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Effects of phosphoinositide 3-kinase on endothelin-1-induced activation of voltage-independent Ca^{2+} channels and vasoconstriction

Yoshifumi Kawanabe[a,b,c,*,1], Nobuo Hashimoto[a], Tomoh Masaki[b]

[a]*Department of Neurosurgery, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan*
[b]*Department of Pharmacology, Kyoto University Graduate School of Medicine, Kyoto 606-8507, Japan*
[c]*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, USA*

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Abstract

We recently demonstrated that endothelin-1 (ET-1) activates two types of Ca^{2+}-permeable nonselective cation channel (designated NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC) in rabbit basilar artery (BA) vascular smooth muscle cells (VSMCs). In this study, we investigated the effects of phosphoinositide 3-kinase (PI3K) on ET-1-induced activation of these channels and BA contraction by using PI3K inhibitors, wortmannin and LY 249002. To determine which Ca^{2+} channels are activated via PI3K, monitoring of intracellular Ca^{2+} concentration was performed. Role of PI3K in ET-1-induced vasoconstriction was examined by tension study using rabbit BA rings. Only NSCC-1 was activated by ET-1 in wortmannin- or LY 294002-pretreated VSMCs. In contrast, addition of these drugs after ET-1 stimulation did not suppress Ca^{2+} influx. Wortmannin inhibited the ET-1-induced contraction of rabbit BA rings that depends on the Ca^{2+} influx through NSCC-2 and SOCC. The IC_{50} values of wortmannin for the ET-1-induced Ca^{2+} influx and vasoconstriction were similar to those for the ET-1-induced PI3K activation. These results indicate that (1) NSCC-2 and SOCC are stimulated by ET-1 via PI3K-dependent cascade, whereas NSCC-1 is stimulated via PI3K-independent cascade; (2) PI3K is required for the activation of the Ca^{2+} entry, but not for its maintenance; and (3) PI3K is involved in the ET-1-induced contraction of rabbit BA rings that depends on the extracellular Ca^{2+} influx through SOCC and NSCC-2.

Keywords: Vasoconstriction; Phosphoinositide 3-kinase; Endothelin-1; Ca^{2+} influx

1. Introduction

Endothelin-1 (ET-1) is a major cause of cerebral vasospasm after subarachnoid hemorrhage (SAH) [1]. The level of ET-1 in bloody cerebrospinal fluid is elevated in patients with SAH [1]. ET-1 produces a potent and long-lasting vasoconstriction of cerebral artery [1]. Endothelin receptor antagonists, endothelin-converting enzyme inhibitors, and ET-1 antisense oligonucleotide prevent cerebral vasospasm in animal models [1–3]. We recently showed that ET-1-induced rabbit basilar artery (BA) contraction is fully dependent on extracellular Ca^{2+} influx [4]. Ca^{2+} channels activated by ET-1 in rabbit BA vascular smooth muscle cells (VSMCs) were characterized using whole-cell patch-clamps and by monitoring the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). ET-1 activates voltage-independent Ca^{2+} channels (VICCs) in addition to voltage-operated Ca^{2+} channels (VOCCs) in BA VSMCs. These VICCs consist of two types of Ca^{2+}-permeable nonselective cation channel (designated NSCC-1 and NSCC-2) and a

Abbreviations: BA, basilar artery; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CHO cell, Chinese hamster ovary cell; ET-1, endothelin-1; ET_ARs, endothelin$_A$ receptor; ET_BRs, endothelin$_B$ receptors; NSCC, nonselective cation channel; PI3K, phosphoinositide 3-kinase; SAH, subarachnoid hemorrhage; SOCC, store-operated Ca^{2+} channel; VICC, voltage-independent Ca^{2+} channel; VOCC, voltage-operated Ca^{2+} channel; VSMCs, vascular smooth muscle cells

* Corresponding author. Tel.: +81-72-696-0571; fax: +81-72-696-1228.
E-mail address: kawanabe@takatsuki.jrc.or.jp (Y. Kawanabe).

