0026-895X/03/6304-808–813$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 63:808–813, 2003

Vol. 63, No. 4
2221/1050235
Printed in U.S.A.

Involvements of Voltage-Independent Ca^{2+} Channels and Phosphoinositide 3-Kinase in Endothelin-1–Induced PYK2 Tyrosine Phosphorylation

YOSHIFUMI KAWANABE, NOBUO HASHIMOTO, and TOMOH MASAKI

Departments of Neurosurgery (Y.K., N.H.) and Pharmacology (T.M.), Kyoto University Graduate School of Medicine, Kyoto, Japan; and Renal Division, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts (Y.K.)

Received October 16, 2002; accepted December 24, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

We demonstrated recently that endothelin-1 (ET-1) activates two types of Ca^{2+}-permeable nonselective cation channels [designated nonselective cation channel (NSCC)-1 and NSCC-2] and a store-operated Ca^{2+} channel (SOCC) in rabbit internal carotid artery vascular smooth muscle cells (ICA VSMCs). 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 LOE 908 and SK&F 96365, and SOCC is resistant to LOE 908 and sensitive to SK&F 96365. The purpose of the present study was to identify the Ca^{2+} channels involved in the ET-1–induced, proline-rich tyrosine kinase 2 (PYK2) phosphorylation in ICA VSMCs. Based on sensitivity to nifedipine, an L-type voltage-operated Ca^{2+} channel (VOCC) blocker, Ca^{2+} influx through VOCC seems to play a minor role in the ET-1–induced PYK2 phosphorylation. In the presence of nifedipine, PYK2 phosphorylation was abolished by blocking Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC. The phosphoinositide 3-kinase (PI3K) inhibitors wortmannin and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY 294002), inhibited ET-1–induced Ca^{2+} influx through NSCC-2 and SOCC. In addition, these inhibitors blocked PYK2 phosphorylation that depends on Ca^{2+} influx through NSCC-2 and SOCC. These results indicate that 1) Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC plays essential roles in ET-1–induced PYK2 phosphorylation, 2) NSCC-2 and SOCC are stimulated by ET-1 via a PI3K-dependent cascade, whereas NSCC-1 is stimulated via a PI3K-independent cascade, and 3) PI3K is involved in the PYK2 phosphorylation that depends on Ca^{2+} influx through SOCC and NSCC-2.

Endothelin-1 (ET-1) exhibits mitogenic activity in vascular smooth muscle cells (VSMCs) (Battistini et al., 1993; Iwasaki et al., 1999; Kawanabe et al., 2002), suggesting a possible role for ET-1 in the pathogenesis of clinical conditions such as hyperlipoproteinemia, atherosclerosis, and restenosis after angioplasty, including that of the carotid artery (Lerman et al., 1991; Douglas et al., 1994; Haak et al., 1994; Burke et al., 1997). The molecular mechanisms of the ET-1–induced mitogenic response, however, remain unclear. The effects of ET-1 are mediated by ligand-dependent activation of the specific G protein-coupled receptors (GPCRs), endothelin$_A$ and endothelin$_B$ receptors (ET_AR and ET_BR, respectively) (Arai et al., 1990; Sakurai et al., 1990). GPCRs are devoid of intrinsic tyrosine kinase activity. Therefore, the protein tyrosine phosphorylation induced by ligands of GPCRs depends ultimately upon subsequent activation of cellular tyrosine kinases. ET-1 activates members of the Src family of cytoplasmic tyrosine kinases and tyrosine phosphorylation seems to be essential for the mitogenic response (Simonson et al., 1996; Cazaubon et al., 1997; Schieffer et al., 1997). One of the typical cellular responses to ligand-dependent GPCR activation shared by ET-1 is the mobilization of intracellular calcium. The cloning of the calcium-regulated cytoplasmic pro-

This study was supported by the Banyu Fellowship Awards in Cardiovascular Medicine (to Y.K.), sponsored by Banyu Pharmaceutical Co., Ltd., and by The Merck Company Foundation (to Y.K.), by a grant from the Smoking Research Foundation, Japan, and by the Uehara Memorial Foundation Fellowship, Tokyo, Japan.

ABBREVIATIONS: ET-1, endothelin-1; VSMC, vascular smooth muscle cell; GPCR, G-protein coupled receptor; ET_xR, endothelin type *x* receptor (*x* = A or B); PYK2, proline-rich tyrosine kinase 2; VICC, voltage-independent Ca^{2+} channels; PI3K, phosphoinositide 3-kinase; VOCC, voltage-operated Ca^{2+} channel; NSCC, nonselective cation channel; SOCC, store-operated 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; ICA, internal carotid artery; PBS, phosphate-buffered saline; BQ 788, *N*-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltrptophanyl-D-Nle; LY 294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; BQ123, cyclo-D-Asp-Pro-D-Val-Leu-D-Trp.

