition was approximately 80% of ET-1–induced AA release (Fig. 2B). Similarly, LOE 908 inhibited ET-1–induced AA release in a concentration-dependent manner with IC_{50} values of approximately 1 μM, and maximal inhibition was observed at concentrations ≥10 μM (Fig. 2A). The extent of maximal inhibition was approximately 60% of ET-1–induced AA release (Fig. 2B). Moreover, the combined treatment with maximal effective concentration (10 μM) of SK&F 96365 and LOE 908 completely inhibited ET-1–induced AA release (Fig. 2B).

Effects of PI3K Inhibitors on ET-1–Induced AA Release in CHO-ET_AR. Wortmannin inhibited ET-1–induced AA release in a concentration-dependent manner with IC_{50} values of approximately 30 nM, and the maximal inhibition (~80% of control) was seen at concentrations ≥1 μM (Fig. 3). ET-1–induced AA release in CHO-ET_AR preincubated with 1 μM wortmannin was inhibited by 10 μM LOE 908 (Fig. 3B). In contrast, 10 μM SK&F 96365 failed to inhibit ET-1–induced AA release in CHO-ET_AR preincubated with 1 μM wortmannin (Fig. 3B). We also used LY 294002, an inhibitor of PI3K, to evaluate the effects of PI3K on ET-1-induced AA release. LY 294002 at 50 μM also inhibited ET-1-induced AA release (Fig. 3B). ET-1-induced AA release was also sensitive to LOE 908 and resistant to SK&F 96365 in CHO-ET_AR preincubated with 50 μM LY 294002 (data not shown).

Effects of ET-1 on AA Release in CHO-$ET_AR\Delta385$ and CHO-$SerET_AR$. ET-1 induced AA release in both CHO-

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$ET_AR\Delta385$ and CHO-SerET_AR (Fig. 4). However, the threshold concentrations of ET-1 for the induction of AA release were different. In CHO-$ET_AR\Delta385$, ET-1 induced AA release in a concentration-dependent manner with EC_{50} values of between 1 and 10 nM, and maximal effects (approximately a 3.5-fold increase) were observed at concentrations ≥10 nM (Fig. 4). Because CHO-$ET_AR\Delta385$ couples with G_q but not with G_S or G_{12} (Kawanabe et al., 2002c), G_q plays essential roles on ET-1–induced AA release in these cells. In CHO-SerET_AR, ET-1 induced AA release in a concentration-dependent manner with EC_{50} values of between 0.01 and 0.1 nM, and maximal effects (approximately a 2-fold increase) were observed at concentrations ≥0.1 nM (Fig. 4).

Effects of G_s and G_{12} in ET-1–Induced AA Release in CHO-SerET_AR. Because CHO-SerET_AR couples with G_S and G_{12} (Kawanabe et al., 2002c), we examined the effects of G_S and G_{12} on ET-1–induced AA release in these cells. Cholera toxin activates G_S via a receptor-independent mechanism (Belevych et al., 2001). Treatment with 1 μg/ml cholera toxin failed to induce AA release (Fig. 5A). Moreover, ET-1–induced AA release was not influenced by cholera toxin (Fig. 5A).

G_{12}G228A was transiently transfected to evaluate the role of G_{12}. For this purpose, we used the MBS Mammalian Transfection Kit (Stratagene). When we transfected green fluorescent protein with this method, approximately 65% of the cells were green fluorescent protein-positive (data not shown). The magnitudes of ET-1–induced AA release in CHO-SerET_AR transfected with G_{12}G228A were approximately 70% of those in CHO-SerET_AR (Fig. 5B). The magnitudes of ET-1–induced AA release in CHO-SerET_AR trans-

