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B103 neuroblastoma cells predominantly express endothelin ET_B receptor; effects of extracellular Ca^{2+} influx on endothelin-1-induced mitogenesis

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Abstract

We sought to examine the effects of endothelin-1 on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and mitogenic response in the neuroblastoma cell line, B103 (B103 cells). The results obtained from an [^{125}I] endothelin-1 binding assay demonstrated that B103 cells express the endothelin receptor. The B_{max} and K_d values for [^{125}I]endothelin-1 binding were 70 ± 36 fmol/mg protein and 52 ± 13 pM, respectively. Endothelin-1 failed to stimulate cAMP formation, but it did inhibit forskolin-induced cAMP formation. Endothelin-1 also stimulated the accumulation of [^{3}H]inositol phosphates. These results indicate that the endothelin receptor in B103 cells couples with G_i and G_q but not with G_s. Monitoring of $[Ca^{2+}]_i$ showed that endothelin-1 evoked a transient increase in $[Ca^{2+}]_i$; this remained even in the absence of extracellular Ca^{2+}. However, no sustained, endothelin-1-induced increase in $[Ca^{2+}]_i$ due to extracellular Ca^{2+} influx was detected. The endothelin B receptor-selective antagonist, 2,6-Dimethylpiperidinecarbonyl-γ-Methyl-Leu-N_{in}-[Methoxycarbonyl]-D-Trp-D-Nle (BQ 788), abolished the endothelin-1-induced increase in $[Ca^{2+}]_i$, while the endothelin ET_A receptor-selective antagonist, cyclo-D-Asp-Pro-D-Val-Leu-D-Trp (BQ 123), failed to inhibit it. These results indicate that B103 cells express endothelin ET_B receptor or an endothelin ET_B-like receptor predominantly and have no Ca^{2+} channels activated by endothelin-1. Endothelin-1 activated mitogen-activated protein kinase in B103 cells. However, based on the data for 3-(4,5-dimethy-2-thiazolyl)-2,5-diphenyl tetrazolium bromide, [^{3}H]thymidine incorporation, and apoptosis screening assays, endothelin-1 induces neither mitogenesis nor apoptosis. These results suggest that endothelin-1 has no role in the mitogenic response in B103 cells, and this is consistent with the notion that an endothelin-1-induced sustained increase in $[Ca^{2+}]_i$ plays a role in endothelin-1-induced cell proliferation.

Keywords: Endothelin; Neuroblastoma cell; Ca^{2+} increase; Mitogenesis; Endothelin ET_B receptor

1. Introduction

Endothelin-1 was characterized as a highly potent vasoconstricting peptide of 21 amino acids (Yanagisawa et al., 1988). However, subsequent studies have described many other biological activities, including a neuromodulatory role in the central nervous system (CNS) (Yanagisawa and Masaki, 1989; Nambi et al., 1990; Koizumi et al., 1992). Endothelin-1 has also been identified as an autocrine/paracrine growth factor for some human cancer cell lines (Shichiri et al., 1991), suggesting a role in oncogenesis or tumor growth. The biological actions of endothelin-1 are mediated by two distinct receptor subtypes, endothelin ET_A receptor and endothelin ET_B receptor, which belong to a family of heptahelical G-protein-coupled receptors (Arai et al., 1990; Sakurai et al., 1990).

Endothelin-1 induces a transient and subsequent sustained increase in $[Ca^{2+}]_i$ in several cell lines (Enoki et al., 1995; Minowa et al., 1997; Iwamuro et al., 1999). It is generally accepted that the transient phase results from the mobilization of Ca^{2+} from intracellular stores, while the sustained phase results from entry of extracellular Ca^{2+} (Gardner, 1989). Moreover, recent reports have demon-

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strated that Ca^{2+} influx plays an important role in mitogenesis induced by endothelin-1 (Chattopadhyay et al., 1999; Kawanabe et al., 2001a,b).

In this study, we sought to characterize the endothelin-1-induced Ca^{2+} influx and mitogenic response in CNS cells. We selected B103 neuroblastoma cells because there was no previous report as to whether they express the endothelin receptor. B103 cells are derived from rat CNS and cannot make the β-amyloid precursor protein (Schubert et al., 1974). Hypoxanthine increases the proliferation of B103 cells (Ma et al., 2001). First, we clarified whether B103 cells express endothelin receptors. We then examined the Ca^{2+} mobilization and mitogenic response induced by endothelin-1 in B103 cells.

2. Materials and methods

2.1. Cell culture

B103 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum and 5% horse serum in a humidified atmosphere of 5% CO_2/95% O_2.