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store-operated Ca^{2+} channel (SOCC) [4]. Importantly, these VICCs can be distinguished in terms of the sensitivity to Ca^{2+} channel blockers, SK&F 96365 and LOE 908. SK&F 96365 and LOE 908 had been developed as an inhibitor of the receptor-mediated Ca^{2+} channels [5–7]. 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; SOCC is resistant to LOE 908 and sensitive to SK&F 96365 [4]. Moreover, LOE 908 inhibits SAH-induced BA vasospasm in rabbit model [8]. These results indicate that if the activation pathways of VICCs induced by ET-1 is revealed, blockade of these pathways may become a new treatment for ET-1-induced vasoconstriction. Biological actions of ET-1 are mediated by two distinct subtypes of receptor, namely $endothelin_A$ and $endothelin_B$ receptors (ET_ARs and ET_BRs, respectively) [9,10]. However, less is known about intracellular signaling pathways that regulate the activation of VICCs by ET-1. In this study, the roles of phosphoinositide 3-kinase (PI3K) in the ET-1-induced VICCs activation and vascular contraction are investigated. Previous reports demonstrate that ET-1 stimulates PI3K in rabbit BA VSMCs and the PI3K inhibitor, wortmannin, abolished ET-1-induced BA contraction in vitro [11]. PI3K plays important roles for stimulation of L-type voltage-dependent Ca^{2+} channels by angiotensin [12,13] and T cell Ca^{2+} signaling via phosphatidylinositol 3,4,5-trisphosphate-sensitive Ca^{2+} entry pathway [14]. Moreover, we demonstrated recently that PI3K is involved in the activation of NSCC-2 and SOCC, but not NSCC-1, in Chinese hamster ovary cells stably expressing $endothelin_A$ receptors (CHO-ET_AR) [15] or $endothelin_B$ receptors (CHO-ET_BR) [16]. Based on these data, we examined whether and which VICCs were activated by ET-1 in BA VSMCs via PI3K-dependent pathway. In addition, because Ca^{2+} influx through VICCs plays an essential role in the ET-1-induced vasoconstriction [4], we examined if stimulation of extracellular Ca^{2+} influx through VICCs is one of the roles of PI3K for ET-1-induced contraction of rabbit BA. Recent report demonstrates that PI3K fails to reduce vasospasm in dog model of experimental SAH [17]. The data of the present study indicate that PI3K seems to be required for the activation of the Ca^{2+} entry, but not for its maintenance. Therefore, the rabbits BA were pretreated with PI3K inhibitor, wortmannin, to examine the effects of PI3K on ET-1-induced BA contraction.

2. Methods

2.1. Cell culture

Isolated VSMCs were prepared from rabbit BA as described previously [4]. The cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum supplemented with 100 μ/ml penicillin G and 100 μg/ml streptomycin under a humidified 5% CO_2/95% air atmosphere.

2.2. Monitoring of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was monitored using the fluorescent probe, fluo-3. The measurement of fluorescence by a CAF 110 spectrophotometer (JASCO, Tokyo, Japan) was performed as described previously [18].

2.3. Tension study

Measurement of tension was performed as described previously [4]. The removed BA was placed in Krebs solution which contained (in mM): NaCl 120; KCl 5.4; $CaCl_2$ 2.2; $MgCl_2$ 1.0; $NaHCO_3$ 25; and glucose 5.6. BA was cut into 3-mm rings in a dissecting chamber filled with Krebs solution bubbled with a 95% O_2 and 5% CO_2 mixture. Endothelial cells were removed from ring specimens by gently rubbing the intimal surface with a cotton bud moistened with Krebs solution. Successful removal of endothelial cells was confirmed by the inability of acetylcholine (1 μM) to induce relaxation. The rings were mounted using a pair of stainless steel hooks under a resting tension of 500 mg in organ baths containing 5 ml of Krebs solution which was maintained at 37 °C and bubbled with a 95% O_2 and 5% CO_2 mixture: one of the hooks was connected to a force transducer (Orientec, Tokyo) and the developed tension was displayed on a Nihon Kohden (Tokyo) RJG4128 polygraph. Bath fluid was changed and resting force readjusted every 20 min until a stable baseline was attained (usually after about 60 min). After readjustment, the preparations were challenged at hourly intervals with 0.3 μM noradrenaline. When two noradrenaline contractions gave reproducible results, the actual experiment was started.

