British Journal of Pharmacology (2003) 139, 1516–1522 © 2003 Nature Publishing Group All rights reserved 0007–1188/03 $25.00
www.nature.com/bjp

Involvement of extracellular Ca^{2+} influx and epidermal growth factor receptor tyrosine kinase transactivation in endothelin-1-induced arachidonic acid release

*,[1,2]Yoshifumi Kawanabe, [1]Kazuhiko Nozaki, [1]Nobuo Hashimoto & [2]Tomoh Masaki

[1]Department of Neurosurgery, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan and [2]Department of Pharmacology, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan

1 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 vascular smooth muscle cells (VSMCs). These channels can be distinguished by their sensitivity to Ca^{2+}-channel blockers, SK&F 96365 and LOE 908. LOE 908 is sensitive to NSCC-1 and NSCC-2, and SK&F 96365 is sensitive to NSCC-2 and SOCC. Moreover, these channels play essential roles in ET-1-induced epidermal growth factor receptor protein tyrosine kinase (EGFR PTK) transactivation. The main purpose of the present study was to demonstrate the involvement of EGFR PTK transactivation in ET-1-induced arachidonic acid release in VSMCs.

2 Both SK&F 96365 and LOE 908 inhibited ET-1-induced arachidonic acid 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 arachidonic acid release.

3 AG1478, a specific inhibitor of EGFR PTK, inhibited ET-1-induced arachidonic acid release and extracellular signal-regulated kinase 1 and 2 (ERK1/2). The IC_{50} values of AG1478 for ET-1-induced arachidonic acid release and ERK1/2 correlated well with those for ET-1-induced EGFR PTK transactivation.

4 Mitogen-activated protein kinase kinase inhibitor, PD 98059, inhibited ET-1-induced arachidonic acid release. The IC_{50} values of PD 98059 for ET-1-induced arachidonic acid release were similar to those for ET-1-induced ERK1/2 activity. In contrast, PD 98059 failed to inhibit ET-1-induced EGFR PTK transactivation.

5 These results indicate that (1) extracellular Ca^{2+} influx through NSCCs and SOCC plays important roles for ET-1-induced arachidonic acid release, (2) EGFR PTK transactivation/ERK1/2 pathways are involved in ET-1-induced arachidonic acid release.
British Journal of Pharmacology (2003) **139**, 1516–1522. doi:10.1038/sj.bjp.0705386

Keywords: Endothelin-1; arachidonic acid release; Ca^{2+} channel; epidermal growth factor receptor

Abbreviations: $AACOCF_3$, arachydonyl trifluoromethyl ketone; $cPLA_2$, cytosolic PLA_2; DMEM, Dulbecco's modified Eagle's medium; EGFR, epidermal growth factor receptor; ERK 1/2, extracellular signal-regulated kinase 1 and 2; ET-1, endothelin-1; ICA, internal carotid artery; NSCC, nonselective cation channel; PBS, phosphate-buffered saline; PLA_2, phospholipase A_2; PTK, protein tyrosine kinase; SOCC, store-operated Ca^{2+} channel; VICC, voltage-independent Ca^{2+} channel; VOCC, voltage-operated Ca^{2+} channel; VSMC, vascular smooth muscle cell

Introduction

Activation of phospholipase A_2 (PLA_2) liberates arachidonic acid from phospholipids. Arachidonic acid metabolites, including prostaglandins, leukotrienes, lipoxins, and hydroxy derivatives, have been implicated in numerous physiological and pathophysiological processes (Brady & Serhan, 1996; Makita *et al*., 1996; Vane & Botting, 1997). This arachidonic acid is preferentially released by the 85-kDa cytosolic PLA_2 ($cPLA_2$) (Sharp *et al*., 1991; Trevisi *et al*., 2002). Both Ca^{2+} and phosphorylation regulate $cPLA_2$ activity. Ca^{2+} is required for $cPLA_2$ to translocate from the cytosol to phospholipid, a membrane that is mediated by its Ca^{2+}-dependent phospholipid binding domain (Nalefski *et al*., 1994). Endothelin-1 (ET-1) induces arachidonic acid release through the activation of $cPLA_2$ in vascular smooth muscle cells (Resnik *et al*., 1989; Trevisi *et al*., 2002). In addition, extracellular Ca^{2+} influx plays critical roles in ET-1-induced arachidonic acid release (Dunican *et al*., 1996; Wu-Wong *et al*., 1996). However, it remains unclear as to what types of Ca^{2+} channels are involved in ET-1-induced arachidonic acid release in vascular smooth muscle cells (VSMCs). These uncertainties are mainly attributable to the lack of specific Ca^{2+}-channel blockers. We have recently shown that ET-1 activates three types of voltage-independent Ca^{2+} channel (VICC), as well as voltage-operated Ca^{2+} channels (VOCCs) in rabbit internal carotid artery (ICA) VSMCs. The VICCs include two types of Ca^{2+}-permeable

*Author for correspondence;
E-mail: ykawanabe@rics.bwh.harvard.edu

Current address: Renal Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Harvard Institutes of Medicine, Room 520, 77 Avenue Louis Pasteur, Boston, MA 02115, U.S.A.

nonselective cation channel (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC) (Kawanabe *et al*., 2002a). Importantly, we have also shown that these channels can be distinguished by their sensitivity to blockers of the receptor-operated Ca^{2+} channel such as SK&F 96365 and LOE 908 (Meritt *et al*., 1990; Encabo *et al*., 1996). 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 SOCC is resistant to LOE 908 and sensitive to SK&F 96365 (Kawanabe *et al*., 2002a). Based on these results, we tried to elucidate which Ca^{2+} channels are involved in ET-1-induced arachidonic acid release using SK&F 96365 and LOE 908 in this study.