line-rich tyrosine kinase (PYK2) suggested a link between GPCRs and the induction of tyrosine phosphorylation via mobilization of intracellular calcium (Rikitake et al., 2001; Beitner-Johnson et al., 2002). Moreover, PYK2 plays an important role in coupling GPCRs with extracellular signal-regulated kinase activation (Dikic et al., 1996; Blaukat et al., 1999). ET-1–induced extracellular signal-regulated kinase activation was found to coincide with PYK2 tyrosine phosphorylation in primary astrocytes (Cazaubon et al., 1997). Therefore, it is important to investigate the intracellular signaling pathways that regulate the activation of PYK2. We have focused on voltage-independent Ca^{2+} channels (VICCs) and phosphoinositide 3-kinase (PI3K) in this context. We recently demonstrated that ET-1 activates three types of VICCs in addition to VOCCs in ICA VSMCs (Kawanabe et al., 2002). The VICCs include two types of Ca^{2+}-permeable nonselective cation channel (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC) (Kawanabe et al., 2002). Importantly, these channels can be distinguished by their sensitivity to receptor-operated Ca^{2+} channel blockers 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., 2002). Previous studies demonstrated that PI3K plays important roles for stimulation of L-type VOCCs by angiotensin (Seki et al., 1999; Viard et al., 1999) and T cell Ca^{2+} signaling via the phosphatidylinositol 3,4,5-triphosphate-sensitive Ca^{2+} entry pathway (Hsu et al., 2000). In this study, we examined whether and which VICCs are activated by ET-1 in VSMCs via PI3K-dependent pathway. We also investigated the effects of PI3K on ET-1–induced PYK2 phosphorylation.

Materials and Methods

Cell Culture. Isolated VSMCs were prepared from rabbit ICA as described previously (Kawanabe et al., 2002). The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with 100 units/ml penicillin G and 100 ug/ml streptomycin under a humidified 5% CO_2/95% air atmosphere.

Measurement of PYK2 Phosphorylation. Measurement of PYK2 phosphorylation was performed using a universal tyrosine kinase assay kit (Takara, Tokyo, Japan). Extraction buffer and kinase reacting solution were provided in this kit. Cells seeded at 5×10^6 cells/well in six-well plates were deprived of serum for 24 h then stimulated with various concentrations of ET-1 for the indicated times. Reaction was terminated by washing cells three times with phosphate-buffered saline (PBS). After the addition of 1 ml of extraction buffer, the cells were scraped off and centrifuged at 14,500 rpm for 10 min at 4°C. The supernatant was incubated with rabbit polyclonal anti-PYK2 antibody (Upstate Biotechnology, Lake Placid, NY) for 1 h at room temperature and subsequently incubated with protein A-agarose for an additional 20 min. The mixture was centrifuged at 10,000*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. PYK2 phosphorylation 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).

Immunoblotting. Samples resuspended in kinase reaction buffer were analyzed by SDS-polyacrylamide gel electrophoresis and transferred electrophoretically to polyvinylidene difluoride membranes (15 V, 90 min). After blocking with 5% bovine serum albumin for 1 h, membranes were reacted with anti phosphotyrosine monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit polyclonal anti-PYK2 antibody for 1 h. The blots were washed and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h. After further washing, immunoreactive proteins were detected by the enhanced chemiluminescence system.

Monitoring of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was monitored using the fluorescent probe, fluo-3, as described previously (Kawanabe et al., 2001). Briefly, cells were loaded with fluo-3 by incubation with 10 μM fluo-3/AM at 37°C in the dark for 30 min. After washing, the cells were suspended at a density of $\sim 2 \times 10^7$ cells/ml, and 0.5-ml aliquots were used for measurement of fluorescence by a CAF 110 spectrophotometer (JASCO, Tokyo, Japan) at an excitation wavelength of 490 nm and an emission wavelength of 540 nm. At the end of the experiment, Triton X-100 and subsequently EGTA were added to a final concentration of 0.1% and 5 mM, respectively, to determine maximum fluorescence (F_{max}) and the minimum fluorescence (F_{min}) values. The $[Ca^{2+}]_i$ was calculated by the equilibrium equation, $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where F is the experimental value of fluorescence and K_d was defined as 0.4 μM (Minta et al., 1989).

Drugs. LOE 908 was kindly provided by Boehringer Ingelheim K.G. (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, and 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 PYK2 Phosphorylation in VSMCs. To investigate whether PYK2 participate in ET-1–induced signaling pathways in ICA VSMCs, we first examined the activation of PYK2 by immunoblot analysis (Fig. 1). Our results indicate that PYK2 is expressed in ICA VSMCs and that the protein is tyrosine-phosphorylated within 5 min (Fig. 1A). PYK2 phosphorylation induced by 10 nM ET-1

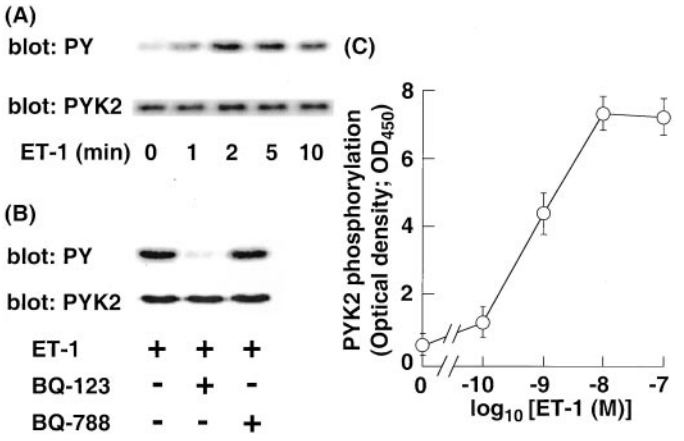

Fig. 1. Effects of ET-1 on PYK2 phosphorylation in VSMCs. A, the cells were incubated with 10 nM ET-1 for the indicated times. B, the cells were pretreated with or without 5 μM BQ123 or BQ788 for 30 min and incubated with 10 nM ET-1 for 2 min. PYK2 was immunoprecipitated from cell lysates with polyclonal anti-PYK2 antibody and analyzed by immunoblotting with either anti-phosphotyrosine (PY20) (top) or polyclonal anti-PYK2 antibody (bottom). C, effects of various concentrations of ET-1 on PYK2 phosphorylation in VSMCs. The cells were stimulated with increasing concentrations of ET-1 for 2 min. PYK2 phosphorylation was determined using a universal tyrosine kinase assay kit as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate.

