Involvement of phospholipase C in endothelin 1–induced stimulation of Ca++ channels and basilar artery contraction in rabbits

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Object. Endothelin 1 (ET-1) is a major cause of cerebral vasospasm after subarachnoid hemorrhage (SAH), and extracellular Ca++ influx plays an essential role in ET-1–induced vasospasm. The authors recently demonstrated that ET-1 activates two types of Ca++-permeable nonselective cation channels (designated NSCC-1 and NSCC-2) and a store-operated Ca++ channel (SOCC) in vascular smooth-muscle cells located in the basilar arteries (BAs) of rabbits. In the present study, they investigate the effects of phospholipase C (PLC) on ET-1–induced activation of these Ca++ channels and BA contraction by using the PLC inhibitor U73122.

Methods. To determine which Ca++ channels are activated via a PLC-dependent pathway, these investigators monitored the intracellular free Ca++ concentration ([Ca++]). The role of PLC in ET-1–induced vascular contraction was examined by performing a tension study of rabbit BA rings. The U73122 inhibited the ET-1–induced transient increase in [Ca++], which resulted from mobilization of Ca++ from the intracellular store. Phospholipase C also inhibited ET-1–induced extracellular Ca++ influx through the SOCC and NSCC-2, but not through the NSCC-1. The U73122 inhibited the ET-1–induced contraction of the rabbit BA rings, which depended on extracellular Ca++ influx through the SOCC and NSCC-2.

Conclusions. These results indicate the following. 1) The SOCC and NSCC-2 are stimulated by ET-1 via a PLC-dependent cascade whereas NSCC-1 is stimulated via a PLC-independent cascade. 2) The PLC is involved in the ET-1–induced contraction of rabbit BA rings, which depends on extracellular Ca++ influx through the SOCC and NSCC-2.

Key Words • vascular contraction • endothelin 1 • phospholipase C • Ca++ channel

Phospholipase C hydrolyzes phosphatidyl inositol 4,5-bisphosphate and produces IP, and diacylglycerol. The IP, mobilizes Ca++ from the intracellular Ca++ stores and elicits rapid contraction of smooth-muscle cells. Diacylglycerol activates PKC and initiates sustained contraction. In addition, PLC is involved in the development of myogenic tone in the posterior cerebral arteries of rats. These results indicate the importance of PLC in cerebral artery contraction, especially cerebral vasospasm after SAH. Findings in recent reports demonstrate that extracellular Ca++ influx plays an important role in sustained vascular contraction. Although PLC is involved in the stimulation of extracellular Ca++ influx, it is unclear what types of Ca++ channels are activated via the PLC pathway. This lack of knowledge is mainly due to a lack of available specific Ca++ channel blockers. Previously we characterized the Ca++ channels that are activated by ET-1 in the VSMCs of rabbit BAs by using whole-cell patch clamps and by monitoring the [Ca++]i. Endothelin 1 is a major cause of cerebral vasospasm after SAH. Endothelin 1 activates three types of voltage-independent Ca++ channels in addition to voltage-operated Ca++ channels in BA VSMCs. Moreover, voltage-operated Ca++ channels play minor roles in ET-1–induced extracellular Ca++ influx. These voltage-independent Ca++ channels include two types of Ca++-permeable NSCCs (designated NSCC-1 and NSCC-2) and an SOCC (Table 1). Importantly, these channels can be discriminated by their sensitivity to two Ca++ channel blockers, SK&F 96365 and LOE 908. Whereas 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

Abbreviations used in this paper: BA = basilar artery; [Ca++]i = intracellular free calcium concentration; EC50 = median effective concentration; ET-1 = endothelin 1; IC50 = median drug concentration for inhibitory effect; IP = inositol phosphate; IP3 = inositol 1,4,5-trisphosphate; NSCC = nonselective cation channel; PKC = protein kinase C; PLC = phospholipase C; SAH = subarachnoid hemorrhage; SEM = standard error of the mean; SOCC = store-operated Ca++ channel; VSMC = vascular smooth-muscle cell.
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SOCC is resistant to LOE 908 and sensitive to SK&F 96365. Moreover, extracellular Ca\(^{2+}\) influx through voltage-independent Ca\(^{2+}\) channels has a major role in ET-1–induced extracellular Ca\(^{2+}\) influx and BA contraction. In the present study, we attempted to evaluate the efficacy of PLC on ET-1–induced extracellular Ca\(^{2+}\) influx through voltage-independent Ca\(^{2+}\) channels through the VSMCs of BAs and in BA contraction. For this purpose, we used U73122, a specific inhibitor of the guanine nucleotide regulation of PLC.

**Materials and Methods**

**Cell Culture**

Isolated VSMCs were prepared from rabbit BAs as described previously. The cells were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum, which was supplemented with 100 μg/ml penicillin G and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO\(_2\)/95% air.

