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Effects of extracellular Ca^{2+} influx on endothelin-1-induced intracellular mitogenic cascades in C6 glioma cells

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Abstract

We have recently shown that endothelin-1 activates two types of Ca^{2+}-permeable nonselective cation channels (NSCC-1 and NSCC-2) in C6 glioma cells. These channels can be distinguished by their sensitivity to blockers of the receptor-operated Ca^{2+} channel, 1-[*b*-(3-[4-methoxyphenyl]propoxy)-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SK&F 96365) and (*R*,*S*)-(3,4-dihydro-6,7-dimethoxy-isoquinoline-1-yl)-2-phenyl-*N*,*N*-di-[2-(2,3,4-trimethoxyphenyl)ethyl]-acetamide (LOE 908). NSCC-1 is sensitive to LOE 908 and resistant to SK&F 96365, whereas NSCC-2 is sensitive to both LOE 908 and SK&F 96365. Moreover, extracellular Ca^{2+} influx through these channels plays an essential role in endothelin-1-induced mitogenesis in C6 glioma cells. The purpose of the present study was to investigate the effects of extracellular Ca^{2+} influx on intracellular pathways of endothelin-1-induced mitogenic responses in C6 glioma cells. We focused on extracellular signal-regulated kinase 1 and 2 (ERK1/2) in this context. An inhibitor of mitogen-activated protein kinase, 2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD 98059), abolished the endothelin-1-induced increase in ERK1/2 activity, but only partially suppressed the mitogenic response. ERK1/2 activation by endothelin-1 was partially suppressed in the absence of extracellular Ca^{2+}. On the basis of the sensitivity to LOE 908 and SK&F 96365, Ca^{2+} influx through NSCC-1 and NSCC-2 plays an essential role in the extracellular Ca^{2+}-dependent component of ERK1/2 activity. In contrast, Ca^{2+} influx through NSCC-2 is involved in the ERK1/2-independent component of endothelin-1-induced mitogenesis. These results indicate that (1) the endothelin-1-induced mitogenic response involves both ERK1/2-dependent and -independent mechanisms, (2) ERK1/2 activation by endothelin-1 involves an extracellular Ca^{2+} influx-dependent cascade as well as an extracellular Ca^{2+} influx-independent cascade, (3) because endothelin-1-induced mitogenesis is completely dependent on extracellular Ca^{2+} influx, extracellular Ca^{2+} influx also plays an important role in mitogenic pathways downstream of ERK1/2, (4) extracellular Ca^{2+} influx through NSCC-1 and NSCC-2 has an important role in the extracellular Ca^{2+} influx-dependent component of ERK1/2-dependent mitogenesis, (5) extracellular Ca^{2+} influx through NSCC-2 has an important role in ERK1/2-independent mitogenesis, and (6) Ca^{2+} influx through each Ca^{2+} channel may play a distinct role in intracellular mitogenic cascades.

Keywords: Endothelin; Mitogenesis; Ca^{2+} influx; Extracellular signal-regulated kinase

1. Introduction

Endothelin-1 possesses mitogenic activity on C6 glioma cells (Sedo et al., 1999; Kawanabe et al., 2001a), and extracellular Ca^{2+} influx plays an important role in this process (Kawanabe et al., 2001a). Endothelin-1 activates two types of Ca^{2+}-permeable nonselective cation channel (designated NSCC-1 and NSCC-2). Importantly, these channels can be distinguished by their sensitivity to blockers of the receptor-operated Ca^{2+} channel such as 1-[*b*-(3-[4-methoxyphenyl]propoxy)-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SK&F 96365) and (*R*,*S*)-(3,4-dihydro-6,7-dimethoxy-isoquinoline-1-yl)-2-phenyl-*N*,*N*-di-[2-(2,3,4-trimethoxyphenyl)ethyl]-acetamide (LOE 908). Thus, NSCC-1 is sensitive to LOE 908 and resistant to SK&F 96365, whereas NSCC-2 is sensitive to both LOE 908 and SK&F 96365 (Kawanabe et al., 2001a). However, the molecular mechanisms of endothelin-1-induced mitogenesis in C6 glioma cells are still unclear. Furthermore, previous reports did not delineate the effects of extracellular Ca^{2+} influx through NSCCs on these mechanisms.