increased with time and reached a peak value after approximately 2 min (Fig. 1A). Thus, the stimulation time was set to 2 min in subsequent experiments. ET-1–induced PYK2 phosphorylation was inhibited significantly by 5 μM BQ 123, a specific blocker of ET_AR, whereas it was not inhibited significantly by 5 μM BQ 788, a specific blocker of ET_BR (Fig. 1B). PYK2 phosphorylation was induced by ET-1 in a concentration-dependent manner with EC_{50} values of ~1 nM (Fig. 1C). The stimulation reached its maximum at concentrations ≥10 nM (Fig. 1C).

Effects of Extracellular Ca^{2+} and Nifedipine on ET-1–Induced PYK2 Phosphorylation. The magnitudes of ET-1–induced PYK2 phosphorylation in the absence of extracellular Ca^{2+} were near the basal level (Fig. 2). Therefore, extracellular Ca^{2+} influx seems to play an important role in ET-1–induced PYK2 phosphorylation.

We examined the effects of extracellular Ca^{2+} influx through VOCCs on ET-1–induced PYK2 phosphorylation using nifedipine, a specific blocker of L-type VOCCs. Nifedipine at 1 μM completely inhibited the ET-1–induced extracellular Ca^{2+} influx through VOCCs in VSMC (data not shown). In contrast, it inhibited ET-1–induced PYK2 phosphorylation by a maximum of only about 10% (Fig. 2B).

Effects of SK&F 96365 and LOE 908 on ET-1–Induced PYK2 Phosphorylation. Using SK&F 96365 and LOE 908, we attempted to determine the effects of extracellular Ca^{2+} influx through VICCs on ET-1–induced PYK2 phosphorylation. 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 ET-1–induced PYK2 phosphorylation in a concentration-dependent manner with an IC_{50} value of ~3 μM (Fig. 3, A and C). Maximal inhibition was observed at concentrations ≥10 μM (Fig. 3C). The extent of maximal inhibition was around 80% of nifedipine-resistant part of PYK2 phosphorylation (Fig. 3D). Similarly, the IC_{50} values of LOE 908 for inhibition of ET-1–induced PYK2 phosphorylation were ~3 μM, and maximal inhibition was observed at concentrations ≥10 μM (Fig. 3, B and C). The extent of maximal inhibition was around 60% of PYK2 phosphorylation (Fig. 3D). Notably, the combined treatment with maximal effective concentration (10 μM) of SK&F 96365 and LOE 908 completely inhibited nifedipine-resistant PYK2 phosphorylation (Fig. 3D).

Effects of Wortmannin and LY 294002 on the ET-1–Induced Increase in $[Ca^{2+}]_i$. Next, we examined the effects of PI3K on the ET-1–induced increase in $[Ca^{2+}]_i$ using wortmannin and LY 294002, inhibitors of PI3K. ET-1 at 10 nM induced a biphasic increase in $[Ca^{2+}]_i$ consisting of an initial transient peak and a subsequent sustained increase in both VSMCs and VSMCs preincubated with wortmannin (Fig. 4, A and B). In experiments performed on cells incubated in a bath depleted of extracellular Ca^{2+}, treatment with 10 nM ET-1 did not affect the transient peak, but abolished the sustained increase (data not shown). The magnitude of the transient increase in $[Ca^{2+}]_i$ caused by 10 nM ET-1 in VSMCs preincubated with wortmannin was similar to that in control VSMCs (Fig. 4, A–C). On the other hand, the magnitude of the sustained increase in $[Ca^{2+}]_i$ caused by 10 nM ET-1 in VSMCs preincubated with wortmannin was ~20% of that in VSMCs (Fig. 4, C and D). In contrast, addition of wortmannin after stimulation with ET-1 did not affect the sustained increases in $[Ca^{2+}]_i$ (Fig. 4B).

We also used LY 294002 to evaluate the effects of PI3K on ET-1–induced extracellular Ca^{2+} influx. LY 294002 at 50 μM inhibited PI3K activation completely in VSMCs (Kusch et al., 2000). The magnitudes of the ET-1–induced transient increase in $[Ca^{2+}]_i$ in VSMCs preincubated with 50 μM LY 294002 were similar to those in VSMCs (Fig. 5, A–C). Con-

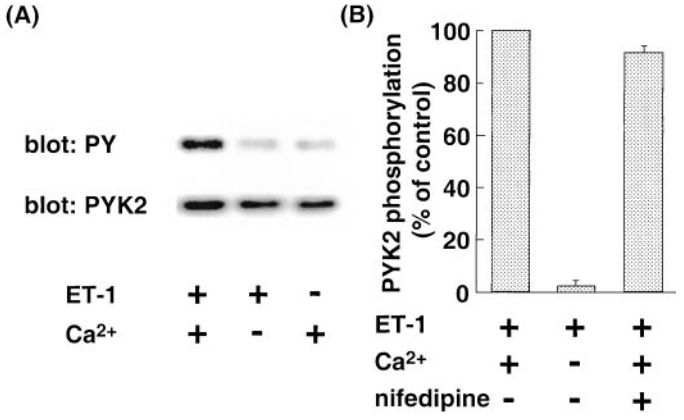

Fig. 2. Effects of extracellular Ca^{2+} and nifedipine on ET-1–induced PYK2 phosphorylation in VSMCs. A, the cells were washed five times with Ca^{2+}-free PBS and incubated with Ca^{2+}-free PBS containing 10 nM ET-1 or Ca^{2+}-plus PBS containing 10 nM ET-1 for 2 min. PYK2 phosphorylation was analyzed as described under *Materials and Methods*. B, the cells incubated for 15 min with nifedipine and then stimulated with 10 nM ET-1. PYK2 phosphorylation was determined using a universal tyrosine kinase assay kit as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate.