**Monitoring of [Ca\(^{2+}\)]\(_i\)**

We monitored [Ca\(^{2+}\)]\(_i\) by using the fluorescent probe, fluo-3, as described previously. Briefly, the cells were loaded with fluo-3 by incubating them with 10 μM fluo-3/AM at 37°C under reduced light for 30 minutes. After they had been washed, the cells were suspended at a density of approximately 2 × 10\(^5\) cells/ml and 0.5-ml aliquots were used for measurement of fluorescence by using a spectrofluorometer (CAF 110; JASCO, Tokyo, Japan) with an excitation wavelength of 490 nm and an emission wavelength of 540 nm. At the end of the experiment, Triton X-100 and later ethylene glycol tetraacetic acid were added at final concentrations of 0.1% and 5 mM, respectively, to obtain maximum and minimum levels of fluorescence. The [Ca\(^{2+}\)]\(_i\) was determined by the following equilibrium equation: [Ca\(^{2+}\)] = K\(_i\)(F - F\(_{\text{min}}\))/(F\(_{\text{max}}\) - F), where F is the experimental value of fluorescence, F\(_{\text{max}}\) is the maximum fluorescence, F\(_{\text{min}}\) is the minimum fluorescence, and K\(_i\) is defined as 0.4 μM.

**Formation of Inositol Phosphates**

The level of formation of IPs was determined in a manner described previously. Briefly, cells that had been placed in 24-well plates were incubated with myo-[\(^3\)H]inositol (final concentration 5 μCi/ml) in 0.3 ml of Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum for 18 hours. After they had been washed, the cells were incubated with or without various concentrations of ET-1 for 30 minutes, and the reaction was terminated by adding ice-cold perchloric acid. After they had been neutralized with KOH and Tris, the samples were applied onto small AG1X8 columns (100–200 mesh, Cl\(^-\) form; Bio-Rad, Hercules, CA) to separate total IPs from myo-[\(^3\)H]inositol. The \(^3\)H-labeled IPs were eluted with 1 N HCl, and the radioactivity was counted using a liquid scintillation counter.

**Tension Study**

Vessel tension was measured in a manner described previously. Briefly, the removed BA was placed in Krebs solution, which contained the following (in mM): NaCl 120, KCl 5.4, CaCl\(_2\) 2.2, MgCl\(_2\) 1, NaHCO\(_3\) 25, and glucose 5.6. The BA was cut into 3-mm-thick rings in a dissecting chamber filled with Krebs solution bubbled with a 95% O\(_2\)/5% CO\(_2\) mixture. Endothelial cells were removed from the ring specimens by gently rubbing the intimal surface with a cotton pad moistened with Krebs solution. Successful removal of the endothelial cells was confirmed by the inability of acetylcholine (1 μM) to inducing relaxation. The rings were mounted by 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\(_2\)/5% CO\(_2\) mixture. One of the hooks was connected to a force transducer (Oriente, Tokyo, Japan) and the tension that developed was displayed on a polygraph (RIG-4128; Nihon Kohden, Tokyo, Japan). The bath fluid was changed and the resting force readjusted every 20 minutes until a stable baseline was attained (usually after ~ 60 minutes). Each preparation was “challenged” with 1 μM noradrenaline to measure the contractile force of each preparation, and the ET-1–induced contractile responses were represented as percentages of the tension induced by 1 μM noradrenaline. Unless otherwise specified, inhibitors were added 30 minutes before stimulation.

**Drugs Used in the Study**

The LOE 908 was kindly provided by Boehringer Ingelheim KG (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); fluo-3/AM from Dojindo Laboratories (Kumamoto, Japan); and nifedipine, U73122, and U73343 from Wako (Osaka, Japan).

**Statistical Analysis**

All results are expressed as the means ± the SEMs. The data were subjected to a two-way analysis of variance. When a significant F value was encountered, the Newman–Keuls multiple range test was used to test for significant differences between treatment groups. A probability level less than 0.05 was considered statistically significant.

**Results**

**Effects of U73122 on the ET-1–Induced Accumulation of \(^3\)H]IPs in BA VSMCs**

Endothelin 1 caused a concentration-dependent stimulation of \(^3\)H]IPs accumulation with EC\(_{50}\) values of approximately 1 nM (Fig. 1A). The maximum effect, an approximately fivefold increase, was obtained at concentrations of 10 nM or greater (Fig. 1A). The U73122 inhibited a 10-nM ET-1–induced accumulation of \(^3\)H]IPs with IC\(_{50}\) values of approximately 0.5 μM, and complete inhibition was observed at concentrations of 3 μM or greater (Fig. 1B). On the other hand, U73343, an inactive analog of U73122 failed to inhibit ET-1–induced \(^3\)H]IPs accumulation (data not shown).