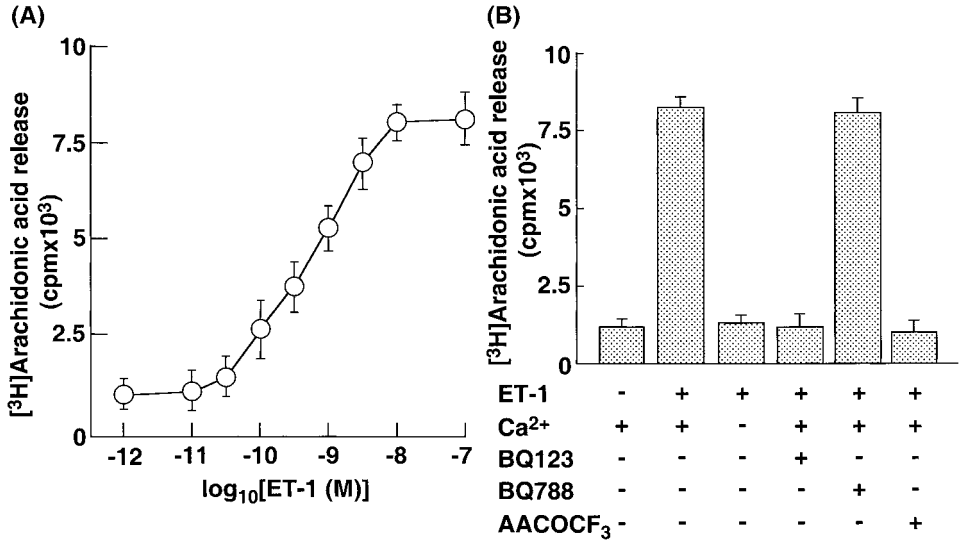

Fig. 1. A, effects of various concentrations of ET-1 on AA release in CHO-ET_AR. The cells were stimulated with increasing concentrations of ET-1 for 5 min. B, effects of extracellular Ca^{2+}, BQ123, BQ788, and $AACOCF_3$ on ET-1–induced AA release in CHO-ET_AR. The cells were pretreated with or without 5 μM BQ123, 5 μM BQ788, or 50 μM $AACOCF_3$ for 30 min and incubated with 10 nM ET-1 for 5 min. AA release was determined as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate.

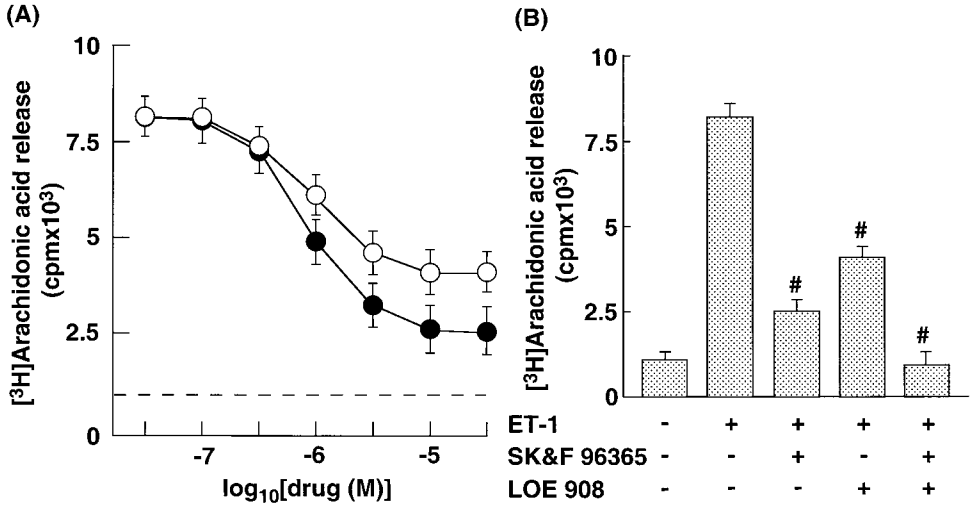

Fig. 2. A, effects of various concentrations of SK&F 96365 and LOE 908 on ET-1–induced AA release in CHO-ET_AR. The cells were incubated for 15 min with various concentrations of SK&F 96365 (●) or LOE 908 (○) and then stimulated with 10 nM ET-1 for 5 min. B, effects of a maximal effective concentration (10 μM) of SK&F 96365 and LOE 908 on ET-1–induced AA release in CHO-ET_AR. AA release was determined as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate. #, $P < 0.05$, significantly different from the control values stimulated by ET-1 in each experiment.

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fected with only vector were similar to those in CHO-SerET$_A$R (data not shown).

