Characterization of Ca\(^{2+}\) channels involved in ET-1-induced transactivation of EGF receptors

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Characterization of Ca\(^{2+}\) channels involved in ET-1-induced transactivation of EGF receptors. Am J Physiol Heart Circ Physiol 283: H2671–H2675, 2002; 10.1152/ajpheart.00350.2002.—The purpose of this study was to demonstrate the involvement of Ca\(^{2+}\) influx through voltage-independent Ca\(^{2+}\) channels (VICCs) in endothelin-1 (ET-1)-induced transactivation of epidermal growth factor receptor protein tyrosine kinase (EGFR PTK) using the Ca\(^{2+}\) channel blockers LOE-908 and SK&F-96365 in rabbit internal carotid artery vascular smooth muscle cells. ET-1-induced EGFR PTK transactivation was completely inhibited by AG-1478, which is a specific inhibitor of EGFR PTK. In the absence of extracellular Ca\(^{2+}\), the magnitude of EGFR PTK transactivation was near the basal level. Based on sensitivity to nifedipine, which is a specific blocker of voltage-operated Ca\(^{2+}\) channels (VOCCs), VOCCs have minor roles in EGFR PTK transactivation. In contrast, Ca\(^{2+}\) influx through VICCs plays an important role in EGFR PTK transactivation. Moreover, based on the sensitivity of VICCs to SK&F-96365 and LOE-908, VICCs were shown to consist of two types of Ca\(^{2+}\)-permeable nonselective cation channels (NSCCs), which are designated NSCC-1 and NSCC-2, and a store-operated Ca\(^{2+}\) channel. In summary, Ca\(^{2+}\) influx through VICCs plays an essential role in ET-1-induced EGFR PTK transactivation in rabbit internal carotid artery vascular smooth muscle cells.

**ENDOTHELIN (ET)-1** exhibits mitogenic activity in vascular smooth muscle cells (VSMCs; Refs. 1, 6, 7), which indicates a possible role for ET-1 in the pathogenesis of clinical conditions such as hyperlipoproteinemia or atherosclerosis (5, 8). However, the molecular mechanisms of the ET-1-induced mitogenic response in VSMCs are still unclear. Recently, a novel aspect of the role of tyrosine kinase signaling in ET-1 action has been revealed by the finding in rat-1 fibroblasts that the ET type A (ET\(_A\)) receptor-mediated mitogenic response as well as other G protein-coupled receptor-mediated responses are accompanied by transactivation of the epidermal growth factor receptor (EGFR) protein tyrosine kinase (PTK). Through the formation of Shc/Grb2/Sos complexes, this event leads to activation of the ras/mitogen-activated protein kinase pathway and transcription of early-response genes (2, 10, 12). Moreover, it was shown that vascular growth by ET-1 fully depends on this EGFR transactivation in VSMCs (6).

We have recently shown (7) that ET-1 activates three types of voltage-independent Ca\(^{2+}\) channels (VICCs) as well as voltage-operated Ca\(^{2+}\) channels (VOCCs) in rabbit internal carotid artery VSMCs. The VICCs include two types of Ca\(^{2+}\)-permeable nonselective cation channels (NSCCs), which are designated NSCC-1 and NSCC-2, and a store-operated Ca\(^{2+}\) channel (SOCC). Importantly, we have also shown that these channels can be distinguished by sensitivity to blockers of the receptor-operated Ca\(^{2+}\) channel such as SK&F-96365 and LOE-908 (4, 9); NSCC-1 is sensitive to LOE-908 and resistant to SK&F-9635, NSCC-2 is sensitive to both LOE-908 and SK&F-96365, and SOCCs are resistant to LOE-908 and sensitive to SK&F-96365 (7). Moreover, Ca\(^{2+}\) influx through NSCC-1, NSCC-2, and SOCCs plays an essential role for ET-1-induced mitogenesis in internal carotid artery VSMCs (7). Previous reports (3, 6, 10, 13) have demonstrated that extracellular Ca\(^{2+}\) influx is important in EGFR PTK transactivation by angiotensin II, bradykinin, and ET-1. Extracellular Ca\(^{2+}\) influx through VOCCs is involved in EGFR PTK transactivation in PC12 cells (10, 13). However, it is totally unknown whether extracellular Ca\(^{2+}\) influx through VICCs is involved in EGFR PTK phosphorylation. In the present study, we attempted to elucidate which Ca\(^{2+}\) channels are involved in ET-1-induced EGFR PTK phosphorylation using SK&F-96365 and LOE-908 in internal carotid artery VSMCs.