Next, we investigated the intracellular mechanisms of the ET-1-induced arachidonic acid release. Stimulation of cells by epidermal growth factor (EGF) results in the activation of $cPLA_2$ (Bonventre *et al*., 1990). Moreover, EGF-induced extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation results in $cPLA_2$ activation (van Rossum *et al*., 2001). ET-1 transactivates EGFR protein tyrosine kinase (PTK) in VSMCs (Iwasaki *et al*., 1999; Kawanabe *et al*., 2002b). Therefore, we focused on investigating whether ET-1-induced EGFR PTK transactivation was involved in the stimulation of arachidonic acid release by ET-1.

Methods

Cell culture

Isolated VSMCs were prepared from rabbit ICA, as described previously (Kawanabe *et al*., 2002a). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum supplemented with 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin, 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^{-1}). After washing, ET-1 was added and, after 5 min, the medium was 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 chloroform, and applied to silica gel plates for thin-layer chromatography (Merck, Darmstadt, Germany). The plates were developed in heptane/diethyl ether/acetic acid (v v^{-1}; 75 : 25 : 4). The distance of movement was visualized with iodine vapor. The plate was scraped, and the radioactivity was counted with a liquid scintillation counter.

Measurement of EGFR PTK transactivation

Measurement of EGFR PTK transactivation was performed using a Universal Tyrosine Kinase Assay Kit (Takara, Tokyo, Japan), as described previously (Kawanabe *et al*., 2002b). Extraction buffer and kinase reacting solution were equipped with this kit. Cells seeded at 5×10^6 cells well^{-1} in six-well plates were starved for 24 h and then stimulated with 10 nM ET-1 for 2 min. The reaction was terminated by washing three times with phosphate-buffered saline (PBS). After the addition of 1 ml of extraction buffer, the cells were scraped off with a scraper and centrifuged at 14,500 rpm for 10 min at 4°C. The supernatant was incubated with mouse monoclonal anti-EGFR antibody (Takara, Tokyo, Japan) for 2 h at room temperature and subsequently incubated with Protein A–agarose for an additional 1 h. The mixture was centrifuged at 10,000 $\times$ *g* for 1 min at 4°C, and the pellets were washed three times with PBS. The washed pellets were resuspended in 150 μl of kinase reaction buffer. EGFR PTK transactivation was determined according to the manufacturer's instructions. The absorbance of the lysate at 450 nm was measured with an EL340 Microtiter Plate Reader (Bio-Tek Instruments, Winooski, VT, USA).

Measurement of ERK1/2 activity

Measurement of ERK1/2 activity was performed as described previously (Sugawara *et al*., 1996; Kawanabe *et al*., 2001). Briefly, ICA VSMCs at 50% confluency in 10-cm dishes were starved for 24 h before being stimulated by 10 nM ET-1 for 5 min in serum-free DMEM. The reaction was terminated by washing once with PBS and twice with 20 mM Tris-HCl (pH 7.4). After the addition of 1 ml of ice-cold extraction buffer (10 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mg ml^{-1} aprotinin, 0.05 mM NaF, 0.5 mM Na_3PO_4, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 5 mM β-glycerophosphate; pH 7.4), the cells were scraped off using a scraper. For partial purification of ERK, the cell suspension was transferred to a 15-ml conical tube, sonicated for 10 s $\times$ 3 at 10-s intervals, and centrifuged at 25,000 $\times$ *g* for 20 min. The supernatant was applied to a DEAE-Sephadex column (bed volume, 0.5 ml) pre-equilibrated with equilibration buffer (extraction buffer containing 100 mM NaCl). The enzyme was eluted with the elution buffer (extraction buffer containing 500 mM NaCl) and concentrated using Centricon YM-30 (Millipore Corporation, Bedford, MA, U.S.A.). The protein concentration of the partially purified enzyme in each sample was determined with a BCA Microprotein Assay Kit (Pierce, Rockford, IL, U.S.A.), and 5 μg of the enzyme was used for each assay. ERK1/2 activity was determined using a MAP Kinase Assay Kit (Amersham, Buckinghamshire, U.K.) according to the manufacturer's instructions.

Drugs

Boehringer Ingelheim K.G. (Ingelheim, Germany) kindly provided LOE 908. Other chemicals were commercially obtained.

Statistical analysis

All results were expressed as mean $\pm$ s.e.m. The data were subjected to a two-way analysis of variance, and when a significant F-value was encountered, the Newman–Keuls' multiplerange 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 arachidonic acid release in VSMCs

ET-1 induced arachidonic acid release in a concentration-dependent manner with an EC_{50} value of around 1 nM, and maximal effects were observed at concentrations of $\geqslant$10 nM (Figure 1a). In the absence of extracellular Ca^{2+}, the magnitudes of ET-1-induced arachidonic acid release were near the basal level (Figure 1b). Therefore, extracellular Ca^{2+} influx appears to play an important role in ET-1-induced arachidonic acid release. ET-1-induced arachidonic acid release was abolished by BQ123, a specific antagonist of endothelin$_A$ receptor, but it was unaffected by BQ788, a specific antagonist of endothelin$_B$ receptor (Figure 1b). Moreover, ET-1-induced arachidonic acid release was inhibited by arachydonyl trifluoromethyl ketone ($AACOCF_3$), a selective inhibitor of $cPLA_2$.