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

$[Ca^{2+}]_i$ was measured using the fluorescent probe fura-2, as described previously (Minowa et al., 1997). Briefly, cells were loaded with fura-2 by incubating them with fura-2/acetoxymethyl ester (10 mM) for 30 min at 37 °C under reduced light. After being washed, the cells were suspended at a density of approximately 2×10^7 cells/ml; 0.5-ml aliquots were used for measuring fluorescence using a CAF 110 spectrophotometer (JASCO, Tokyo, Japan) with excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm at 25 °C. At the end of the experiment, Triton X-100 and then EGTA were added to final concentrations of 0.1% and 5 mM, respectively, in order to measure the fluorescence maximum (F_{max}) and minimum (F_{min}). $[Ca^{2+}]_i$ was determined from the ratio of fura-2 fluorescence at 340 nm to that at 380 nm by use of external standards (De Erausquin et al., 1990).

2.3. $[^{125}I]$ET-1 binding assay

Assays with membrane preparations were performed as described previously (Sakamoto et al., 1993).

2.4. Cyclic AMP formation

Cells (10^4/well) in 96-well plates were washed with phosphate-buffered saline (PBS) and then incubated at 37 °C for 10 min with 0.1 ml PBS containing 3-isobutyl-1-methylzanthine (1 mM). The cells were stimulated for 10 min in the same medium with various concentrations of endothelin-1, as indicated, with or without forskolin (100 μM). The cAMP content of the solution was measured using a cAMP enzyme immunoassay system (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions.

2.5. Formation of inositol phosphates

Formation of inositol phosphates was determined as described previously (Suzuki et al., 1999). Briefly, cells in 24-well plates were incubated with *myo*-[^{3}H]inositol (final concentration, 5 μCi/ml) for 18 h in 0.3 ml of DMEM supplemented with 10% fetal calf serum and 5% horse serum. After being washed, the cells were incubated with or without various concentrations of endothelin-1 for 30 min; the addition of ice-cold perchloric acid terminated the reaction. After neutralization with potassium hydroxide solution and Tris, the samples were applied to small columns of AG1X8 (100–200 mesh, Cl^- form; Bio-Rad, Hercules, CA) to separate total IPs from *myo*-[^{3}H]inositol. [^{3}H]inositol phosphates were eluted with 1 N HCl, and radioactivity was counted using a liquid scintillation counter.

2.6. Measurement of mitogen-activated protein kinase (MAPK) activity

Cells at 50–80% confluency in 10-cm dishes were cultured in serum-free DMEM for 24 h. Then, they were stimulated with endothelin-1 for various times in serum-free DMEM. The reaction was terminated by washing the cells once with PBS and twice with 20 mM Tris–HCl (pH 7.4). After addition of ice-cold extraction buffer (1 ml; 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, 5 mM β-glycerophosphate; pH 7.4), cells were scraped off the dishes with a scraper. For partial purification of MAPK, the cell suspension was transferred to a 15-ml conical tube, sonicated for 10 s three times with 10-s intervals, and centrifuged ($25{,}000 \times g$, 20 min). The supernatant was applied to a DEAE-Sephadex column (bed volume, 0.5 ml) and pre-equilibrated with equilibration buffer (extraction buffer containing 100 mM NaCl). The enzyme was eluted with elution buffer (extraction buffer containing 500 mM NaCl) and was concentrated using a Centricon YM-30 (Millipore Corporation, Bedford, MA,USA). The protein concentration of the partially purified enzyme in each sample was determined with the bicinchoninic acid Microprotein Assay Kit (Pierce, Rockford, IL, USA), and 5 μg enzyme was used for each assay. MAPK activity was determined using a MAP Kinase Assay Kit (Amersham), according to the manufacturer's instructions.

2.7. Cell proliferation assay

Cells were seeded into 96-well plates at 5×10^3 cells/well for the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) incorporation assay and into 24-well plates at 4×10^4 cells/well for the [^{3}H]thymidine incorporation assay. They were incubated overnight in DMEM supplemented with 10% fetal calf serum and 5% horse serum at 37 °C. The cells were deprived of serum for 24 h, washed with PBS, and incubated with endothelin-1 for a further 48 h in serum-free DMEM.