2.4. Drugs

LOE 908 was kindly provided by Boehringer Ingelheim K.G. (Ingelheim, Germany). Other chemicals were commercially obtained from the following sources: ET-1 from Peptide Institute (Osaka, Japan); SK&F 96365 from Biomol (Plymouth Meeting, PA, USA); fluo-3/AM from Dojindo Laboratories (Kumamoto, Japan); wortmannin and nifedipine from Wako (Osaka); LY 294002 and LY303511 from Sigma Chemical Co. (St. Louis, MO, USA).

2.5. 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.

3. Results

3.1. Effects of wortmannin on the ET-1-induced increase in $[Ca^{2+}]_i$ in BA VSMCs

In the presence of 1 μM nifedipine, a blocker of L-type VOCC, 10 nM ET-1 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. 1A and B). These increases in $[Ca^{2+}]_i$ were not detected in BA VSMCs treated with BQ123, a specific ET_AR blocker, but not BQ788, a specific ET_BR blocker (data not shown). In addition, vehicles failed to induce increases in $[Ca^{2+}]_i$ (data not shown). In experiments performed on cells incubated in a bath in which the extracellular Ca^{2+} had been removed, upon treatment with 10 nM ET-1, the transient peak was not affected, but the sustained increase was abolished (data not shown). The magnitudes of ET-1-induced transient increase in $[Ca^{2+}]_i$ in VSMCs preincubated with various concentrations of wortmannin were similar to those in VSMCs (Fig. 1C). On the other hand, wortmannin inhibited ET-1-induced sustained increase in $[Ca^{2+}]_i$ in a concentration-dependent manner with an IC_{50} values of ~30 nM, and the maximal inhibition (~80% of control) was obtained at concentrations ≥ 1 μM (Fig. 1C and D). In contrast, addition of wortmannin after stimulation with ET-1 did not affect the sustained increase in $[Ca^{2+}]_i$ (Fig. 1B).

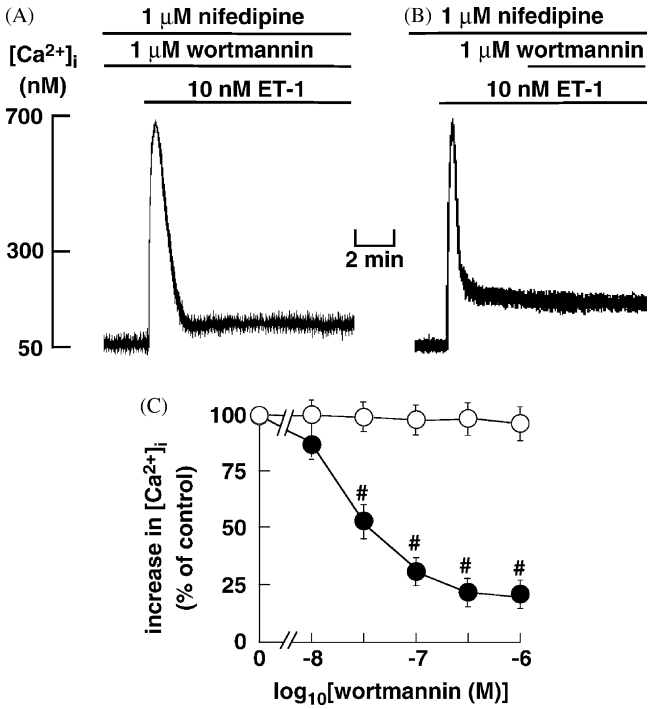

Fig. 1. (A and B) Original tracings illustrating the effects of wortmannin on the endothelin-1 (ET-1)-induced increase in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in basilar artery (BA) vascular smooth muscle cells (VSMCs). In the presence of 1 μM nifedipine, the cells loaded with fluo-3 were incubated with 1 μM wortmannin before (A) or after (B) 10 nM ET-1 stimulation. (C) Effects of wortmannin on the ET-1-induced transient (open circles) and sustained (closed circles) increase in $[Ca^{2+}]_i$ in BA VSMCs. The cells were incubated with various concentrations of wortmannin before stimulation with 10 nM ET-1. The increases in $[Ca^{2+}]_i$ in the presence of wortmannin are presented as a percentage of values in its absence. Each point represents the mean ± S.E.M. of 10 experiments. $^{\#}P < 0.05$; significantly different from the control values (in the absence of wortmannin) in each experiment.