Effects of SK&F 96365, LOE 908, and Wortmannin on ET-1–Induced AA Release in CHO-ET$_A$RΔ385 and CHO-SerET$_A$R. In CHO-ET$_A$RΔ385, ET-1–induced AA release was inhibited by SK&F 96365 in a concentration-dependent manner with IC_{50} values of approximately 1 μM, and complete inhibition was observed at concentrations ≥10 μM (Fig. 6). On the other hand, LOE 908 failed to inhibit ET-1–induced AA release in CHO-ET$_A$RΔ385 (Fig. 6). In addition, ET-1 failed to induce AA release in CHO-ET$_A$RΔ385 pretreated with 1 μM wortmannin (Fig. 6B). In CHO-SerET$_A$R, ET-1–induced AA release was inhibited by LOE 908 in a concentration-dependent manner with IC_{50} values of approximately 1 μM, and a complete inhibition was observed at concentrations ≥10 μM (Fig. 7). On the other hand, SK&F 96365 failed to inhibit ET-1–induced AA release in CHO-ET$_A$RΔ385 (Fig. 7). Moreover, the magnitudes of ET-1–induced AA release in CHO-SerET$_A$R pretreated with 1 μM wortmannin were similar to those observed in CHO-SerET$_A$R (Fig. 7B). LOE 908 also inhibited this wortmannin-resistant part of ET-1–induced AA release (Fig. 7B).

Discussion

ET-1 induces AA release in CHO-ET$_A$R (Fig. 1A). BQ123 inhibited ET-1–induced AA release, whereas BQ788 failed to inhibit it (Fig. 1B). Therefore, ET-1–induced AA release is

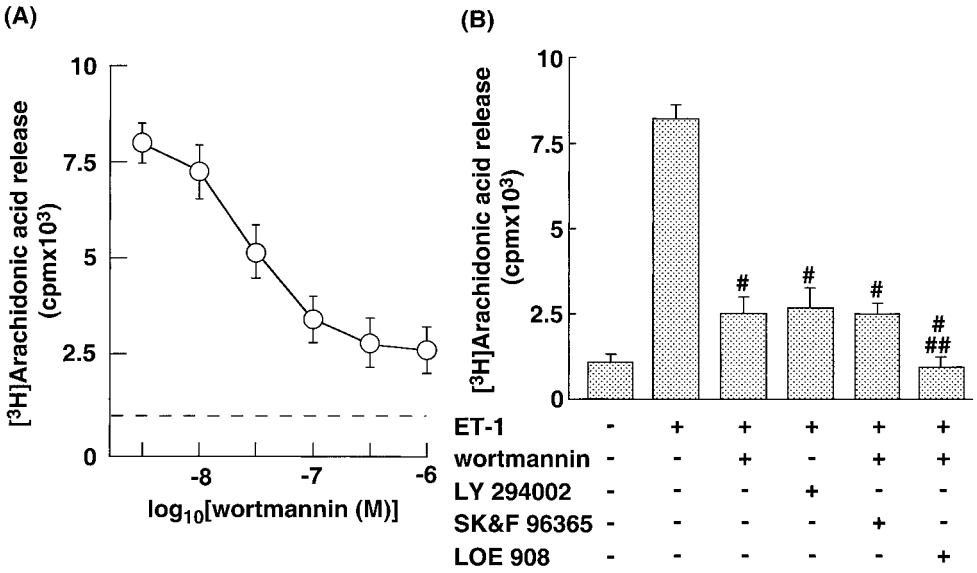

Fig. 3. A, effects of various concentrations of wortmannin on ET-1–induced AA release in CHO-ET$_A$R. The cells were incubated for 15 min with various concentrations of wortmannin and then stimulated with 10 nM ET-1 for 5 min. B, effects of 50 μM LY294002 on ET-1–induced AA release in CHO-ET$_A$R and maximal effective concentrations (10 μM) of SK&F 96365 and LOE 908 on ET-1–induced AA release in CHO-ET$_A$R treated with 1 μM wortmannin. AA release was determined as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate. #, $P < 0.05$, significantly different from the control values stimulated by ET-1 in each experiment. ##, $P < 0.05$, significantly different from the control values stimulated by ET-1 in the presence of wortmannin in each experiment.