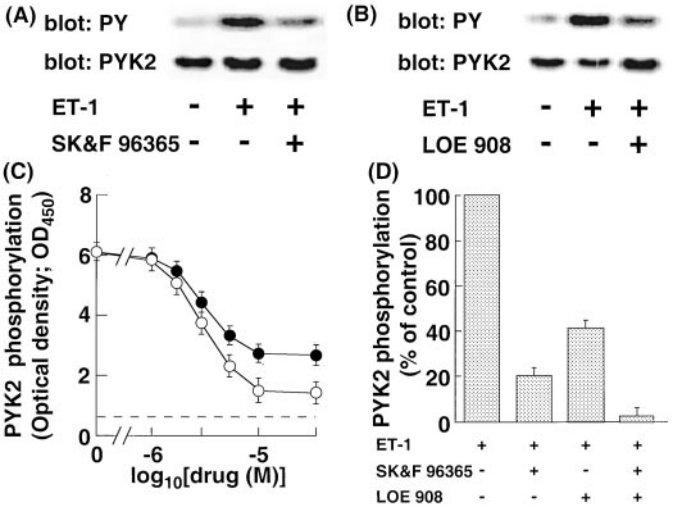

Fig. 3. Effects of SK&F 96365 and LOE 908 on ET-1–induced PYK2 phosphorylation in VSMCs. A, B and D, effects of a maximal effective concentration (10 μM) of SK&F 96365 and LOE 908 on ET-1–induced PYK2 phosphorylation in VSMCs. PYK2 was immunoprecipitated from cell lysates and analyzed by immunoblotting with either anti-phosphotyrosine (PY20) (top) or polyclonal anti-PYK2 antibody (bottom). C, effects of various concentrations of SK&F 96365 and LOE 908 on ET-1–induced PYK2 phosphorylation in VSMCs. Starved cells were incubated for 15 min with various concentrations of SK&F 96365 (○) or LOE 908 (●) in addition to 1 μM nifedipine and then stimulated with 10 nM ET-1 for 2 min. PYK2 phosphorylation was determined using a universal tyrosine kinase assay kit as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of five determinations, each done in triplicate. #, $P < 0.05$; significantly different from the control values in each experiment.

versely, 50 μM LY 294002 affected approximately 80% inhibition of the ET-1–induced sustained increase in $[Ca^{2+}]_i$ (Fig. 5, B and D). Addition of LY 294002 after stimulation with ET-1 did not affect the sustained increases in $[Ca^{2+}]_i$ (Fig. 5B).

Effects of SK&F 96365 and LOE 908 on the ET-1–Induced Sustained Increase in $[Ca^{2+}]_i$ in VSMCs Preincubated with Wortmannin. As described previously (Kawanabe et al., 2002), the ET-1–induced sustained increase in $[Ca^{2+}]_i$ was partially suppressed by LOE 908 or SK&F 96365 in VSMCs. Moreover, combined treatment with these drugs abolished ET-1–induced sustained increase in $[Ca^{2+}]_i$ in the presence of nifedipine On the other hand, the ET-1–induced sustained increase in $[Ca^{2+}]_i$ in VSMCs preincubated with 1 μM wortmannin was inhibited by LOE 908; complete inhibition was observed at concentrations $\geq$10 μM (Fig. 6). Moreover, SK&F 96365, up to 10 μM, failed to inhibit the ET-1–induced sustained increase in $[Ca^{2+}]_i$ in VSMCs preincubated with 1 μM wortmannin (Fig. 6). These results suggest that NSCC-1 is activated by ET-1 via a wortmannin-independent pathway, whereas NSCC-2 and SOCC are activated via a wortmannin-dependent pathway. In VSMCs preincubated with 50 μM LY 294002, the ET-1–induced sustained increase in $[Ca^{2+}]_i$ was also sensitive to LOE 908 and resistant to SK&F 96365 (data not shown).