3.2. Effects of LY 294002 on the ET-1-induced increase in $[Ca^{2+}]_i$ in BA VSMCs

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 [19]. The magnitudes of ET-1-induced transient increase in $[Ca^{2+}]_i$ in VSMCs preincubated with 50 μM LY 294002 were similar to those in VSMCs (Fig. 2A-C). On the other hand, 50 μM LY 294002 inhibited ET-1-induced sustained increase in $[Ca^{2+}]_i$, and ~80% inhibition was obtained (Fig. 2A and D). Addition of LY294002 after stimulation with ET-1 did not affect the sustained increases in $[Ca^{2+}]_i$ (Fig. 2B). We also examined the effects of LY303511, an inactive analogue of LY294002, on ET-1-induced sustained increase in $[Ca^{2+}]_i$. LY 303511 at 50 μM failed to inhibit ET-1-induced sustained increase in $[Ca^{2+}]_i$ (data not shown).

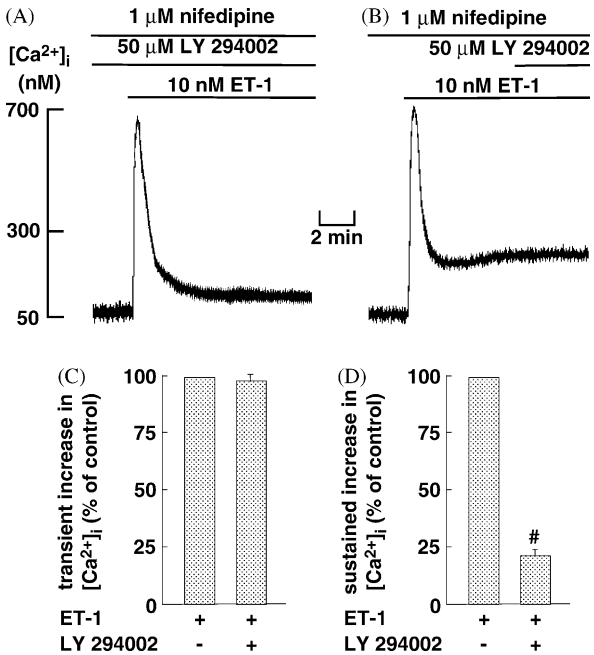

Fig. 2. (A and B) Original tracings illustrating the effects of LY 294002 on the ET-1-induced increase in $[Ca^{2+}]_i$ in BA VSMCs. In the presence of 1 μM nifedipine, the cells loaded with fluo-3 were incubated with 50 μM LY 294002 before (A) or after (B) 10 nM ET-1 stimulation. (C and D) Effects of LY 294002 on the ET-1-induced transient (C) and sustained (D) increase in $[Ca^{2+}]_i$ in BA VSMCs. The cells were incubated with 50 μM LY 294002 before stimulation with 10 nM ET-1. The increases in $[Ca^{2+}]_i$ in the presence of LY 294002 are presented as a percentage of values in its absence. Each point represents the mean ± S.E.M. of 10 experiments. $^{\#}P < 0.05$; significantly different from the control values (in the absence of LY 294002) in each experiment.