The MTT assay, a measure of the number of viable cells, was performed as described previously (Sugawara et al., 1996). Briefly, the cells were exposed to 1 mg/ml MTT solution during the last 4 h of the incubation period with endothelin-1, washed, and then lysed with acidic lysis buffer (200 g/l sodium dodecyl sulfate in 50% *N*,*N*-dimethyl formamide solution; pH 4.7). The optical densities of the lysate at 562 and 630 nm were measured with an EL340 Microtiter Plate Reader (Bio-Tek Instruments, Winooski, VT); the difference between the values at 562 and 630 nm was used as an index of the number of viable cells.

For measurement of [^{3}H]thymidine incorporation, [^{3}H]thymidine (1 mCi/ml) was added during the last half of the 48-h incubation period with endothelin-1. To stop the reaction, the cells were washed three times with ice-cold PBS, incubated with 100 g/l trichloroacetic acid at 4 °C for 30 min, and subsequently washed three times with ice-cold PBS to remove trichloroacetic acid-soluble material. Radioactivity incorporated into the trichloroacetic acid-insoluble fraction was recovered in 0.1 N NaOH and counted using a liquid scintillation counter (Aloka) and a solid scintillator Luma-Cap (Packard, Groningen, Netherlands).

2.8. Apoptosis screening assay

B103 cells were seeded into 96-well plates at 10^4 cells/well and incubated overnight in DMEM supplemented with 10% FCS and 5% HS at 37 °C. The cells were deprived of serum for 24 h, washed with PBS, and incubated with ET-1 for a further 48 h in serum-free DMEM. Apoptosis screening was performed using an Apoptosis Screening Kit (Wako, Osaka, Japan).

2.9. Drugs

Materials were obtained from the following sources: ET-1 from the Peptide Institute (Osaka, Japan); fura-2/AM from Dojindo Laboratories (Kumamoto, Japan); [^{125}I]ET-1 and *myo*-[^{3}H]inositol from Amersham Pharmacia Biotech; MTT, 2,6-Dimethylpiperidinecarbonyl-g-Methyl-Leu-N_{in}-[Methoxycarbonyl]-D-Trp-D-Nle (BQ 788) and cyclo-D-Asp-Pro-D-Val-Leu-D-Trp (BQ 123) from Sigma (St. Louis, MO, USA); [^{3}H]thymidine from NEN (Boston, MA, USA); 1-[*b*-3-[4-Methoxyphenyl]propoxy)-4-methoxyphenethyl]-1*H*-imidazole hydrochloride (SK&F 96365) from Biomol (Plymouth Meeting, PA, USA). All other chemicals were of reagent grade and were obtained commercially.

2.10. Statistical analysis

All results are expressed as means ± S.E.M.

3. Results

3.1. [^{125}I]endothelin-1 binding assays with B103 cells

The [^{125}I] endothelin-1 binding assays with membrane preparations gave a K_d value of 52 ± 13 pM and a B_{max} value of 70 ± 36 fmol/mg protein.

3.2. Effect of endothelin-1 on cAMP formation in B103 cells

Endothelin-1 failed to stimulate cAMP formation at concentrations between 10 pM and 10 nM (Fig. 1). Endothelin-1 inhibited forskolin-induced cAMP formation with an IC_{50} value of 22.4 ± 4.4 pM; the maximal inhibition, amounting to about 60%, was observed at concentrations of ≥ 10 nM (Fig. 1).

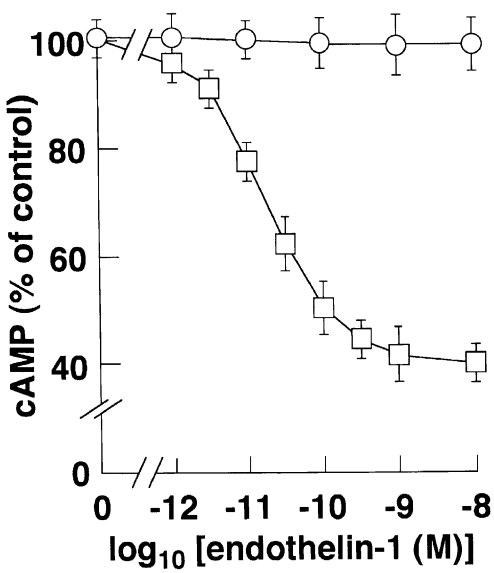

Fig. 1. Effects of endothelin-1 on cAMP accumulation in B103 cells. In the presence of 3-isobutyl-1-methylxanthine, cells were stimulated with various concentrations of endothelin-1 for 10 min in the presence (square) or absence (circle) of forskolin (100 μM). The cAMP concentration in the solution was measured using a cAMP enzyme immunoassay system, as described in the Materials and methods. Values are expressed as fold increases above basal values. Each data point represents mean ± S.E.M. of five experiments.