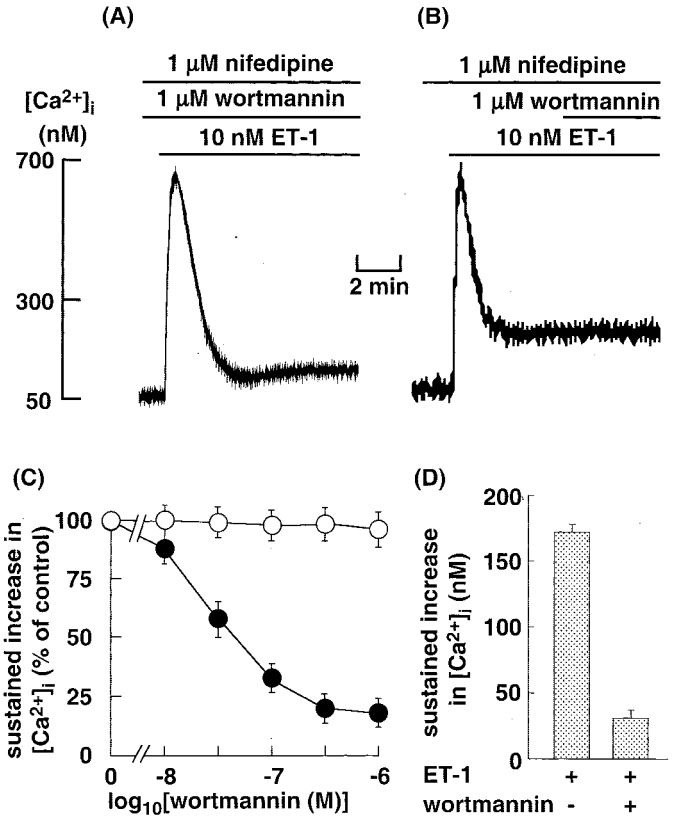

Fig. 4. A and B, original tracings illustrating the effects of wortmannin on the ET-1–induced increase in $[Ca^{2+}]_i$ in VSMCs. The cells loaded with fluo-3 were incubated with 1 μM wortmannin before (A) or after (B) 10 nM ET-1 stimulation. C, effects of various concentrations of wortmannin on the ET-1–induced transient (○) and sustained (●) increase in $[Ca^{2+}]_i$ in VSMCs. Wortmannin was added 15 min before stimulation with ET-1. D, effects of a maximal effective concentration (1 μM) of wortmannin on ET-1–induced sustained increase in $[Ca^{2+}]_i$. The cells loaded with fluo-3 were incubated with 1 μM wortmannin before stimulation with 10 nM ET-1. Each point represents the mean ± S.E.M. of five experiments.

Effects of Wortmannin and LY 294002 on the ET-1–Induced PYK2 Phosphorylation. The magnitudes of PYK2 phosphorylation caused by 10 nM ET-1 in VSMCs preincubated with wortmannin or LY 294002 were smaller than those in VSMCs (Fig. 7, A and B). Wortmannin inhibited ET-1–induced PYK2 phosphorylation in a concentration-dependent manner with an IC_{50} value of ~30 nM (Fig. 7C). Maximal inhibition was observed at concentrations $\geq$1 μM (Fig. 7C). The extent of maximal inhibition was around 80% of nifedipine-resistant PYK2 phosphorylation (Fig. 7D). The

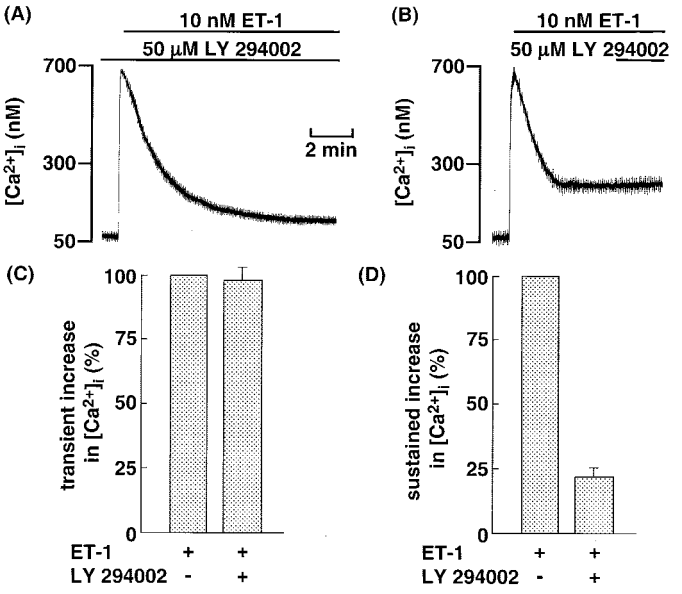

Fig. 5. A and B, original tracings illustrating the effects of LY 294002 on the ET-1–induced increase in $[Ca^{2+}]_i$ in VSMCs. The cells loaded with fluo-3 were incubated with 50 μM LY 294002 before (A) or after (B) 10 nM ET-1 stimulation. C and D, effects of a maximal effective concentration (50 μM) of LY 294002 on ET-1–induced transient (C) or sustained (D) increase in $[Ca^{2+}]_i$. The cells loaded with fluo-3 were incubated with 50 μM LY 294002 before stimulation with 10 nM ET-1. Each point represents the mean ± S.E.M. of five experiments.

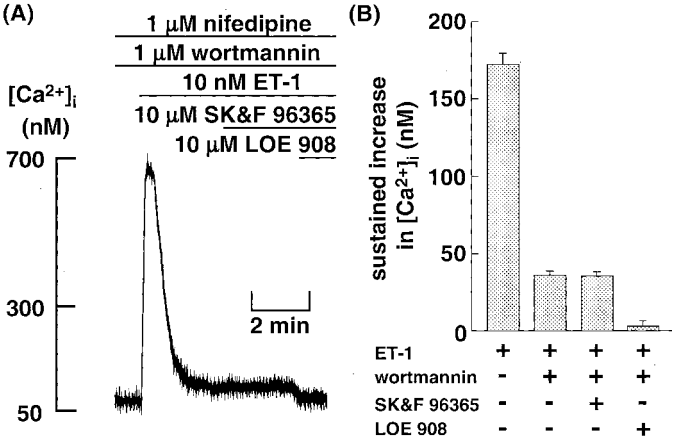

Fig. 6. A, original tracings illustrating the effects of SK&F 96365 and LOE 908 on the ET-1–induced sustained increase in $[Ca^{2+}]_i$ in VSMCs pretreated with wortmannin. The cells loaded with fluo-3 were incubated with 1 μM wortmannin before 10 nM ET-1 stimulation. After $[Ca^{2+}]_i$ reached a steady-state, 10 μM SK&F 96365 or 10 μM LOE 908 was added at the time indicated by horizontal bars. B, effects of a maximal effective concentration (10 μM) of SK&F 96365 or LOE 908 on the 10 nM ET-1–induced sustained increase in $[Ca^{2+}]_i$ in VSMCs pretreated with 1 μM wortmannin. Each point represents the mean ± S.E.M. of five experiments.