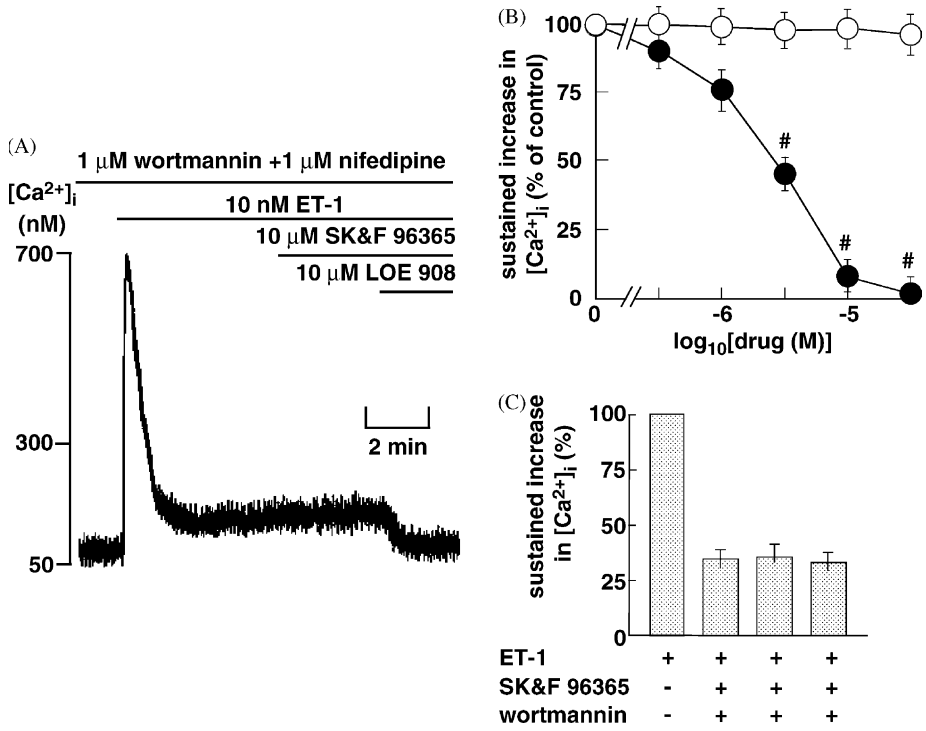

Fig. 3. (A) Original tracing illustrating the effects of SK&F 96365 and LOE 908 on the ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs pretreated with wortmannin. In the presence of 1 μM nifedipine, the cells loaded with fluo-3 were incubated with 1 μM wortmannin before 10 nM ET-1 stimulation. 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 various concentrations of SK&F 96365 (open circles) and LOE 908 (closed circles) on the ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs pretreated with wortmannin. $P < 0.05$; significantly different from the control values (in the absence of LOE 908) in each experiment. (C) Effects of maximal effective concentrations of SK&F 96365 (10 μM) and wortmannin (1 μM) on the ET-1-induced ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs. The cells were pretreated with SK&F 96365 and/or wortmannin before 10 nM ET-1 stimulation. The sustained increases in $[Ca^{2+}]_i$ in the presence of drugs are presented as a percentage of values in their absences. Each point represents the mean ± S.E.M. of 10 experiments.

3.3. Effects of SK&F 96365 and LOE 908 on ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs preincubated with wortmannin

In the presence of 1 μM nifedipine, ET-1-induced sustained increase in $[Ca^{2+}]_i$ in VSMCs preincubated with 1 μM wortmannin was inhibited by LOE 908 in a concentration-dependent manner, and complete inhibition was observed at concentrations ≥ 10 μM (Fig. 3A and B). In contrast, SK&F 96365 up to 30 μM failed to inhibit ET-1-induced sustained increase in $[Ca^{2+}]_i$ in VSMCs preincubated with 1 μM wortmannin (Fig. 3A and B). In addition, the magnitudes of ET-1-induced sustained increase in $[Ca^{2+}]_i$ in VSMCs pretreated with 10 μM SK&F 96365 were similar to those in VSMCs pretreated with 1 μM wortmannin alone or with 10 μM SK&F 96365 (Fig. 3C). In VSMCs preincubated with 50 μM LY 294002, ET-1-induced sustained increase in $[Ca^{2+}]_i$ was also sensitive to LOE 908 and resistant to SK&F 96365 (data not shown).