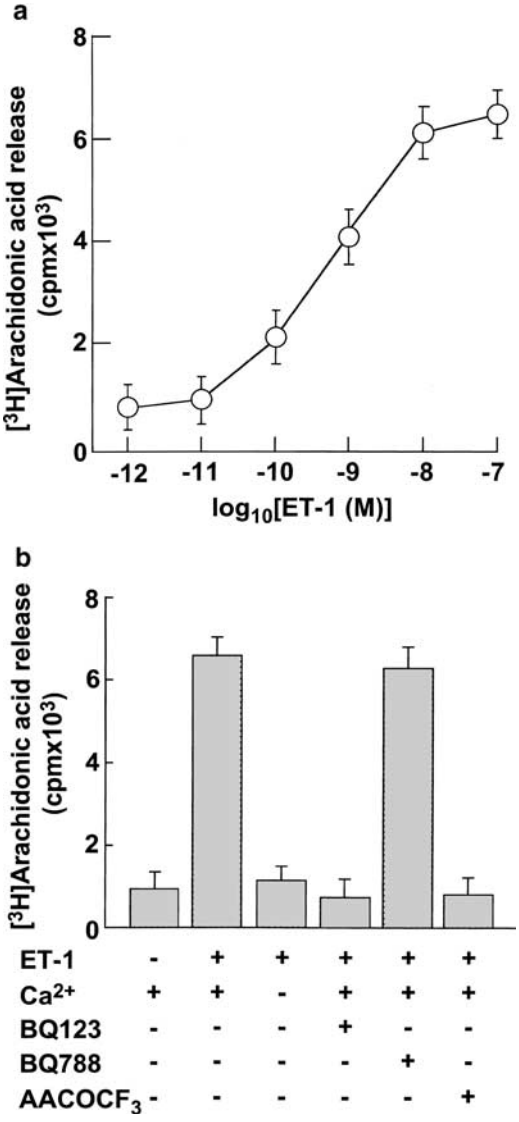

Figure 1 (a) Effects of various concentrations of ET-1 on arachidonic acid release in vascular smooth muscle cells. The cells were stimulated with increasing concentrations of ET-1 for 5 min. (b) Effects of extracellular Ca^{2+}, BQ123, BQ788, and $AACOCF_3$ on ET-1-induced arachidonic acid release in VSMCs. 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. Arachidonic acid release was determined as described in Methods. Data presented are the mean$\pm$s.e.m. of six determinations, each done in triplicate.

Effects of SK&F 96365 and LOE 908 on ET-1-induced arachidonic acid release

We examined the effects of extracellular Ca^{2+} influx through VOCCs on ET-1-induced arachidonic acid release using nifedipine, a specific blocker of L-type VOCCs. Nifedipine at 1 μM completely inhibited 10 nM ET-1-induced extracellular Ca^{2+} influx through VOCCs in VSMC (data not shown). In contrast, it inhibited 10 nM ET-1-induced arachidonic acid release by a maximum of only about 10% (Figure 2a). Using SK&F 96365 and LOE 908, we attempted to determine the effects of extracellular Ca^{2+} influx through VICCs on ET-1-induced arachidonic acid release. In the following experiments, nifedipine was added to the incubation media at a final concentration of 1 μM to analyze the role of Ca^{2+} channels other than L-type VOCC. SK&F 96365 inhibited 10 nM ET-1-induced arachidonic acid release in a concentration-dependent manner with IC_{50} values of around 1 μM (Figure 2b). Maximal inhibition was observed at concentrations $\geqslant$10 μM. The extent of maximal inhibition was around 80% of 10 nM ET-1-induced arachidonic acid release (Figure 2). Similarly, LOE 908 inhibited 10 nM ET-1-induced arachidonic acid release in a concentration-dependent manner with IC_{50} values of around 1 μM, and maximal inhibition was observed at concentrations $\geqslant$10 μM (Figure 2). The extent of maximal inhibition was around 60% of 10 nM ET-1-induced arachidonic acid release (Figure 2b). Moreover, the combined treatment with maximal effective concentration (10 μM) of SK&F 96365 and LOE 908 completely inhibited 10 nM ET-1-induced arachidonic acid release (Figure 2a).

Effects of AG1478 on ET-1-induced arachidonic acid release and ERK1/2 stimulation

AG1478, a specific inhibitor of EGFR PTK, inhibited 10 nM ET-1-induced arachidonic acid release (Figure 3a). The inhibitory effect of AG1478 on ET-1-induced arachidonic acid release was in a concentration-dependent manner with IC_{50} values of around 10 nM (Figure 3a). Maximal inhibition was observed at concentrations $\geqslant$1 μM (Figure 3a). The extent of maximal inhibition was around 55% of ET-1-induced arachidonic acid release (Figure 3a). Similarly, AG1478 inhibited 10 nM ET-1-induced ERK1/2 stimulation (Figure 3b). The inhibitory effect of AG1478 on ET-1-induced ERK1/2 was in a concentration-dependent manner with IC_{50} values of around 10 nM (Figure 3b). Maximal inhibition was observed at concentrations $\geqslant$1 μM (Figure 3b). The extent of maximal inhibition was around 60% of ET-1-induced ERK1/2 stimulation (Figure 3a).