3.3. Effect of endothelin-1 on accumulation of [^{3}H]inositol phosphates in B103 cells

Endothelin-1 caused a concentration-dependent stimulation of [^{3}H]inositol phosphates accumulation with an EC_{50} value of 1.5 ± 0.4 nM; the maximal effect, about sixfold increase, was obtained at concentrations of ≥ 10 nM (Fig. 2).

3.4. Measurement of $[Ca^{2+}]_i$ in B103 cells

Endothelin-1 at 10 nM evoked a transient increase in $[Ca^{2+}]_i$, but no subsequent sustained increase in $[Ca^{2+}]_i$ was detected (Fig. 3A). In the absence of external Ca^{2+}, the transient increase in $[Ca^{2+}]_i$ persisted (Fig. 3B). The transient increase in $[Ca^{2+}]_i$ increased in a concentration-dependent manner; the maximum peak was obtained at ≥ 10 nM (data not shown). The endothelin-1-induced increase in $[Ca^{2+}]_i$ was abolished by BQ788 (10 μM), a specific antagonist of the endothelin B receptor (Peter and Davenport, 1996), while it was unaffected by BQ123 (10 μM), a specific antagonist of the endothelin A receptor (Peter and Davenport, 1996) (Fig. 3C and D).

To assess the existence of the store-operated Ca^{2+} channel (SOCC), B103 cells were treated with thapsigargin, an inhibitor of sarcoplasmic reticulum Ca^{2+}-ATPase. Generally, treating cells with thapsigargin depletes intracellular Ca^{2+} stores and thereby activates a Ca^{2+} channel on the plasma membrane, called the SOCC, causing a sustained increase in $[Ca^{2+}]_i$ (Thastrup et al., 1990). As a result, the sustained increase in $[Ca^{2+}]_i$ is regarded as an index of the activity of the SOCC. Thapsigargin (0.1 μM) induced both transient and sustained increases in $[Ca^{2+}]_i$ (Fig. 4A). In the absence of external Ca^{2+}, the sustained increase in $[Ca^{2+}]_i$ was abolished, while the transient increase in $[Ca^{2+}]_i$ remained (Fig. 4B). SK&F 96365, an inhibitor of receptor-mediated Ca^{2+} entry (Merritt et al., 1990), in concentrations up to 10 μM, inhibited the thapsigargin-induced sustained increase in $[Ca^{2+}]_i$ in A7r5 cells (Iwamuro et al., 1999). In contrast, SK&F 96365 had no effect on the sustained increase in $[Ca^{2+}]_i$ in B103 cells (Fig. 4A). Moreover, a thapsigargin-induced sustained increase in $[Ca^{2+}]_i$ was detected even in cells pre-incubated with endothelin-1 (Fig. 4C).

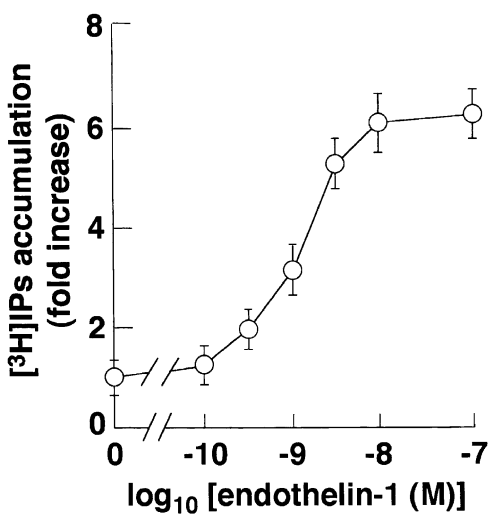

Fig. 2. Effects of endothelin-1 on accumulation of inositol phosphates in B103 cells. The cells, which had been incubated with *myo*-[^{3}H]inositol for 18 h, were stimulated with various concentrations of endothelin-1 for 30 min. Total inositol phosphates in the cell extract were measured as described in the Materials and methods. Each point represents mean ± S.E.M. of five experiments.