3.4. Effects of wortmannin on ET-1-induced contraction of rabbit BA rings without endothelium

In the presence of 1 μM nifedipine, ET-1-induced contraction of rabbit BA rings without endothelium was inhibited by pretreatment with wortmannin. In rabbit BA rings without endothelium pretreated with BQ123, ET-1 failed to induce vascular contraction (data not shown). Vehicles also failed to induce vascular contraction of rabbit BA rings without endothelium (data not shown). Wortmannin inhibited contractions evoked by 10 nM ET-1 in a concentration-dependent manner with IC_{50} values of ∼30 nM, and the maximal inhibition (∼80% of control) was observed at concentrations ≥ 1 μM (Fig. 4A). In contrast, addition of wortmannin after stimulation with ET-1 did not affect to the contraction of rabbit BA rings without endothelium (Fig. 4A).

3.5. Effects of SK&F 96365 and LOE 908 on ET-1-induced contraction of rabbit BA rings without endothelium pretreated with wortmannin

In rabbit BA rings without endothelium pretreated with wortmannin, LOE 908 inhibited ET-1-induced contractions in a concentration-dependent manner with an IC_{50} value of ∼3 μM, and complete inhibition was observed at concentrations ≥ 10 μM (Fig. 4B). In contrast, SK&F 96365 up to 30 μM did not affect the wortmannin-resistant part of contractions caused by ET-1 (Fig. 4B).

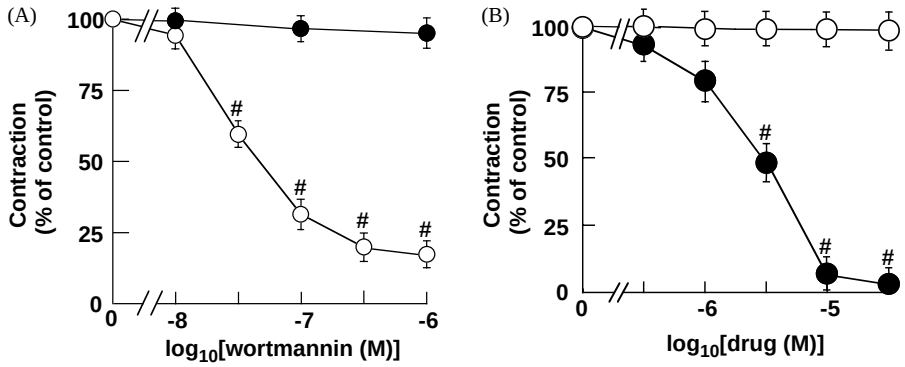

Fig. 4. (A) Effects of various concentrations of wortmannin on the ET-1-induced contractions of BA rings without endothelium. In the presence of 1 μM nifedipine, various concentrations of wortmannin were added 30 min before (open circles) or after (closed circles) stimulation with 10 nM ET-1. The contraction in the presence of wortmannin is represented as the percentage of the contraction induced by ET-1. Data presented are means ± S.E.M. of 10 experiments, each done in triplicate. $^{\#}P < 0.05$; significantly different from the control values (in the absence of wortmannin) in each experiment. (B) Inhibitory effect of SK&F 96365 and LOE 908 on the ET-1-induced contractions of BA rings without endothelium pretreated with wortmannin. Concentration-response curves for inhibition of ET-1-induced contractions of BA rings without endothelium pretreated with 1 μM wortmannin by SK&F 96365 (open circles) or LOE 908 (closed circles). The contraction in the presence of drugs is represented as the percentage of the contraction induced by ET-1 in the presence of 1 μM wortmannin. $^{\#}P < 0.05$; significantly different from the control values (in the absence of LOE 908) in each experiment. Data presented are means ± S.E.M. of 10 experiments, each done in triplicate.

4. Discussion

The present study aimed to determine whether activation of PI3K is involved in contractile responses of cerebral arteries to ET-1 and whether this effect is associated with alteration in $[Ca^{2+}]_i$, thereby giving us some insight into the potential contribution of this cellular signaling pathway to ET-1-induced vasoconstriction. Based on the data using endothelin receptor blocker, BQ123 and BQ788, ET_AR plays critical roles for increase in $[Ca^{2+}]_i$ as well as vascular contraction in BA VSMCs.