ET-1–induced PYK2 phosphorylation in VSMCs preincubated with 1 μM wortmannin was inhibited by LOE 908, concentrations $\geq$10 μM effecting complete inhibition (Fig. 7D). In contrast, SK&F 96365, up to 10 μM, failed to inhibit ET-1–induced PYK2 phosphorylation in VSMCs preincubated with 1 μM wortmannin (Fig. 7D).

Discussion

ET-1 induces PYK2 phosphorylation in rabbit ICA VSMCs (Fig. 1). Based on the sensitivity to BQ123 and BQ788, ET_AR plays essential roles in this ET-1–induced PYK2 phosphorylation (Fig. 1B). The magnitudes of ET-1–induced PYK2 phosphorylation in the absence of extracellular Ca^{2+} were near the basal level (Fig. 2). These results indicate that extracellular Ca^{2+} influx plays important roles in ET-1–induced PYK2 phosphorylation in ICA VSMCs. Therefore, we tried to characterize the Ca^{2+} channels involved in ET-1–induced PYK2 phosphorylation in ICA VSMCs. Our recent study indicated that NSCC-1, NSCC-2, and SOCC mediate a major part of the ET-1–induced extracellular Ca^{2+} influx in ICA VSMCs (Kawanabe et al., 2002). Moreover, extracellular Ca^{2+} influx through these Ca^{2+} channels plays essential roles in the ET-1–induced mitogenesis (Kawanabe et al., 2002). Thus, we examined the involvement of NSCC-1, NSCC-2, and SOCC in ET-1–induced PYK2 phosphorylation using SK&F 96365 and LOE 908. According to the nifedipine sensitivity of ET-1–induced PYK2 phosphorylation, involvement of VOCC in this response was estimated to be minor (~10%) (Fig. 2B). 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., 2002). This suggests that Ca^{2+} channels other than VOCC may play an important role in ET-1–induced PYK2 phosphorylation in addition to mediating extracellular Ca^{2+} influx in rabbit ICA VSMCs.

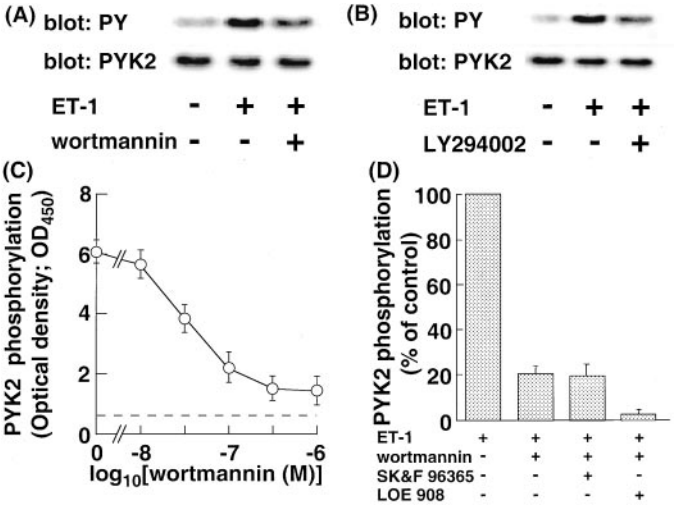

Fig. 7. Effects of wortmannin and LY 294002 on ET-1–induced PYK2 phosphorylation in VSMCs. Starved cells were incubated for 15 min with 1 μM wortmannin (A) or 50 μM LY 294002 (B) in addition to 1 μM nifedipine and then stimulated with 10 nM ET-1 for 2 min. PYK2 was immunoprecipitated from cell lysates and analyzed by immunoblotting with either anti-phosphotyrosine (PY20) (top) or polyclonal anti-PYK2 antibody (bottom). C, effects of various concentrations of wortmannin on ET-1–induced PYK2 phosphorylation. D, effects of SK&F 96365 or LOE 908 on ET-1–induced PYK2 phosphorylation in VSMCs pretreated with wortmannin. PYK2 phosphorylation was determined using a universal tyrosine kinase assay kit as described under *Materials and Methods*. Data presented are the mean ± S.E.M. of three determinations, each done in triplicate.

The inhibitory action of SK&F 96365 and LOE 908 on ET-1–induced PYK2 phosphorylation is considered to be mediated by blockage of Ca^{2+} entry through VICCs for the following reasons. 1) In our recent work using whole-cell patch-clamp and $[Ca^{2+}]_i$ monitoring, ET-1 was found to activate three types of VICCs in VSMCs: 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., 2002). 2) The IC_{50} values of these blockers for ET-1–induced PYK2 phosphorylation (Fig. 3) correlated well with those for ET-1–induced extracellular Ca^{2+} influx (Kawanabe et al., 2002). Moreover, because SK&F 96365 and LOE 908 failed to inhibit the ET-1–induced transient increase in $[Ca^{2+}]_i$ resulting from the release of Ca^{2+} from intracellular Ca^{2+} stores (Kawanabe et al., 2002), the release of sarcoplasmic reticulum Ca^{2+} is insufficient to stimulate PYK2 phosphorylation. Three types of VICC seem to be involved in the ET-1–induced PYK2 phosphorylation in terms of its sensitivity to SK&F 96365 and LOE 908 (Fig. 3). 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 believed to be NSCC-1, NSCC-2, and SOCC, respectively. The magnitudes of the ET-1–induced PYK2 phosphorylation that were inhibited by combined treatment with nifedipine, SK&F 96365, and LOE 908 were similar to those observed in the absence of extracellular Ca^{2+} (Fig. 3D). Extracellular Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC plays important roles for ET-1–induced PYK2 phosphorylation in rabbit ICA VSMCs.