Effects of PD 98059 on ET-1-induced arachidonic acid release and EGFR PTK transactivation

PD 98059, an inhibitor of mitogen-activated protein kinase kinase, inhibited 10 nM ET-1-induced arachidonic acid release (Figure 4a). The inhibitory effect of PD 98059 on ET-1-

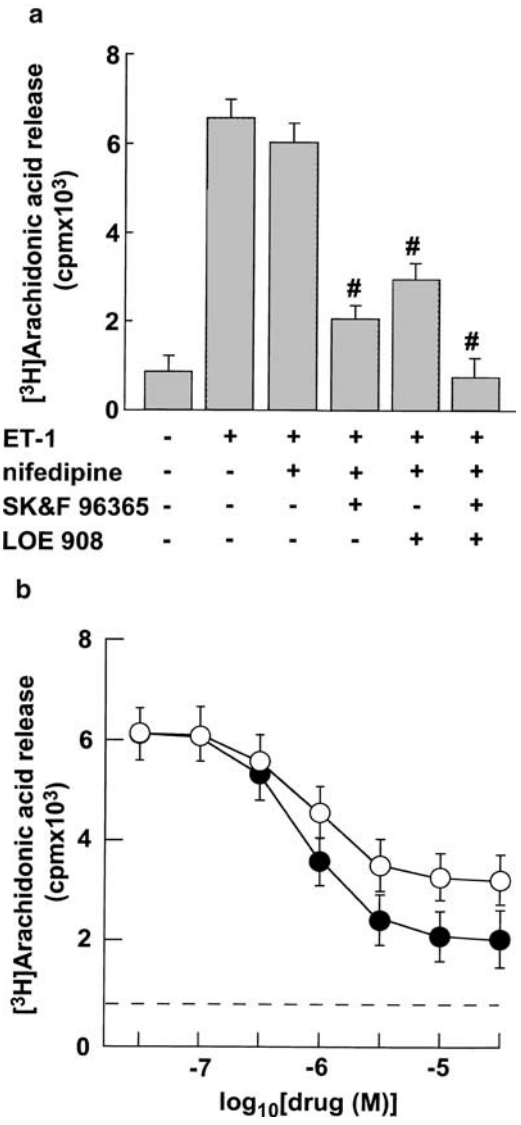

Figure 2 (a) Effects of a maximal effective concentration of nifedipine (1 μM), SK&F 96365 (10 μM), and LOE 908 (10 μM) on ET-1-induced arachidonic acid release in VSMCs. (b) Effects of various concentrations of SK&F 96365 and LOE 908 on ET-1-induced arachidonic acid release in VSMCs. The cells were incubated for 15 min with various concentrations of SK&F 96365 (closed circles) or LOE 908 (open circles) and then stimulated with 10 nM ET-1 for 5 min in the presence of 1 μM nifedipine. Arachidonic acid release was determined as described in Methods. Data presented are the mean $\pm$ s.e.m. of six determinations, each done in triplicate. #$P<0.05$; significantly different from the control values stimulated by ET-1 in each experiment.

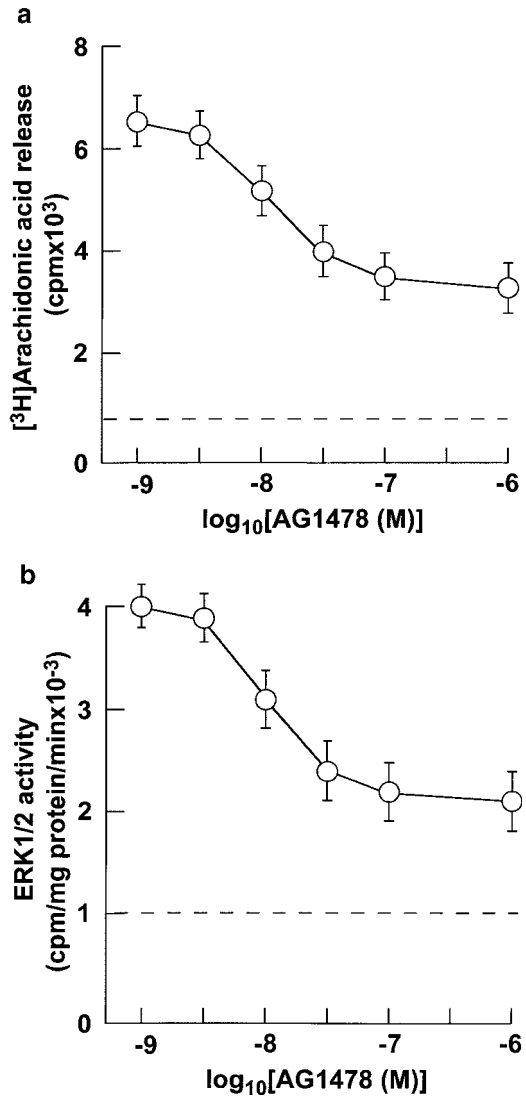

Figure 3 (a) Effects of various concentrations of AG1478 on ET-1-induced arachidonic acid release in VSMCs. The cells were incubated for 15 min with various concentrations of AG1478 and then stimulated with 10 nM ET-1 for 5 min. Arachidonic acid release was determined as described in Methods. (b) Effects of various concentrations of AG1478 on ET-1-induced ERK1/2 in VSMCs. ERK1/2 activity was determined as described in Methods. Data presented are the mean $\pm$ s.e.m. of six determinations, each done in triplicate.