In the presence of nifedipine, wortmannin and LY 294002 inhibited ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs (Figs. 1 and 2) like CHO-ET_AR and CHO-ET_BR [15,16]. The inhibitory effects of wortmannin on ET-1-induced sustained increase in $[Ca^{2+}]_i$ may be due to its inhibitory effects on PI3K, judging from the following data: (1) wortmannin is generally accepted as PI3K inhibitor [20]. Moreover, at nanomolar concentrations, wortmannin acts specifically on PI3K [21]; (2) another PI3K inhibitor, LY 294002, also inhibited wortmannin-sensitive part of ET-1-induced sustained increase in $[Ca^{2+}]_i$ (Fig. 2); (3) the IC_{50} values (~30 nM) and

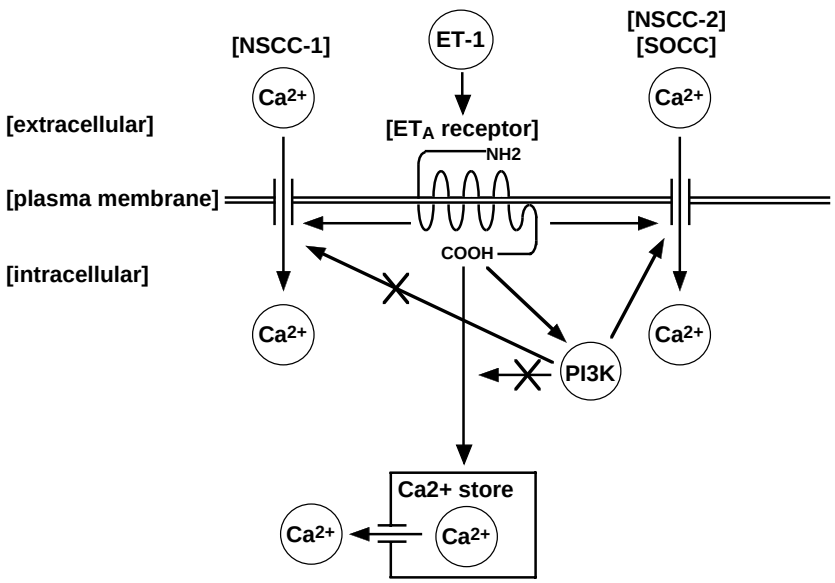

Fig. 5. Schematic representation of phosphoinositide 3-kinase (PI3K) signaling pathways for voltage-independent Ca^{2+} channels activated by ET-1 in BA VSMCs. ET-1 activates two types of Ca^{2+}-permeable nonselective cation channels (NSCC-1 and NSCC-2) and a store-operated Ca^{2+} channel (SOCC). NSCC-2 and SOCC are activated by ET-1 via PI3K-dependent pathway, while NSCC-1 is activated by ET-1 via PI3K-independent pathway. PI3K plays no role in the intracellular Ca^{2+}-release induced by ET-1. See text for details.