**MATERIALS AND METHODS**

**Cell culture.** Rabbit internal carotid artery VSMCs were prepared as described previously (7). Briefly, male Japan White rabbits (body wt, 2–3 kg) were anesthetized by injection of thiopental sodium (20 mg/kg iv) and killed by exsanguination. The internal carotid artery was removed, cleaned of surrounding tissue, dissected into small (2 × 5 mm) strips, and kept in Ca\(^{2+}\)-free Krebs-HEPES solution that contained...
(in mM) 140 NaCl, 3 KCl, 1 MgCl2, 11 glucose, and 10 HEPES (pH 7.3, adjusted with NaOH). The strips were incubated overnight (12–24 h) at 4°C in Ca2+-free Krebs-HEPES solution that contained papain (0.25–0.5 mg/ml) and 0.5 mM dithiothreitol. Thereafter, the strips were resuspended and incubated in Ca2+-free Krebs-HEPES solution that contained collagenase (0.25–0.75 mg/ml) at 35°C for 10 min. The digested strips were cut into pieces with fine scissors and triturated with a blunt-tipped pipette until a sufficient number of single cells were released. Cells were routinely maintained in Dulbecco’s modified Eagle’s medium that contained 10% fetal bovine serum in a humidified atmosphere of 5% CO2-95% air.

Measurement of EGFR PTK transactivation. Transactivation of EGFR PTK was measured using a universal tyrosine kinase assay kit (Takara; Tokyo, Japan). Extraction buffer and kinase-reacting solution were included with this kit. Cells seeded at 5 × 10^5 cells/well in six-well plates were starved for 24 h and then stimulated with various concentrations of ET-1 for the indicated times. The reaction was terminated by washing the cells three times with phosphate-buffered saline. For the addition 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) 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 phosphate-buffered saline. 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 using an EL340 microtiter plate reader (Bio-Tek Instruments; Winooski, VT). The stimulation reached a maximum 1 nM (Fig. 1B). Thus the stimulation time was set at 2 min. ET-1 activated EGFR PTK in a concentration-dependent manner. IC50 values were ~1 nM (Fig. 1C). The stimulation reached a maximum at concentrations ≥10 nM (Fig. 1C).

AG-1478, which is a specific inhibitor of EGFR PTK, inhibited ET-1-induced EGFR PTK transactivation (Fig. 2A). The inhibitory effects of AG-1478 on ET-1 induced EGFR PTK transactivation in a concentration-dependent manner. IC50 values were ~10 nM, and ≤1 μM AG-1478 provided complete inhibition (Fig. 2B).

Effects of extracellular Ca2+ and nifedipine on ET-1-induced EGFR PTK transactivation. In the absence of extracellular Ca2+, the magnitude of ET-1-induced EGFR PTK transactivation was near the basal level (Fig. 3). Therefore, extracellular Ca2+ influx plays an important role in the ET-1-induced EGFR PTK transactivation.

Next, we examined the effects of extracellular Ca2+ influx through VOCCs on the ET-1-induced EGFR PTK
transactivation using nifedipine, which is a specific blocker of L-type VOCCs. At a concentration of 1 μM, nifedipine inhibited the ET-1-induced extracellular Ca\(^{2+}\) influx through VOCCs in VSMCs (7); in contrast, nifedipine inhibited ET-1-induced EGFR PTK transactivation by a maximum of only \(\frac{1}{10}\%\) (Fig. 3B).

Effects of SK&F-96365 and LOE-908 on ET-1-induced EGFR PTK transactivation. Using SK&F-96365 and LOE-908, we attempted to determine the effects of extracellular Ca\(^{2+}\) influx through VICCs on the ET-1-induced EGFR PTK transactivation. In these 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 VOCCs. SK&F-96365 inhibited ET-1-induced EGFR PTK transactivation in a concentration-dependent manner, and the IC\(_{50}\) value was \(\sim 3\) μM (Fig. 4A). Maximal inhibition was observed at concentrations \(\leq 10\) μM (Fig. 4A). The extent of maximal inhibition was \(\sim 80\%\) of the nifedipine-resistant part of EGFR PTK transactivation (Fig. 4B). Similarly, the IC\(_{50}\) values of LOE-908 for inhibition of ET-1-induced EGFR PTK phosphorylation were \(\sim 3\) μM, and maximal inhibition was observed at concentrations \(\sim 10\) μM (Fig. 4A). The extent of maximal inhibition was \(\sim 60\%\) of EGFR PTK transactivation (Fig. 4B). Notably, the combined treatment with the maximally effective concentrations (10 μM) of SK&F-96365 and LOE-908 completely inhibited the nifedipine-resistant part of EGFR PTK transactivation (Fig. 4B).