induced arachidonic acid release was in a concentration-dependent manner with IC_{50} values of around 3 μM (Figure 4a). Maximal inhibition was observed at concentrations $\geqslant 30$ μM (Figure 4a). The extent of maximal inhibition was around 60% of ET-1-induced arachidonic acid release (Figure 4a). In contrast, 30 μM PD 98059 failed to inhibit 10 nM ET-1-induced EGFR PTK transactivation (Figure 4b).

Discussion

Based on the sensitivity to BQ123 and BQ788, endothelin$_A$ receptor plays essential roles in the ET-1-induced arachidonic acid release in ICA VSMCs (Figure 1b). Based on the sensitivity to $AACOCF_3$ (Figure 1b), ET-1 induces arachidonic acid release through $cPLA_2$ activation. These results are in agreement with the observations that agonist-induced AA release is mainly mediated by $cPLA_2$ in many cell types (Wu-Wong *et al*., 1996; Trevisi *et al*., 2002). Previous reports demonstrated that extracellular Ca^{2+} influx plays important roles in the arachidonic acid release (Dunican *et al*., 1996; Wu-Wong *et al*., 1996). We tried to characterize the Ca^{2+} channels involved in the ET-1-induced arachidonic acid release in ICA VSMCs. The magnitudes of ET-1-induced arachidonic acid release in the absence of extracellular Ca^{2+} were near the basal level (Figure 1b). These results indicate that extracellular Ca^{2+} influx plays important roles in ET-1-induced arachidonic acid release in ICA VSMCs. Our recent study indicated that

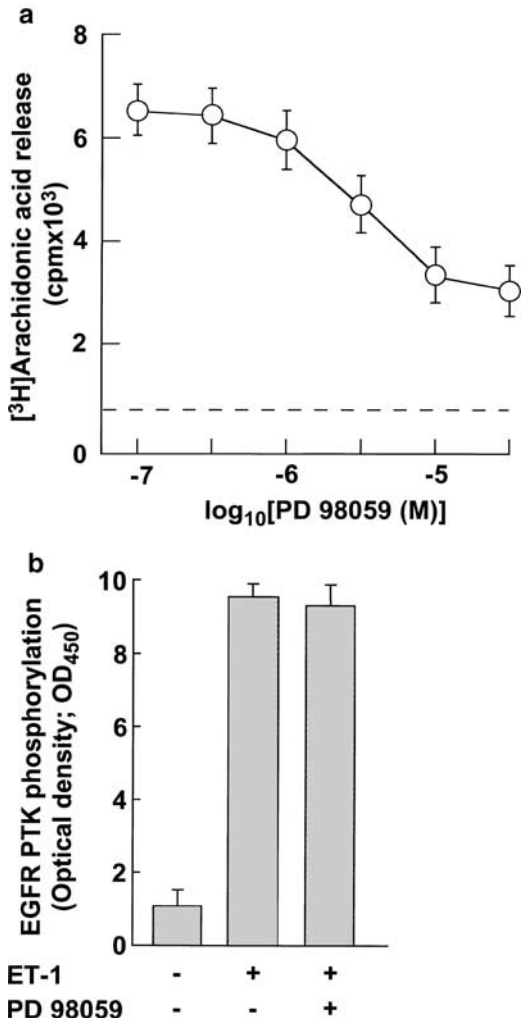

Figure 4 (a) Effects of PD 98059 on ET-1-induced arachidonic acid release in VSMCs. The cells were incubated for 15 min with various concentrations of PD 98059 and then stimulated with 10 nM ET-1 for 5 min. ERK1/2 activity was determined as described in Methods. Data presented are the mean ± s.e.m. of six determinations, each done in triplicate. (b) Effects of PD 98059 on ET-1-induced EGFR PTK transactivation. The cells were incubated for 15 min with or without 30 μM PD 98059 and then stimulated with 10 nM ET-1 for 2 min. EGFR PTK activity was determined as described in Methods. Data presented are the mean ± s.e.m. of six determinations, each done in triplicate.