maximal effective concentration (1 μM) of wortmannin for ET-1-induced sustained increase in $[Ca^{2+}]_i$ (Fig. 1) were similar to those for ET-1-induced phosphatidylinositol trisphosphate (PIP_3) formation as an index of PI3K activity [22]. The magnitudes of ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs treated with wortmannin were similar to those in BA VSMCs treated with wortmannin and nifedipine (data not shown). These results are in agreement with the previous observation that wortmannin blocks VOCC [12,13]. As wortmannin partially suppresses 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 in BA VSMCs. SK&F 96365 and LOE 908 inhibit ET-1-induced sustained increase in $[Ca^{2+}]_i$ partially in BA VSMCs [4]. Moreover, the ET-1-induced sustained increase in $[Ca^{2+}]_i$ was abolished by combined treatment with SK&F 96365 and LOE 908 [4]. On the other hand, the ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs treated with wortmannin, was only sensitive to LOE 908 (Fig. 3). Based on these sensitivity to SK&F 96365 and LOE 908, wortmannin-resistant part of sustained increase in $[Ca^{2+}]_i$ is due to Ca^{2+} influx through NSCC-1 (Fig. 5). Therefore, Ca^{2+} influx through NSCC-2 and SOCC are composed of wortmannin sensitive part (Fig. 5). These results indicate that PI3K may play important roles for ET-1-induced activation of NSCC-2 and SOCC in BA VSMCs (Fig. 5). Moreover, the results that the magnitudes of ET-1-induced sustained increase in $[Ca^{2+}]_i$ in BA VSMCs treated with SK&F 96365 are similar to those in BA VSMCs treated with wortmannin and/or SK&F 96365 (Fig. 3C) may support this indication. In addition, from the data that addition of wortmannin or LY 294002 after stimulation with ET-1 did not suppress sustained increase in $[Ca^{2+}]_i$ (Figs. 1 and 2), PI3K seems to be required for the activation of the Ca^{2+} entry, but not for its maintenance. On the other hand, wortmannin failed to inhibit 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 PLC/IP_3/mobilization of Ca^{2+} from the intracellular store [23]. Therefore, PI3K does not seem to affect this cascade (Fig. 5). The operation points of PI3K on NSCC-2 and SOCC activation by ET-1 are still unclear in this study. PI3K are composed of the p85 regulatory subunit and the p110 catalytic subunit, which contains a $p21^{ras}$-binding domain [24]. Recently, $p21^{ras}$ was reported to stimulate Ca^{2+} channels [25]; it therefore may be involved in the Ca^{2+} influx stimulated by ET-1 via activation of PI3K. Moreover, other report demonstrates that PI3K activation leads to synthesis of PI3K products (e.g., PIP_3) that may directly act on Ca^{2+} channels [26]. Further study is necessary to clarify intracellular molecules involved in NSCC-2 and SOCC activation by ET-1.

ET-1 induces contraction of rabbit BA rings without endothelium as described previously [4]. The IC_{50} values (~30 nM) and maximal effective concentration (1 μM) of wortmannin for ET-1-induced contraction (Fig. 4A) were similar to those for ET-1-induced sustained increase in $[Ca^{2+}]_i$ (Fig. 1). Therefore, the inhibitory effects of wortmannin on ET-1-induced contraction may also be due to its inhibitory effects on PI3K. Based on the data that (1) LOE 908, but not SK&F 96365, inhibited the wortmannin-resistant part of ET-1-induced contraction in a concentration-dependent manner (Fig. 4B); (2) maximal effective concentration (10 μM) of LOE 908 abolished the wortmannin-resistant part of ET-1-induced contraction (Fig. 4B); and (3) the IC_{50} values of LOE 908 for the wortmannin-resistant part of ET-1-induced contraction (Fig. 4B) were similar to those for the wortmannin-resistant part of ET-1-induced sustained increase in $[Ca^{2+}]_i$ (Fig. 3), the wortmannin-resistant part of contraction by ET-1 was dependent on the extracellular Ca^{2+} influx through NSCC-1. These results indicate that the inhibitory effects of wortmannin on ET-1-induced contraction may be mediated by blockade of Ca^{2+} entry through NSCC-2 and SOCC. Moreover, the addition of wortmannin after stimulation with ET-1 did not suppress contraction (Fig. 4A) just like sustained increase in $[Ca^{2+}]_i$ (Fig. 1). Previous report demonstrates that PI3K inhibitor treatment 2 days after cisternal injection of blood fails to inhibit vasospasm in dog model of SAH [17]. PI3K may have been activated before starting PI3K treatment in this experimental model. Inhibition of PI3K by wortmannin seems to be involved in activation of Ca^{2+} channels and contraction of BA rings, but not for their maintenance (Figs. 1 and 4).

In conclusion, PI3K plays important roles for activation of NSCC-2 and SOCC by ET-1. In addition, PI3K is required for the activation of the Ca^{2+} entry, but not for its maintenance. PI3K is involved in ET-1-induced rabbit BA contraction. Based on tension study, stimulation of extracellular Ca^{2+} influx through SOCC and NSCC-2 is one of the roles of PI3K for ET-1-induced contraction of rabbit BA rings.

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