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Extracellular signal-regulated kinase 1 and 2 (ERK1/2), members of the mitogen-activated protein (MAP) kinase family, are considered to represent major signaling pathways mediating endothelin-1-induced mitogenesis (Malarkey et al., 1995; Sugawara et al., 1996; Iwasaki et al., 1999; Suzuki et al., 1999). The existence of an ERK1/2-independent cascade for endothelin-1-induced mitogenesis has not been examined, although other mitogens such as basic fibroblast growth factor and insulin-like growth factor stimulate cell proliferation via MAP kinase-dependent and -independent pathways (Milasincic et al., 1996). The purpose of the present study was to examine the role, if any, of an ERK1/2-independent cascade in endothelin-1-induced mitogenesis, in addition to the ERK1/2-dependent cascade. Moreover, we examined the effects of extracellular Ca^{2+} influx on these cascades, using SK&F 96365 and LOE 908.

2. Materials and methods

2.1. Cell culture

C6 glioma cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum under a humidified atmosphere of 5% CO_2/95% air.

2.2. Measurement of ERK1/2 activity

Measurement of ERK1/2 activity was performed as described previously (Kawanabe et al., 2001b). C6 glioma cells at 50–80% confluency in 10-cm dishes were starved for 24 h before being stimulated with endothelin-1 for various times in serum-free DMEM in the absence or presence of Ca^{2+} channel blockers. The reaction was terminated by washing once with phosphate-buffered saline (PBS) and twice with 20 mM Tris–HCl (pH 7.4). After the addition of 1 ml of ice-cold extraction buffer (10 mM Tris–HCl, 0.5 mM EDTA, 0.5 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5 mg/ml aprotinin, 0.05 mM NaF, 0.5 mM Na_3PO_4, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride, and 5 mM β-glycerophosphate; pH 7.4), the cells were scraped off with a scraper. For partial purification of ERK, the cell suspension was transferred to a 15-ml conical tube, sonicated for 10 s × 3 at 10-s intervals, and centrifuged at 25,000 × *g* for 20 min. The supernatant was applied to a DEAE-Sephadex column (bed volume, 0.5 ml) pre-equilibrated with equilibration buffer (extraction buffer containing 100 mM NaCl). The enzyme was eluted with the elution buffer (extraction buffer containing 500 mM NaCl) and concentrated using Centricon YM-30 (Millipore, Bedford, MA, USA). The protein concentration of the partially purified enzyme in each sample was determined with a BCA Microprotein Assay Kit (Pierce, Rockford, IL, USA), and 5 μg of the enzyme was used for each assay. ERK1/2 activity was determined using a MAP Kinase Assay Kit (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

2.3. [^{3}H]thymidine incorporation

[^{3}H]Thymidine incorporation was performed as described previously (Kawanabe et al., 2001a). Briefly, C6 glioma cells were seeded into 24-well plates at 4×10^4 cells/well for [^{3}H]thymidine incorporation and were incubated overnight in DMEM supplemented with 10% fetal calf serum at 37 °C. The cells were deprived of serum for 24 h, washed with PBS, and incubated with endothelin-1 for an additional 48 h in serum-free DMEM. For measurement of [^{3}H]thymidine incorporation, [^{3}H]thymidine (1 μCi/μl) was added during the last half of the 48-h incubation. To stop the reaction, cells were washed three times with ice-cold PBS, incubated with 10% (w/v) trichloroacetic acid at 4 °C for 30 min, and subsequently washed three times with ice-cold PBS to remove the trichloroacetic acid-soluble material. The radioactivity incorporated into the trichloroacetic acid-insoluble fraction was recovered in 0.1 N NaOH and counted with a liquid scintillation counter (Aloka, Tokyo, Japan) using the solid scintillator Luma-Cap (Packard, Groningen, Netherlands).

2.4. Drugs

Boehringer Ingelheim (Ingelheim, Germany) kindly provided LOE 908. Materials were obtained from the following sources: ET-1 from the Peptide Institute (Osaka, Japan); SK&F 96365 from Biomol (Plymouth Meeting, PA, USA); [^{3}H]thymidine from NEN (Boston, MA, USA); and 2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD 98059) from Wako (Osaka, Japan). All other chemicals were of reagent grade and were obtained commercially.