NSCC-1, NSCC-2, and SOCC play a major part in the ET-1-induced extracellular Ca^{2+} influx in ICA VSMCs (Kawanabe *et al*., 2002a). Moreover, extracellular Ca^{2+} influx through these Ca^{2+} channels plays essential roles in the ET-1-induced PYK2 protein tyrosine kinase activation and cell proliferation (Kawanabe *et al*., 2002a, c; 2003). Thus, we examined the involvement of NSCC-1, NSCC-2, and SOCC in the ET-1-induced arachidonic acid release using SK&F 96365 and LOE 908. According to the nifedipine sensitivity of ET-1-induced arachidonic acid release, involvement of VOCC in this response was estimated to be minor, at around only 10% (Figure 1b). We demonstrated in a recent report that nifedipine suppressed the 10 nM ET-1-induced sustained increase in $[Ca^{2+}]_i$ by a maximum of no more than 10% (Kawanabe *et al*., 2002a). Therefore, Ca^{2+} channels other than VOCC may play important roles in ET-1-induced arachidonic acid release in addition to extracellular Ca^{2+} influx in ICA VSMCs. The inhibitory action of SK&F 96365 and LOE 908 on the ET-1-induced arachidonic acid release is considered to be mediated by blockade of Ca^{2+} entry through VICCs for the following reasons. (1) In our recent work using patch-clamp and $[Ca^{2+}]_i$ monitoring, ET-1 was found to activate three types of VICCs in VSMCs, namely NSCC-1, NSCC-2, and SOCC. In addition, LOE 908 was found to be a blocker of both NSCC-1 and NSCC-2, whereas SK&F 96365 was found to be a blocker of NSCC-2 and SOCC (Kawanabe *et al*., 2002a). (2) The IC_{50} values of these blockers for the ET-1-induced arachidonic acid release (Figure 2b) correlated well with those for the ET-1-induced extracellular Ca^{2+} influx (Kawanabe *et al*., 2002a). Moreover, because SK&F 96365 and LOE 908 failed to inhibit ET-1-induced transient increase in $[Ca^{2+}]_i$ due

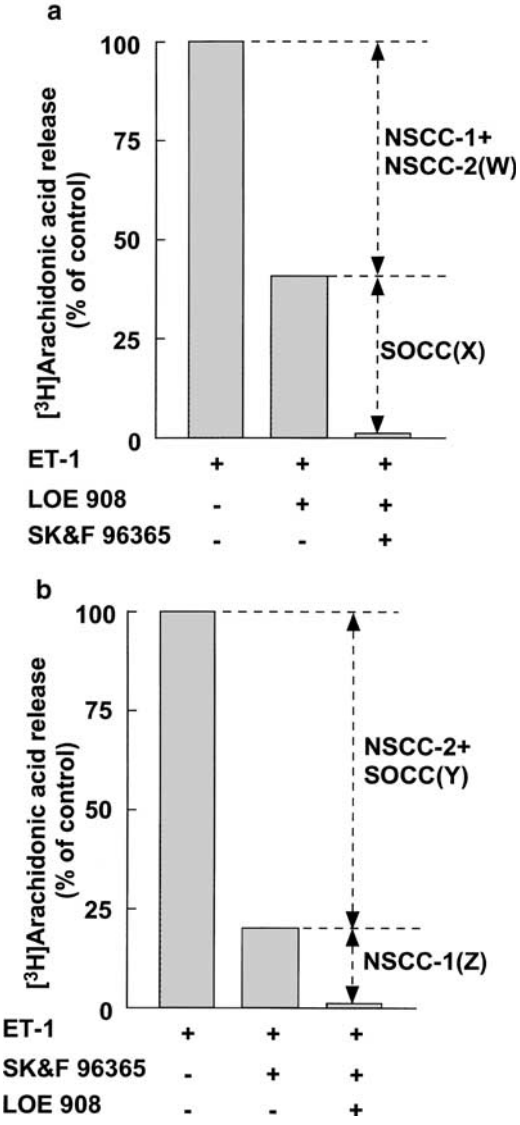

Figure 5 (a, b) Calculation for contribution of Ca^{2+} influx through three types of VICCs to ET-1-induced arachidonic acid release in VSMCs in the presence of 1 μM nifedipine. The ET-1-induced arachidonic acid release in the presence of 10 μM LOE 908 and/or 10 μM SK&F 96365 is represented as a percentage 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.

to the release of intracellular Ca^{2+} store (Kawanabe *et al*., 2002a), the release of salcoplasmic reticulum Ca^{2+} is not sufficient to stimulate arachidonic acid release. Three types of VICC seem to be involved in the ET-1-induced arachidonic acid release in terms of its sensitivity to SK&F 96365 and LOE 908 (Figure 5). One type of Ca^{2+} channel is sensitive to LOE 908 and resistant to SK&F 96365, another type is sensitive to both LOE 908 and SK&F 96365, and the third type is resistant to LOE 908 and sensitive to SK&F 96365. Based on pharmacological criteria, these channels are considered to be NSCC-1, NSCC-2, and SOCC, respectively. Moreover, the percent contribution of NSCC-1, NSCC-2, and SOCC to the ET-1-induced arachidonic acid release is calculated to be about 20, 40, and 40%, respectively, of nifedipine-resistant part of arachidonic acid release caused by 10 nM ET-1 (Figure 5). The magnitudes of the ET-1-induced arachidonic acid release that were inhibited by the combined treatment with nifedipine, SK&F 96365, and LOE 908 were similar to those in the absence of extracellular Ca^{2+} (Figures 1b, 2a). Therefore, extracellular Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC plays important roles for ET-1-induced arachidonic acid release in ICA VSMCs.