Next, we investigated the effects of PI3K on ET-1–induced PYK2 phosphorylation in ICA VSMCs. For this purpose, at first, we examined whether PI3K was involved in the ET-1–mediated activation of Ca^{2+} channels. The inhibitory effects of wortmannin on the ET-1–induced sustained increase in $[Ca^{2+}]_i$ may be because of its inhibitory effects on PI3K, judging 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, LY 294002, also inhibited the wortmannin-sensitive ET-1–induced sustained increase in $[Ca^{2+}]_i$ (Fig. 5). 3) The IC_{50} values (~30 nM) and maximal effective concentration (1 μM) of wortmannin for the ET-1–induced sustained increase in $[Ca^{2+}]_i$ (Fig. 4) were similar to those for ET-1–induced phosphatidylinositol triphosphate formation, which was measured as an index of PI3K activity (Sugawara et al., 1996). Just as wortmannin and LY 294002 partially suppressed the ET-1–induced sustained increase in $[Ca^{2+}]_i$ (Fig. 1), ET-1 induces extracellular Ca^{2+} influx through VICCs via both PI3K-dependent and PI3K-independent pathways. Based on the sensitivity to SK&F 96365 and LOE 908 (Fig. 4), the wortmannin resistant sustained increase in $[Ca^{2+}]_i$ was caused by Ca^{2+} influx through NSCC-1 (LOE 908-sensitive and SK&F 96365-resistant). Therefore, Ca^{2+} influx through NSCC-2 and SOCC are wortmannin-sensitive. These results indicate that PI3K may play important roles for ET-1–in-

duced activation of NSCC-2 and SOCC. Moreover, from the data demonstrating that addition of wortmannin or LY 294002 after stimulation with ET-1 does not suppress the sustained increase in $[Ca^{2+}]_i$ (Figs. 4 and 5), PI3K seems to be required for the activation of Ca^{2+} entry but not for its maintenance. On the other hand, wortmannin and LY 294002 failed to inhibit the ET-1–induced transient increase in $[Ca^{2+}]_i$ (Fig. 1). The ET-1–induced transient increase in $[Ca^{2+}]_i$ involves intracellular cascade such as phospholipase C/inositol trisphosphate/mobilization of Ca^{2+} from intracellular stores (Berridge, 1993). PI3K does not seem to affect this cascade.

To evaluate the effects of PI3K on ET-1–induced PYK2 phosphorylation, we investigated the effects of wortmannin on ET-1–induced PYK2 phosphorylation. The IC_{50} values (~30 nM) and maximal effective concentration (1 μM) of wortmannin for the ET-1–induced PYK2 phosphorylation (Fig. 7) were similar to those for ET-1–induced sustained increase in $[Ca^{2+}]_i$ (Fig. 4). Therefore, the inhibitory effects of wortmannin on ET-1–induced PYK2 phosphorylation may be also caused by its inhibitory effects on PI3K. The wortmannin-resistant, ET-1–induced PYK2 phosphorylation was dependent on extracellular Ca^{2+} influx through NSCC-1, based on the resistance to SK&F 96365 and sensitivity to LOE 908 (SK&F 96365-resistant and LOE 908-sensitive) (Fig. 6). These results indicate that the inhibitory effects of wortmannin on ET-1–induced PYK2 phosphorylation might be mediated by blockage of Ca^{2+} entry through NSCC-2 and SOCC. The detailed mechanisms of NSCC-1–dependent PYK2 phosphorylation caused by ET-1 remain unclear. The signaling pathways involved in ET-1–induced PYK2 phosphorylation remain unknown.

In conclusion, extracellular Ca^{2+} influx through NSCC-1, NSCC-2, and SOCC plays an essential role for ET-1–induced PYK2 phosphorylation in rabbit ICA VSMCs. In addition, PI3K seems to be involved in ET-1–induced PYK2 phosphorylation that depends on the extracellular Ca^{2+} influx through SOCC and NSCC-2.

Acknowledgments

We thank Boehringer Ingelheim K.G. (Ingelheim, Germany) for the kind donation of LOE 908.