2.5. Statistical analysis

All results are expressed as means ± S.E.M. The data were subjected to a two-way analysis of variance. When a significant F value was detected, Newman–Keuls' multiple range test was used to test for significant differences between treatment means. A probability level of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of endothelin-1 on ERK1/2 activity

After stimulation with 10 nM endothelin-1, ERK1/2 activity in the cytosolic fraction increased with time and at 2 min reached a peak value 4-fold higher than pre-stimulation values (Fig. 1(A)). Thereafter, activity rapidly decreased and by 30 min had returned to the control level (Fig. 1(A)). Thus, in subsequent experiments, the stimulation time was set at 2 min.

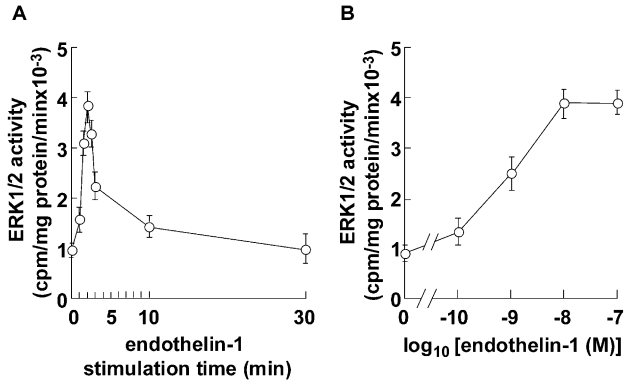

Fig. 1. (A) Time course of ERK1/2 activity following stimulation with endothelin-1 in C6 glioma cells. After cells had been cultured in serum-free medium for 24 h, they were stimulated with 10 nM endothelin-1 for the indicated time periods. (B) Effects of various concentrations of endothelin-1 on ERK1/2 activity in C6 glioma cells. After cells had been deprived of serum for 24 h, they were stimulated with increasing concentrations of endothelin-1 for 2 min. ERK1/2 activity was determined as described in Materials and methods. Data are the means ± S.E.M. of three determinations, each done in triplicate.

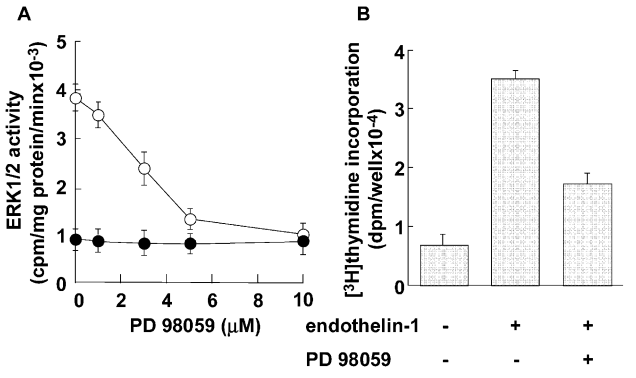

Fig. 2. (A) Inhibitory effects of PD 98059 on endothelin-1-induced ERK1/2 activity in C6 glioma cells. Starved cells were incubated for 15 min with increasing concentrations of PD 98059 and then stimulated with (open circles) or without (closed circles) 10 nM endothelin-1. ERK1/2 activity was determined as described in Materials and methods. (B) Inhibitory effects of PD 98059 on endothelin-1-induced [^{3}H]thymidine incorporation in C6 glioma cells. Starved cells were incubated for 15 min with or without 10 μM PD 98059 and then stimulated with 10 nM endothelin-1. Data are the means ± S.E.M. of three determinations, each done in triplicate.

Endothelin-1 stimulated ERK1/2 activity in a concentration-dependent manner with an EC_{50} value of about 1 nM; the maximal effect was observed at a concentration $\geq$ 10 nM (Fig. 1(B)).

3.2. Effects of PD 98059 on endothelin-1-induced ERK1/2 activity and [^{3}H]thymidine incorporation

PD 98059 inhibited the endothelin-1-induced increase in ERK1/2 activity in a concentration-dependent manner with an IC_{50} value of about 3 μM, with complete inhibition being observed at a concentration $\geq$ 10 μM (Fig. 2(A)). Thus, this concentration of PD 98059 was used in the following experiments.

Endothelin-1 at 10 nM (the maximally effective concentration) increased [^{3}H]thymidine incorporation to 5-fold control values. Treatment of the cells with 10 μM PD 98059 suppressed this endothelin-1-induced [^{3}H]thymidine incorporation to 2.5-fold control values (Fig. 2(B)).