EGF stimulates arachidonic acid release (Bonventre *et al*., 1990). Based on the data that ET-1 transactivates EGFR PTK in VSMCs (Iwasaki *et al*., 1999; Kawanabe *et al*., 2002b), and both EGFR PTK transactivation and arachidonic acid release by ET-1 are dependent on extracellular Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC (Kawanabe *et al*., 2002b; Figure 5), we hypothesized that EGFR PTK transactivation was involved in ET-1-induced arachidonic acid release. The inhibitory effects of AG1478 on ET-1-induced arachidonic acid release may be due to its inhibitory effects on EGFR PTK transactivation, judging from the following data: (1) AG 1478 is generally accepted as a EGFR PTK inhibitor (Iwasaki *et al*., 1999). (2) The IC_{50} values (around 10 nM) and maximal effective concentration (1 μM) of AG1478 for ET-1-induced arachidonic acid release (Figure 3a) were similar to those for ET-1-induced EGFR PTK transactivation (Kawanabe *et al*., 2002b). These results indicate that EGFR PTK transactivation plays important roles in ET-1-induced arachidonic acid release. As we mentioned above, extracellular Ca^{2+} influx through voltage-independent Ca^{2+} channels is involved in ET-1-induced arachidonic acid release. Modulation of ET-1-induced EGFR PTK transactivation may be at least one target of extracellular Ca^{2+} influx. Based on the sensitivity to AG1478 (Figure 3), ET-1 induces arachidonic acid release *via* EGFR PTK transactivation-independent pathway, as well as EGFR PTK transactivation-dependent pathway. Recent reports demonstrate that protein kinase C and phosphoinositide 3-kinase play important roles for arachidonic acid release (Silfani & Freeman, 2002; Trevisi *et al*., 2002). Protein kinase C and phosphoinositide 3-kinase pathways may be involved in EGFR PTK transactivation-independent parts of ET-1-induced arachidonic acid release. ET-1-induced arachidonic acid release by $cPLA_2$ activation is dependent on ERK1/2 pathway in VSMCs (Trevisi *et al*., 2002). PD 98059 inhibited ET-1-induced arachidonic acid release (Figure 4). The IC_{50} values (around 3 μM) of PD 98059 for ET-1-induced arachidonic acid release (Figure 4a) were similar to those for ET-1-induced ERK1/2 activation (Kawanabe *et al*., 2002c). In contrast, PD 98059 failed to inhibit ET-1-induced EGFR PTK transactivation (Figure 4b). These results indicate that ERK1/2 plays some roles in ET-1-induced arachidonic acid release downstream of EGFR PTK transactivation in ICA VSMCs. Some reports demonstrated that extracellular Ca^{2+} influx plays important roles for phosphorylation and translocation of $cPLA_2$ (Crawford & Jacobson, 1998; Fatima *et al*., 2001). We have preliminary data that Ca^{2+} influx through NSCCs and SOCCs plays important roles for translocation of $cPLA_2$. The effects of EGFR PTK and ERK1/2 on ET-1-induced phosphorylation and translocation of $cPLA_2$ are now under investigation in our laboratory.

In summary, extracellular Ca^{2+} influx through voltage-independent Ca^{2+} channels such as NSCC-1, NSCC-2, and SOCC plays important roles for ET-1-induced arachidonic acid release. In addition, EGFR PTK transactivation is involved in ET-1-induced arachidonic acid release. Finally, ERK1/2 has important roles in the EGFR PTK transactivation-dependent component of ET-1 -induced arachidonic acid release.

We thank Boehringer Ingelheim K.G. for the kind donation of LOE 908. This study was supported by a grant from the Smoking Research Foundation, Japan and by the Uehara Memorial Foundation Fellowship, Tokyo, Japan.

References

BONVENTRE, J.V., GRONICH, J.H. & NEMENOFF, R.A. (1990). Epidermal growth factor enhances glomerular mesangial cell soluble phospholipase A2 activity. *J. Biol. Chem.,* **265,** 4934–4938.

BRADY, H.R. & SERHAN, C.N. (1996). Lipoxins: putative braking signals in host defense, inflammation and hypersensitivity. *Curr. Opin. Nephrol. Hypertens.,* **5,** 20–27.

CRAWFORD, J.R. & JACOBSON, B.S. (1998). Extracellular calcium regulates HeLa cell morphology during adhesion to gelatin: role of translocation and phosphorylation of cytosolic phospholipase A2. *Mol. Biol. Cell.,* **9,** 3429–3443.

DUNICAN, D.J., GRIFFITHS, R., WILLIAMS, D.C. & O'NEILL, L.A. (1996). Endothelin-1 increases arachidonic acid release in C6 glioma cells through a potassium-modulated influx of calcium. *J. Neurochem.,* **67,** 830–837.

ENCABO, A., ROMANIN, C., BIRKE, F.W., KUKOVETZ, W.R. & GROSCHINER, K. (1996). Inhibition of a store-operated Ca^{2+} entry pathway in human endothelial cells by the isoquinoline derivative LOE 908. *Br. J. Pharmacol.,* **119,** 702–706.

FATIMA, S., KHANDEKAR, Z., PARMENTIER, J.H. & MALIK, K.U. (2001). Cytosolic phospholipase A2 activation by the p38 kinase inhibitor SB203580 in rabbit aortic smooth muscle cells. *J. Pharmacol. Exp. Ther.,* **298,** 331–338.

IWASAKI, H., EGUCHI, S., UENO, H., MARUMO, F. & HIRATA, Y. (1999). Endothelin-mediated vascular growth requires p42/p44 mitogen-activated protein kinase and p70 S6 kinase cascades *via* transactivation of epidermal growth factor receptor. *Endocrinology,* **140,** 4659–4668.