References

Arai H, Hori S, Aramori I, Ohkubo H, and Nakanishi S (1990) Cloning and expression of a cDNA encoding an endothelin receptor. *Nature (Lond)* **348:**730–732.
Battistini B, Chailler P, D'orlrans-Juste P, Briere N, Sirois P (1993) Growth regulatory properties of endothelins. *Peptides* **14:**385–399.
Beitner-Johnson D, Ferguson T, Rust RT, Kobayashi S, and Millhorn DE (2002) Calcium-dependent activation of Pyk2 by hypoxia. *Cell Signal* **14:**133–137.
Berridge MJ (1993) Cell signalling. A tale of two messengers. *Nature (Lond)* **365:** 388–389.
Blaukat A, Ivankovic-Dikic I, Gronroos E, Dolfi F, Tokiwa G, Vuori K, and Dikic I (1999) Adaptor proteins Grb2 and Crk couple Pyk2 with activation of specific mitogen-activated protein kinase cascades. *J Biol Chem* **274:**14893–14901.
Burke SE, Lubbers NL, Gagne GD, Wessale JL, Dayton BD, Wegner CD, and Opgenorth TJ (1997) Selective antagonism of the ET_A receptor reduces neointimal hyperplasia after balloon-induced vascular injury in pigs. *J Cardiovasc Pharmacol* **30:**33–41.
Cazaubon S, Chaverot N, Romero IA, Girault JA, Adamson P, Strosberg AD, and Couraud PO (1997) Growth factor activity of endothelin-1 in primary astrocytes mediated by adhesion-dependent and -independent pathways. *J Neurosci* **17:** 6203–6212.
Dikic I, Tokiwa G, Lev S, Courtneidge SA, and Schlessinger J (1996) A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. *Nature (Lond)* **383:**547–550.
Douglas SA, Louden C, Vickery-Clark LM, Storer BL, Hart T, Feuerstein GZ, Elliott JD, and Ohlstein EH (1994) A role for endogenous endothelin-1 in neointimal formation after rat carotid artery balloon angioplasty. Protective effects of the novel nonpeptide endothelin receptor antagonist SB 209670. *Circ Res* **75:**190–197.
Encabo A, Romanin C, Birke FW, Kukovetz WR, and Groschner 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.
Haak T, Marz W, Jungmann E, Hausser S, Siekmeier R, Gross W, and Usadel KH (1994) Elevated endothelin levels in patients with hyperlipoproteinemia. *Clin Investig* **72:**580–584.
Hsu AL, Ching TT, Sen G, Wang DS, Bondada S, Authi KS, and Chen CS (2000) Novel function of phosphoinositide 3-kinase in T cell Ca^{2+} signaling. *J Biol Chem* **275:**16242–16250.
Iwasaki H, Eguchi S, Ueno H, Marumo F, and 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, Okamoto Y, Enoki T, Hashimoto N, and Masaki T (2001) Ca^{2+} channels activated by endothelin-1 in CHO cells expressing endothelin-A or endothelin-B receptors. *Am J Physiol* **281:**C1676–C1685.
Kawanabe Y, Hashimoto N, and Masaki T (2002) Ca^{2+} channels involved in endothelin-induced mitogenic response in carotid artery vascular smooth muscle cells. *Am J Physiol* **282:**C330–C337.
Kusch A, Tkachuk S, Haller H, Dietz R, Gulba DC, Lipp M, and Dumler I (2000) Urokinase stimulates human vascular smooth muscle cell migration via a phosphatidylinositol 3-kinase-Tyk2 interaction. *J Biol Chem* **275:**39466–39473.
Lerman A, Edwards BS, Hallett JW, Heublein DM, Soderg SM, and Burnett JC (1991) Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis. *N Engl J Med* **325:**997–1001.
Meritt JE, Airmstrong WP, Benham CD, Hallam TJ, Jacob R, Jaxa-Chamiec A, Leigh BK, Mccarthy SA, Moores KE, and Rink TJ (1990) SK&F 96365, a novel inhibitor of receptor-mediated calcium entry. *Biochem J* **271:**515–522.
Minta A, Kao JPY, and Tsien RY (1989) Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J Biol Chem* **264:**8171–8178.
Rikitake Y, Kawashima S, Takahashi T, Ueyama T, Ishido S, Inoue N, Hirata K, and Yokoyama M (2001) Regulation of tyrosine phosphorylation of PYK2 in vascular endothelial cells by lysophosphatidylcholine. *Am J Physiol* **281:**H266–H274.
Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, and Masaki T (1990) Cloning of cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature (Lond)* **348:**732–735.
Schieffer B, Drexler H, Ling BN, and Marrero MB (1997) G protein-coupled receptors control vascular smooth muscle cell proliferation via pp60c-src and p21ras. *Am J Physiol* **272:**C2019–C2030.
Seki T, Yokoshiki H, Sunagawa M, Nakamura M, and Sperelakis N (1999) Angiotensin II stimulation of Ca^{2+}-channel current in vascular smooth muscle cells is inhibited by lavendustin-A and LY-294002. *Pfleug Arch Eur J Physiol* **437:**317–323.
Simonson MS, Wang Y, and Herman WH (1996) Nuclear signaling by endothelin-1 requires Src protein-tyrosine kinases. *J Biol Chem* **271:**77–82.
Sugawara F, Ninomiya H, Okamoto Y, Miwa S, Mazda O, Katsura Y, and Masaki T (1996) Endothelin-1-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.
Ui M, Okada T, Hazeki K, and Hazeki O (1995) Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem Sci* **20:**303–307.
Viard P, Exner T, Maier U, Mironneau J, Nurnberg B, and Macrez N (1999) G$\beta\gamma$ dimers stimulate vascular L-type Ca^{2+} channels via phosphoinositide 3-kinase. *FASEB J* **13:**685–694.
Yano H, Nakanishi S, Kimura K, Hanai N, Saitoh Y, Fukui Y, Nonomra Y, and Matsuda Y (1993) Inhibition of histamine secretion by wortmannin through the blockade of phosphatidyl 3-kinase in RBL-2H3 cells. *J Biol Chem* **268:**13–16.

Address correspondence to: Yoshifumi Kawanabe, M.D., Ph.D., 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. E-mail: ykawanabe@rics.bwh.harvard.edu