3.3. Effects of LOE 908 and SK&F 96365 on endothelin-1-induced ERK1/2 activity

Next, we examined the dependence of ERK1/2 activation by endothelin-1 on extracellular Ca^{2+} influx, and pharmacologically characterized the Ca^{2+} channels involved in the activation of ERK1/2, using LOE 908 and SK&F 96365. The magnitude of endothelin-1-induced ERK1/2 activity in the absence of extracellular Ca^{2+} was 40% of that in the presence of extracellular Ca^{2+} (Fig. 3(B)). LOE 908 and SK&F 96365 inhibited endothelin-1-induced ERK1/2 activity in a concentration-dependent manner with IC_{50} values of about 3 μM (Fig. 3(A)). The maximal effective concentrations of these drugs were $\geq$ 10 μM (Fig. 3(A)). The magnitude of endothelin-1-induced increases in ERK1/2 activity in the presence of maximally effective concentrations of LOE 908 and SK&F 96365 alone or in combination was 40%, 60% and 40% of that in the absence of these drugs, respectively (Fig. 3(B)).

3.4. Effect of SK&F 96365 and LOE 908 on ERK1/2-independent mitogenic responses induced by endothelin-1

We also examined which Ca^{2+} channels were involved in the ERK1/2-independent pathways of endothelin-1-induced mitogenesis, using SK&F 96365 and LOE 908. Endothelin-1-induced [^{3}H]thymidine incorporation in the presence of

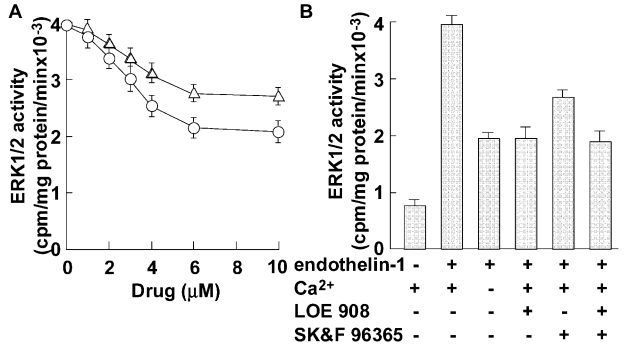

Fig. 3. (A) Effects of SK&F 96365 and LOE 908 on endothelin-1-induced ERK1/2 activity in C6 glioma cells. (B) Effects of extracellular Ca^{2+} and a maximally effective concentration (10 μM) of SK&F 96365 and LOE 908 on endothelin-1-induced ERK1/2 activity in C6 glioma cells. Starved cells were incubated for 15 min with increasing concentrations of LOE 908 (circles) or SK&F 96365 (triangles) and then stimulated with or without 10 nM endothelin-1. ERK1/2 activity was determined as described in Materials and methods. Data are the means ± S.E.M. of three determinations, each done in triplicate.

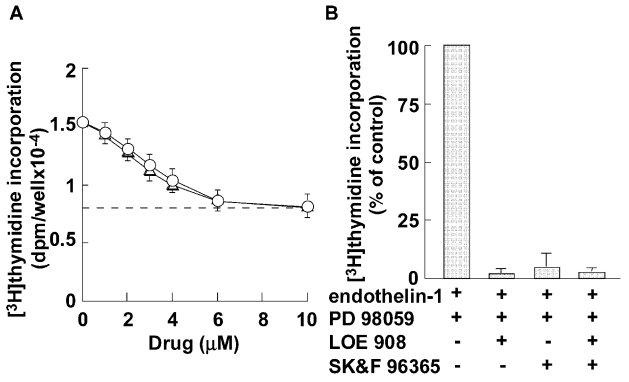

Fig. 4. (A) Effects of SK&F 96365 and LOE 908 on endothelin-1-induced [^{3}H]thymidine incorporation in the presence of 10 μM PD 98059 in C6 glioma cells. (B) Effects of a maximally effective concentration (10 μM) of SK&F 96365 and LOE 908 on endothelin-1-induced [^{3}H]thymidine incorporation in the presence of 10 μM PD 98059 in C6 glioma cells. Starved cells were incubated for 15 min with increasing concentrations of LOE 908 (circles) or SK&F 96365 (triangles) in addition to PD 98059 and then stimulated with or without 10 nM endothelin-1. ERK1/2 activity was determined as described in Materials and methods. Data presented are the means ± S.E.M. of three determinations, each done in triplicate.