KAWANABE, Y., HASHIMOTO, N. & MASAKI, T. (2001). B103 neuroblastoma cells predominantly express endothelin ET_B receptor; effects of extracellular Ca^{2+} influx on endothelin-1-induced mitogenesis. *Eur. J. Pharmacol.,* **425,** 173–179.

KAWANABE, Y., HASHIMOTO, N. & MASAKI, T. (2002a). Ca^{2+} channels involved in endothelin-induced mitogenic response in carotid artery vascular smooth muscle cells. *Am. J. Physiol. Cell. Physiol.,* **282,** C330–C337.

KAWANABE, Y., HASHIMOTO, N. & MASAKI, T. (2002b). Characterization of Ca^{2+} channels involved in ET-1-induced transactivation of EGF receptors. *Am. J. Physiol. Heart Circ. Physiol.,* **283,** H2671–H2675.

KAWANABE, Y., HASHIMOTO, N. & MASAKI, T. (2002c). Extracellular Ca^{2+} influx and endothelin-1-induced intracellular mitogenic cascades in rabbit internal carotid artery vascular smooth muscle cells. *J. Cardiovasc. Pharmacol.,* **40,** 307–314.

KAWANABE, Y., HASHIMOTO, N. & MASAKI, T. (2003). Involvements of voltage-independent Ca^{2+} channels and phosphoinositide 3-kinase in endothelin-1-induced PYK2 tyrosine phosphorylation. *Mol. Pharmacol.,* **63,** 808–813.

MAKITA, K., FALCK, J.R. & CAPDEVILA, J.H. (1996). Cytochrome *P*450, the arachidonic acid cascade, and hypertension: new vistas for an old enzyme system. *FASEB J.,* **10,** 1456–1463.

MERITT, J.E., AIRMSTRONG, W.P, BENHAM, C.D., HALLAM, T.J., JACOB, R., JAXA-CHAMIEC, A., LEIGH, B.K., MCCARTHY, S.A., MOORES, K.E. & RINK, T.J. (1990). SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem. J.,* **271,** 515–522.

NALEFSKI, E.A., SULTZMAN, L.A., MARTIN, D.M., KRIZ, R.W., TOWLER, PS., KNOPF, J.L. & CLARK, J.D. (1994). Delineation of two functionally distinct domains of cytosolic phospholipase A2, a regulatory Ca^{2+}-dependent lipid-binding domain and a Ca^{2+}-independent catalytic domain. *J. Biol. Chem.,* **269,** 18239–18249.

PEREZ, D.M., DEYOUNG, M.B. & GRAHAM, R.M. (1993). Coupling of expressed alpha 1B- and alpha 1D-adrenergic receptor to multiple signaling pathways is both G protein and cell type specific. *Mol. Pharmacol.,* **44,** 784–795.

RESNIK, T.J., SCOTT-BURDEN, T. & BUHLER, F.R. (1989). Activation of phospholipase A2 by endothelin in cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.,* **158,** 279–286.

SHARP, J.D., WHITE, D.L., CHIOU, X.G., GOODSON, T., GAMBOA, G.C., MCCLURE, D., BURGETT, S., HOSKINS, J., SKATRUD, P.L. & SPORTSMAN, J.R. (1991). Molecular cloning and expression of human Ca^{2+}-sensitive cytosolic phospholipase A2. *J. Biol. Chem.,* **266,** 14850–14853.

SILFANI, T.N. & FREEMAN, E.J. (2002). Phosphatidylinositoide 3-kinase regulates angiotensin II-induced cytosolic phospholipase A2 activity and growth in vascular smooth muscle cells. *Arch. Biochem. Biophys.,* **402,** 84–93.

SUGAWARA, F., NINOMIYA, H., OKAMOTO, Y., MIWA, S., MAZDA, O., KATSURA, Y. & MASAKI, T. (1996). Endothelin-I-induced mitogenic responses of Chinese hamster ovary cells expressing human endothelin A: the role of a wortmannin-sensitive signaling pathway. *Mol. Pharmacol.,* **49,** 447–457.

TREVISI, L., BOVA, S., CARGNELI, G., CEOLOTTO, G. & LUCIANI, S. (2002). Endothelin-1-induced arachidonic acid release by cytosolic phospholipase A2 activation in rat vascular smooth muscle *via* extracellular signal-regulated kinases pathway. *Biochem. Pharmacol.,* **64,** 425–431.

VANE, J.R. & BOTTING, R.M. (1997). Mechanism of action of aspirin-like drugs. *Semin. Arthritis. Rheum.,* **26** (Suppl 1), 2–10.

VAN ROSSUM, G.S., KLOOSTER, R., VAN DEN BOSCH, H., VERKLEIJ, A.J. & BOONSTRA, J. (2001). Phosphorylation of $p42/44^{MAPK}$ by various signal transduction pathways activates cytosolic phospholipase A_2 to variable degrees. *J. Biol. Chem.,* **276,** 28976–28983.

WU-WONG, J.R., DAYTON, B.D. & OPGENORTH, T.J. (1996). Endothelin-1-evoked arachidonic acid release: a Ca^{2+}-dependent pathway. *Am. J. Physiol.,* **271,** C869–C877.

(Received February 17, 2003
Revised April 16, 2003
Accepted May 14, 2003)