**Effects of U73122 on the ET-1–Induced Increase in [Ca\(^{2+}\)]\(_i\), in BA VSMCs**

At 10 nM ET-1 induced a biphasic increase in [Ca\(^{2+}\)], consisting of an initial transient peak and a subsequent sustained increase in the VSMCs (Fig. 2A). The sustained phase was abolished by the removal of extracellular Ca\(^{2+}\), whereas the transient peak remained unaffected. These results indicate that the sustained phase was due to extracellu-

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U73122 inhibits ET-1–induced sustained increase in [Ca\(^{++}\)], with IC\(_50\) values of approximately 1 \(\mu\)M, and complete inhibition was observed at concentrations of 10 \(\mu\)M or greater (Fig. 2B and D). In contrast, SK&F 96365 failed to inhibit the U73122-resistant part of the sustained increase in [Ca\(^{++}\)], caused by the ET-1 (Fig. 2B and D).

**Effects of U73122 on ET-1–Induced Contraction of Rabbit BA Rings Without Endothelium**

Endothelin 1 induces contractile responses in rabbit BA rings without endothelium in a concentration-dependent manner, and the maximum effect was obtained at concentrations of 10 nM or greater. The U73122 inhibited ET-1–induced BA contraction with IC\(_50\) values of approximately 0.5 \(\mu\)M, and the maximum inhibition (70%) was observed at concentrations of 3 \(\mu\)M or greater (Fig. 3A). In contrast, U73343 failed to inhibit ET-1–induced BA contraction (Fig. 3A). At 10 \(\mu\)M LOE 908 abolished the U73122-resistant part of BA contraction caused by the ET-1, whereas at concentrations as high as 10 \(\mu\)M SK&F 96365 failed to affect this contraction (Fig. 3B).

**Discussion**

The U73122 inhibition of both transient and sustained increases in [Ca\(^{++}\)], caused by ET-1 (Fig. 2) indicates the importance of PLC for extracellular Ca\(^{++}\) influx as well as Ca\(^{++}\) release from intracellular stores. These data are consistent with the fact that PLC plays an important role in the changes in [Ca\(^{++}\)]. Although U73122 has some effects, such as inhibition of PLC stimulation, phospholipase D stimulation, and PKC\(\alpha\) translocation, the inhibitory effects of U73122 on the ET-1–induced increase in [Ca\(^{++}\)] may be due to its inhibitory effects on PLC stimulation. This assessment is based on the following data. 1) The IC\(_50\) values of U73122 for ET-1–induced transient and sustained increases in [Ca\(^{++}\)] (Fig. 2) are similar to those for ET-1–induced [H\(^{+}\)] accumulation (Fig. 1). 2) The U73343 fails to affect the ET-1–induced increase in [Ca\(^{++}\)], or [H\(^{+}\)] accumulation (Figs. 1 and 2), whereas it inhibits phospholipase D stimulation and PKC\(\alpha\) translocation. As U73122 partially suppresses ET-1–induced sustained increase in [Ca\(^{++}\)] (Fig. 2), ET-1 induces extracellular Ca\(^{++}\) influx in BA VSMCs via two different pathways—one U73122-sensitive and the other U73122-resistant. Given the sensitivity to LOE 908 and SK&F 96365, the U73122-resistant part of the sustained increase in [Ca\(^{++}\)], is due to Ca\(^{++}\) influx through NSCC-1 (LOE 908-sensitive and SK&F 96365-resistant) (Fig. 2). Thus Ca\(^{++}\) influx through NSCC-2 and the SOCC is the U73122-sensitive part. These results indicate that U73122 plays an important role in the activation of NSCC-2 and the SOCC by ET-1. Judging from the data on [H\(^{+}\)] accumulation (Fig. 1), ET-1 stimulates G\(_{i}\) in BA VSMCs. It is generally accepted that PLC and IP\(_3\) are activated downstream of G\(_{i}\). Therefore, the G\(_{i}/PLC/IP\(_3\) cascade is involved in the activation of NSCC-2 and the SOCC.

Findings from previous reports have shown that extracellular Ca\(^{++}\) influx through NSCCs and the SOCCs is responsible for a significant component of SAH-induced vasospasm as well as ET-1–induced vascular contraction.
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In contrast, the extracellular Ca\(^{++}\) influx through voltage-operated Ca\(^{++}\) channels plays a minor role in SAH-induced vasospasm as well as in ET-1–induced vascular contraction.\textsuperscript{11,24} To confirm the involvement of PLC in ET-1–induced BA contraction, we performed a tension study using rabbit BA rings without endothelium. Based on the data that ET-1–induced BA contraction is partially suppressed by the maximally effective concentration of U73122 (Fig. 3), ET-
The blockade of PLC may be a possible new treatment for cerebral vasospasm after SAH.

**Conclusions**

The G/PLC/IP3 cascade plays an essential role in the activation of NSCC-2 and the SOCC by ET-1 in BA VSMCs. Phospholipase C is involved in ET-1–induced contraction of rabbit BAs. Stimulation of extracellular Ca\(^{2+}\) influx through NSCC-2 and the SOCC is one of the roles PLC plays in the ET-1–induced contraction of rabbit BA rings.

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