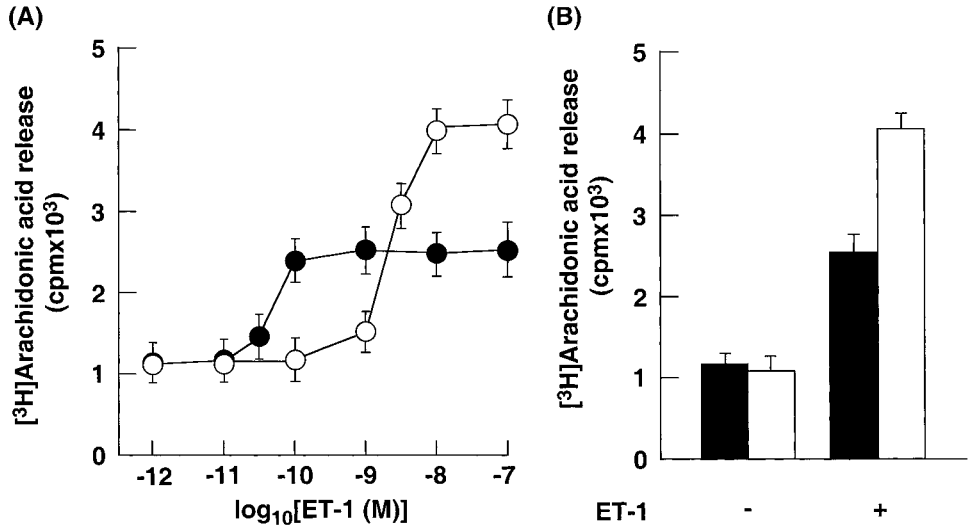

Fig. 4. A, effects of various concentrations of ET-1 on AA release in CHO-ET$_A$RΔ385 or CHO-SerET$_A$R. The cells were stimulated with increasing concentrations of ET-1 for 5 min. B, effects of a maximal effective concentration (10 nM) ET-1 on AA release in CHO-ET$_A$RΔ385 (□) and CHO-SerET$_A$R (■). AA release was determined as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate.

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mediated by ET_AR. Based on the sensitivity to $AACOCF_3$ (Fig. 1B), ET-1 induces AA release through $cPLA_2$ activation. These results are in agreement with the observations in many cell types that agonist-induced AA release is mainly mediated by $cPLA_2$ (Ui et al., 1995; Wu-Wong et al., 1996; Kramer and Sharp, 1997; Trevisi et al., 2002). In the absence of extracellular Ca^{2+}, the magnitudes of ET-1–induced AA release were near the basal level (Fig. 1B). Therefore, extracellular Ca^{2+} influx plays a critical role in ET-1–induced AA release in CHO-ET_AR as was also seen in vascular smooth muscle cells (Wu-Wong et al., 1996). With the use of SK&F 96365 and LOE 908, we attempted to determine the effects of extracellular Ca^{2+} influx through VICCs on ET-1–induced AA release. The inhibitory actions of SK&F 96365 and LOE 908 on ET-1–induced AA release are considered to be mediated by the blockade of Ca^{2+} entry through VICCs for the following two reasons. First, in our recent work using patch-clamp and $[Ca^{2+}]_i$ monitoring, ET-1 activates three types of VICCs in CHO-ET_AR, namely NSCC-1, NSCC-2, and SOCC. In addition, LOE 908 was able to block both NSCC-1 and NSCC-2, whereas SK&F 96365 blocked NSCC-2 and SOCC (Kawanabe et al., 2001). Second, the IC_{50} values of these blockers for ET-1–induced AA release (Fig. 2A) correlated well with those for ET-1–induced extracellular Ca^{2+} influx (Kawanabe et al., 2001). Three types of VICC seem to be involved in ET-1–induced AA release in terms of its sensitivity to SK&F 96365 and LOE 908 (Fig. 2B): the first type of Ca^{2+} channel is sensitive to LOE 908 and is resistant to SK&F 96365; the second type is sensitive to both LOE 908 and SK&F 96365; and the third type is resistant to LOE 908