In contrast, EGF-induced EGFR PTK activation (Fig. 5) and phorbol 12-myristate 13-acetate-induced EGFR PTK transactivation (data not shown) were unaffected by SK&F-96365 or LOE-908.

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Fig. 2. Effects of AG-1478 on ET-1-induced EGFR PTK activity in VSMCs. A: cells were pretreated with or without 1 μM AG-1478 for 30 min and incubated with 10 nM ET-1 for 2 min. B: cells were pretreated with various concentrations of AG-1478 and stimulated with (+) or without (●) 10 nM ET-1. Data are means ± SE of three determinations (each done in triplicate).

Fig. 3. Effects of extracellular Ca\(^{2+}\) and nifedipine on ET-1-induced EGFR PTK phosphorylation in VSMCs. A: cells were washed five times with Ca\(^{2+}\)-free phosphate-buffered saline and incubated for 2 min with either Ca\(^{2+}\)-free or Ca\(^{2+}\)-containing phosphate-buffered saline that contained 10 nM ET-1. B: cells were incubated for 15 min with nifedipine and then stimulated with 10 nM ET-1. Data are means ± SE of three determinations (each done in triplicate).

Fig. 4. A: effects of SK&F-96365 (●) and LOE-908 (○) on ET-1-induced EGFR PTK phosphorylation in VSMCs. B: effects of a maximal effective concentration (10 μM) of SK&F-96365 and LOE-908 on ET-1-induced EGFR PTK 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 were then stimulated with 10 nM ET-1 for 2 min. Data are means ± SE of five determinations (each done in triplicate); #P < 0.05, significantly different from the control values in each experiment; ##P < 0.05, significantly different from the inhibition by SK&F-96365; ###P < 0.05, significantly different from the inhibition by LOE-908.

Fig. 5. A: effects of SK&F-96365 and LOE-908 on EGF-induced EGFR PTK phosphorylation in VSMCs. B: starved cells were incubated for 15 min with 10 μM SK&F-96365 or LOE-908 in addition to 1 μM nifedipine and then stimulated with 5 ng/ml EGF for 3 min. Data are means ± SE of three determinations (each done in triplicate).
DISCUSSION

As described in previously (6), ET-1 induces EGFR PTK phosphorylation in rabbit internal carotid artery VSMCs (see Fig. 1A). Based on its sensitivity to BQ-123 and BQ-788, ETA receptors play essential roles in the ET-1-induced EGFR PTK phosphorylation (Fig. 1A). In the present study, we used a universal tyrosine kinase assay kit and mouse monoclonal anti-EGFR antibody for measurement of EGFR PTK phosphorylation. The ET-1-induced PTK phosphorylation obtained using this method was completely inhibited by AG-1478, which is a specific EGFR PTK inhibitor (Ref. 6; see Fig. 2B). Moreover, the magnitude of ET-1-induced EGFR PTK phosphorylation in internal carotid artery VSMCs treated with AG-1478 was near basal level in the immunoblotting analysis (Fig. 2A). Therefore, we concluded that this method was suitable for measurement of EGFR PTK phosphorylation.