PD 98059 was inhibited by LOE 908 or SK&F 96365 in a concentration-dependent manner with an IC_{50} value of about 3 μM (Fig. 4(A)). Complete inhibition was observed at concentrations ≥ 10 μM (Fig. 4).

4. Discussion

As described previously (Leach et al., 1999), endothelin-1 stimulates ERK1/2 in C6 glioma cells (Fig. 1). Judging from the partial sensitivity to PD 98059, endothelin-1-induced mitogenesis may involve two different pathways, one dependent on ERK1/2 and one independent of ERK1/2 (Fig. 2(B)). The involvement of ERK1/2 in endothelin-1-induced mitogenesis is consistent with previous reports (Leach et al., 1999). To the best of our knowledge, this is the first report showing the involvement of an ERK1/2-independent pathway in endothelin-1-induced mitogenesis.

Because previous reports had indicated that extracellular Ca^{2+} influx activates ERK1/2 (Egea et al., 1999), we examined the effects of Ca^{2+} influx on the stimulation of ERK1/2 by endothelin-1 in C6 glioma cells. There were at least two independent pathways for stimulation of ERK1/2. One was dependent on extracellular Ca^{2+} and the other was not, based on the inhibition of enzyme activity after removal of extracellular Ca^{2+} (Fig. 3). The maximally effective concentration of LOE 908 for completely blocking extracellular Ca^{2+} influx abolished endothelin-1-induced mitogenesis (Kawanabe et al., 2001a). Therefore, endothelin-1-induced mitogenesis via the extracellular Ca^{2+} influx-independent component of ERK1/2 cascades is nonetheless also dependent on extracellular Ca^{2+} influx. These results suggest the possibility that extracellular Ca^{2+} influx also plays an important role in mitogenic pathways downstream of ERK1/2.

The inhibitory action of LOE 908 or SK&F 96365 on endothelin-1-induced ERK1/2 activation is considered to be mediated by blockade of Ca^{2+} influx through NSCC-1 and/or NSCC-2 for the following reasons: (1) the IC_{50} value (3 μM) and maximal effective concentration (10 μM) of these blockers of ERK1/2 activity (Fig. 3) correlated well with those values for endothelin-1-induced extracellular Ca^{2+} influx (Kawanabe et al., 2001a). (2) The extent of inhibition of the extracellular Ca^{2+} influx-dependent component of ERK1/2 induced by these blockers (Fig. 3) was similar to that of the extracellular Ca^{2+} influx (Kawanabe et al., 2001a). Moreover, the extent of ERK1/2 inhibition by the maximally effective concentration of LOE 908 was similar to that seen in the absence of extracellular Ca^{2+} (Fig. 3). (3) Endothelin-1 activates NSCC-1 and NSCC-2. NSCC-1 is sensitive to LOE 908 and resistant to SK&F 96365, whereas NSCC-2 is sensitive to both LOE 908 and SK&F 96365 (Kawanabe et al., 2001a). Therefore, NSCC-1 and NSCC-2 may have important roles in the Ca^{2+}-dependent component of ERK1/2 activation by endothelin-1. On the basis of the sensitivity to LOE 908 and SK&F 96365, Ca^{2+} influx through NSCC-2, but not NSCC-1, seems to be involved in ERK1/2-independent mitogenesis caused by endothelin-1 (Fig. 4). These results suggest that extracellular Ca^{2+} influx through different Ca^{2+} channels may at least partially have different specific roles for intracellular mechanisms.

Ca^{2+} influx regulates the activation of p34cdc2 kinase and subsequent phosphorylation of pRB (the dephosphorylated form of the retinoblastoma protein), leading to DNA synthesis (Takuwa et al., 1993). Phosphorylation of pRB occurs in the mid- to late-G_1 phase and is required for entry into the S phase (Weinberg, 1995). Thus, it is currently thought that a Ca^{2+}-dependent process acting relatively far downstream in the intracellular signaling pathway plays a pivotal role in the regulation of cell cycle progression. However, it is unknown whether the same signaling pathways are operating in C6 glioma cells. It remains to be determined which signaling cascades downstream of ERK1/2 are involved in endothelin-1-induced mitogenesis and at which step(s) extracellular Ca^{2+} influx is required.

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