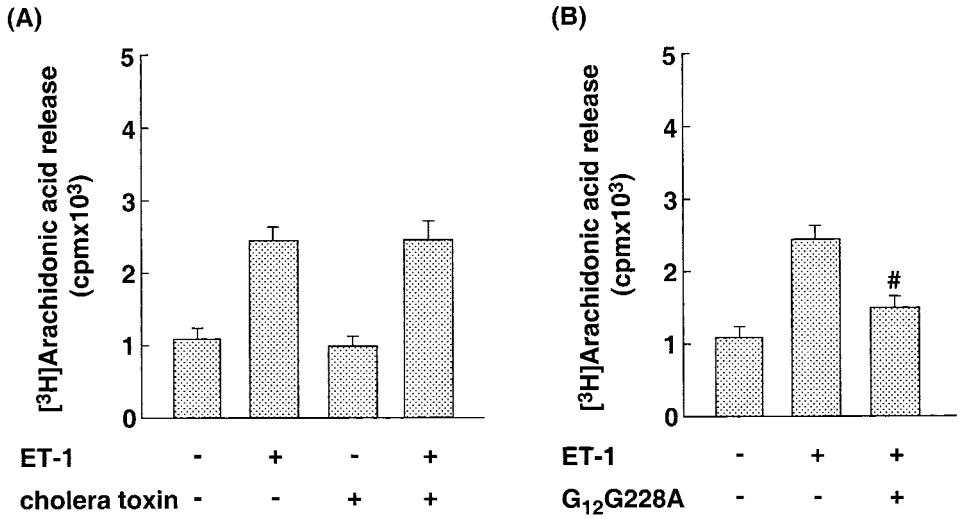

Fig. 5. A, effects of cholera toxin on AA release in resting CHO-SerET_AR and CHO-SerET_AR treated with ET-1. The cells were incubated for 60 min with 1 μg/ml cholera toxin and then stimulated with or without 10 nM ET-1 for 5 min. B, effects of $G_{12}G228A$ on ET-1–induced AA release in CHO-SerET_AR. CHO-SerET_AR cells were transfected with $G_{12}G228A$ transiently as described under *Materials and Methods*. The cells were incubated with 10 nM ET-1 for 5 min. AA release was determined as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate. #, $P < 0.05$, significantly different from the control values stimulated by ET-1 in the absence of $G_{12}G228A$ in each experiment.

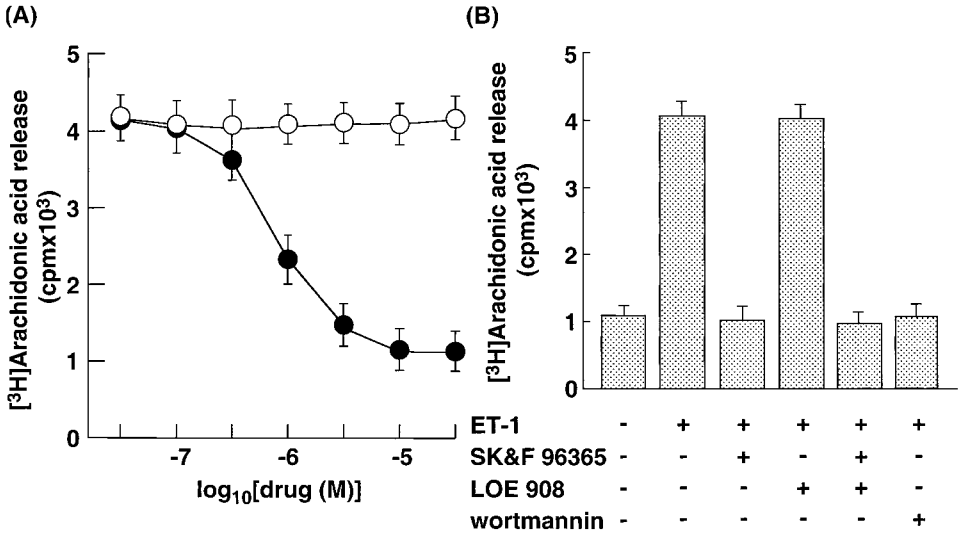

Fig. 6. A, effects of various concentrations of SK&F 96365 and LOE 908 on ET-1–induced AA release in CHO-$ET_AR\Delta385$. The cells were incubated for 15 min with various concentrations of SK&F 96365 (●) or LOE 908 (○) and then stimulated with 10 nM ET-1 for 5 min. B, effects of a maximal effective concentration of SK&F 96365 (10 μM), LOE 908 (10 μM), and/or wortmannin (1 μM) on ET-1–induced AA release in CHO-$ET_AR\Delta385$. AA release was determined as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate.