Previous reports (3, 6) demonstrated that extracellular Ca\(^{2+}\) influx plays important roles in the EGFR PTK transactivation. We tried to characterize the Ca\(^{2+}\) channels involved in the ET-1-induced EGFR PTK transactivation in internal carotid artery VSMCs. The magnitudes of ET-1-induced EGFR PTK transactivation in the absence of extracellular Ca\(^{2+}\) were near the basal level (see Fig. 3). These results indicate that extracellular Ca\(^{2+}\) influx is also important in ET-1-induced EGFR PTK transactivation in internal carotid artery VSMCs. Our recent study indicated that NSCC-1, NSCC-2, and SOCCs play major parts in ET-1-induced extracellular Ca\(^{2+}\) influx in internal carotid artery VSMCs (7). Moreover, extracellular Ca\(^{2+}\) influx through these Ca\(^{2+}\) channels is essential in ET-1-induced mitogenesis (7). Thus we examined the involvement of NSCC-1, NSCC-2, and SOCCs in ET-1-induced EGFR PTK transactivation using SK&F-96365 and LOE-908. According to the nifedipine sensitivity of ET-1-induced EGFR PTK transactivation, involvement of VOCCs in this response was estimated to be minor at around ~10% (see Fig. 3B). We demonstrated in a recent report (7) that nifedipine suppressed the 10 nM ET-1-induced sustained increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) by a maximum of 10%. Therefore, Ca\(^{2+}\) channels that play important roles in ET-1-induced EGFR PTK transactivation in internal carotid artery VSMCs are different from those in PC12 cells (10, 13). Ca\(^{2+}\) channels other than VOCCs may be important in ET-1-induced EGFR PTK transactivation in addition to extracellular Ca\(^{2+}\) influx in rabbit internal carotid artery VSMCs.

For several reasons, the inhibitory actions of SK&F-96365 and LOE-908 on the ET-1-induced EGFR PTK transactivation are considered to be mediated by blockade of Ca\(^{2+}\) entry through VOCCs. First, in our recent work using patch-clamp and [Ca\(^{2+}\)]\(_i\) monitoring, ET-1 was found to activate three types of VOCCs in VSMCs: NSCC-1, NSCC-2, and SOCCs. 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 SOCCs (7). Second, the IC\(_{50}\) values of these blockers for the ET-1-induced EGFR PTK transactivation (see Fig. 4A) correlated well with those for the ET-1-induced extracellular Ca\(^{2+}\) influx (7). Third, these blockers failed to inhibit EGF-induced EGFR PTK activation (Fig. 5). Fourth, these blockers failed to inhibit phorbol 12-myristate 13-acetate-induced EGFR PTK transactivation (data not shown) that was independent of extracellular Ca\(^{2+}\) influx (11). Fifth, neither SK&F-96365 nor LOE-908 is considered to exert cytotoxic effects on the quiescent cells (7). Moreover, because SK&F-96365 and LOE-908 failed to inhibit the ET-1-induced transient increase in [Ca\(^{2+}\)]\(_i\), due to the release of intracellular Ca\(^{2+}\) stores (7), the release of sarcoplasmic reticulum Ca\(^{2+}\) was not sufficient to stimulate EGFR PTK transactivation. Three types of VICCs seem to be involved in the ET-1-induced EGFR PTK transactivation in terms of its sensitivity to SK&F-96365 and LOE-908 (see Figs. 4 and 6). 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 SOCCs, respectively. Moreover, the percent contributions of NSCC-1, NSCC-2, and SOCCs to the ET-1-induced EGFR PTK transactivation are calculated to be ~20, 40, and 40%, respectively, of the nifedipine-resistant part of EGFR PTK transactivation caused by a 10 nM concentration of ET-1 (Fig. 6). The magnitudes of the ET-1-induced EGFR PTK transactivations 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\(^{2+}\) (see Figs. 3B and 4B). Therefore, extracellular Ca\(^{2+}\) influx through NSCC-1, NSCC-2, and SOCCs plays an important role in ET-1-induced EGFR PTK transactivation in rabbit internal carotid artery VSMCs.

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**Fig. 6.** Calculations for contributions of Ca\(^{2+}\) influx through three types of voltage-independent Ca\(^{2+}\) channels to ET-1-induced EGFR PTK transactivation in VSMCs. A, B: ET-1-induced EGFR PTK phosphorylation in the presence of 10 μM LOE-908 or 10 μM SK&F-96365 is represented as a percentage of values in its absence. Contributions of store-operated Ca\(^{2+}\) channel (SOCCs) and Ca\(^{2+}\)-permeable nonselective cation channel (NSCC-1) are represented as X and Z, respectively. Contributions of NSCC-2 are represented as W – Z or Y – X.
In conclusion, extracellular Ca\textsuperscript{2+} influx through NSCC-1, NSCC-2, and SOCCs plays an essential role for ET-1-induced EGFR PTK transactivation in rabbit internal carotid artery VSMCs.

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REFERENCES