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and sensitive to SK&F 96365. Because of their pharmacological characteristics, these channels are considered to be NSCC-1, NSCC-2, and SOCC, respectively. The magnitudes of ET-1–induced AA release that were inhibited by the combined treatment with SK&F 96365 and LOE 908 were similar to those in the absence of extracellular Ca^{2+} (Figs. 1B and 2B). Therefore, extracellular Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC plays an important role in ET-1–induced AA release in CHO-ET_AR.

PI3K is involved in the activation of NSCC-2 and SOCC by ET-1 in CHO-ET_AR (Kawanabe et al., 2002a). Therefore, we investigated the effects of PI3K on ET-1–induced AA release in CHO-ET_AR. The inhibitory effects of wortmannin on ET-1–induced AA release may be caused by its inhibitory effects on PI3K, as determined from the following data: 1) wortmannin is generally accepted as a PI3K inhibitor (Ui et al., 1995). Moreover, at nanomolar concentrations, wortmannin acts specifically on PI3K (Yano et al., 1993); 2) Another PI3K inhibitor, LY294002, also inhibited the wortmannin-sensitive ET-1–induced AA release (Fig. 3B); and 3) the IC_{50} values (~30 nM) and maximal effective concentration (1 μM) of wortmannin for ET-1–induced AA release (Fig. 3A) were similar to those for ET-1–induced phosphatidylinositol triphosphate formation, which was measured as an index of PI3K activity (Sugawara et al., 1996). Moreover, the IC_{50} values and maximal effective concentration of wortmannin for ET-1–induced AA release (Fig. 3A) were also similar to those for ET-1–induced Ca^{2+} influx (Kawanabe et al., 2001). The wortmannin-resistant part of ET-1–induced AA release is dependent on extracellular Ca^{2+} influx through NSCC-1, which is determined by the sensitivity to SK&F 96365 and LOE 908 (SK&F 96365-resistant and LOE 908-sensitive) (Fig. 3B). Therefore, the wortmannin-sensitive part of ET-1–induced AA release is dependent on extracellular Ca^{2+} influx through NSCC-2 and SOCC. These results indicate that PI3K is involved in the ET-1–induced AA release, which depends on NSCC-2 and SOCC.

To identify the G proteins involved in the AA release by ET-1, we used CHO-$ET_AR\Delta385$ and CHO-SerET_AR. CHO-$ET_AR\Delta385$ and CHO-SerET_AR couple with G_q and with G_s/G_{12}, respectively (Kawanabe et al., 2002c). ET-1 induced AA release in CHO-$ET_AR\Delta385$ (Fig. 4). This result indicates that the G_q pathway is involved in ET-1–induced AA release. In addition, ET-1 also induced AA release in CHO-SerET_AR (Fig. 4). Therefore, either G_s and/or G_{12} is required for ET-1–induced AA release. Cholera toxin had no effect on the resting AA release and in ET-1–induced AA release in CHO-SerET_AR (Fig. 5A). These results indicate that ET-1–induced AA release is not mediated by the G_s-dependent pathway. Disruption of signaling through endogenous G_{12} by G_{12}G228A inhibited ET-1–induced AA release in CHO-Ser-ET_AR (Fig. 5B), indicating that the activation of AA release is mediated by G_{12}. Therefore, G_{12} and G_q play important roles in ET-1–induced AA release. These results are consistent with the previous report, which demonstrated that the GTPase-deficient activated mutant of G_{12} stimulates AA release in NIH 3T3 cells (Dermott et al., 1999). ET-1–induced AA release was not inhibited completely by G_{12}G228A in this study (Fig. 5B). We believe that this is because G_{12}G228A is not transfected to all cells. However, another possibility is that ET-1 induces AA release with another unknown pathway in CHO-SerET_AR. Further research is necessary to confirm this. As determined from the sensitivity to SK&F 96365 and LOE 908 (SK&F 96365-sensitive and LOE 908-resistant), ET-1–induced AA release in CHO-$ET_AR\Delta385$ is dependent on extracellular Ca^{2+} influx through SOCC (Fig. 6). On the other hand, ET-1–induced AA release is dependent on extracellular Ca^{2+} influx through NSCC-1 in CHO-SerET_AR (SK&F 96365-resistant and LOE 908-sensitive) (Fig. 7). These results are in agreement with the previous observations that ET-1 activates SOCC in CHO-$ET_AR\Delta385$ or NSCC-1 in CHO-SerET_AR (Kawanabe et al., 2002b) and that ET-1–induced SOCC or NSCC-1 activation is dependent on the G_q-dependent pathway or the G_{12}-dependent pathway, respectively (Kawanabe et al., 2002b). The EC_{50} values and maximal effects of ET-1 for AA release between CHO-

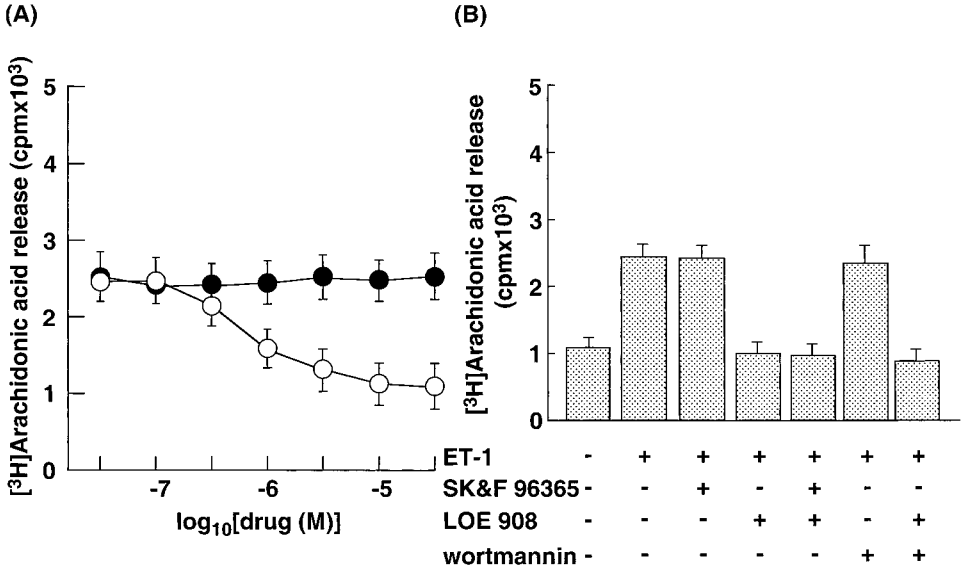

Fig. 7. A, Effects of various concentrations of SK&F 96365 and LOE 908 on ET-1–induced AA release in CHO-SerET_AR. The cells were incubated for 15 min with various concentrations of SK&F 96365 (●) or LOE 908 (○) and then stimulated with 10 nM ET-1 for 5 min. B, effects of a maximal effective concentration of SK&F 96365 (10 μM), LOE 908 (10 μM), and/or wortmannin (1 μM) on ET-1–induced AA release in CHO-SerET_AR. AA release was determined as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate.

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$ET_AR\Delta385$ and CHO-SerET_AR are different (Fig. 4A). These differences seem to be the result of the sensitivity of NSCC-1 and SOCC to ET-1. NSCC-1 is activated by 0.1 nM ET-1, whereas SOCC is activated by 10 nM ET-1 in CHO-ET_AR (Kawanabe et al., 2001). These data also support the conclusion that extracellular Ca^{2+} influx plays an essential role in ET-1–induced AA release. Because both the G_q and G_{12} pathways are necessary for NSCC-2 activation by ET-1 (Kawanabe et al., 2002b), ET-1 failed to activate NSCC-2 in CHO-$ET_AR\Delta385$ and CHO-SerET_AR. Therefore, the involvement of NSCC-2 in ET-1–induced AA release was not detected in these cells. However, taken from the data using CHO-ET_AR, we concluded that NSCC-2 was also involved in ET-1–induced AA release.

In conclusion, extracellular Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC plays an essential role in ET-1–induced AA release in CHO-ET_AR. G_q and G_{12} are involved in ET-1–induced AA release through ET_AR. In addition, PI3K acts as a regulator of ET-1–induced AA release, which depends on the extracellular Ca^{2+} influx through SOCC and NSCC-2.

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