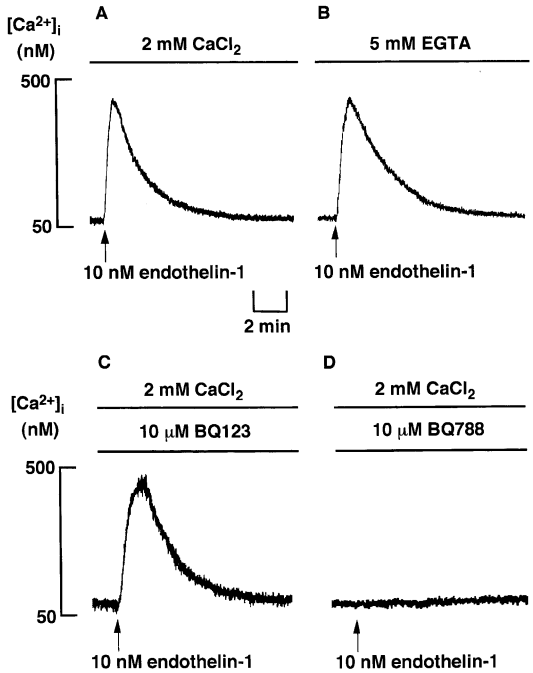

Fig. 3. (A, B) Original tracings illustrating the effects of 10 nM endothelin-1 on the increase in $[Ca^{2+}]_i$ in B103 cells in the presence (A) or absence (B) of extracellular Ca^{2+}. The cells were loaded with the Ca^{2+} indicator fura-2 and subjected to $[Ca^{2+}]_i$ monitoring. Endothelin-1 was added to the bath solution at the times indicated by the arrows. (C, D) Original tracings illustrating the effects of BQ123 (C) or BQ788 (D) on the increase in $[Ca^{2+}]_i$ in B103 cells induced by endothelin-1 (10 nM) in the presence of extracellular Ca^{2+}. Cells were incubated with BQ123 or BQ788 before addition of endothelin-1.

3.5. Effects of endothelin-1 on MAPK activation

After stimulation with 10 nM endothelin-1, MAPK activity in the cytosolic fraction increased with time, and at 2 min, it reached a peak value, amounting to about three times that of control values before stimulation (Fig. 5A). Thereafter, MAPK activity rapidly decreased and it returned to the control level at 10 min (Fig. 5A). As a result, the stimulation time was set at 2 min in subsequent experiments.

Endothelin-1 stimulated MAPK activity in a concentration-dependent manner with an EC_{50} value of ~ 1 nM; the maximal effect was obtained at a concentration of ≥ 10 nM (Fig. 5B).

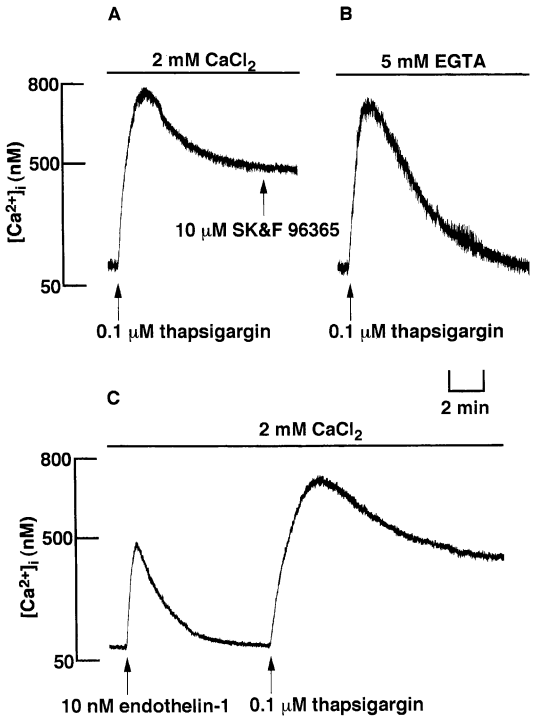

Fig. 4. (A, B) Original tracings illustrating the effects of thapsigargin (0.1 μM) on the increase in $[Ca^{2+}]_i$ in B103 cells as an index of the activity of SOCC in the presence (A) or absence (B) of extracellular Ca^{2+}. (C) Original tracings illustrating the effects of thapsigargin (0.1 μM) on the increase in $[Ca^{2+}]_i$ in B103 cells in the presence of ET-1 (10 nM). The cells were loaded with fura-2 and subjected to $[Ca^{2+}]_i$ monitoring. Cells were stimulated with thapsigargin at the times indicated by arrows. After $[Ca^{2+}]_i$ reached a steady state, SK and F 96365 was added to the bath solution at the times indicated by arrows.

3.6. Effects of endothelin-1 on B103 cell proliferation

To estimate cell proliferation, MTT and [^{3}H] thymidine incorporation assays were performed. Endothelin-1 up to 10 nM had no effect on B103 cell proliferation, in either the MTT or the [^{3}H] thymidine incorporation assays (Table 1). Moreover, endothelin-1 up to 10 nM had no effect on the number of apoptotic cells (Table 1).

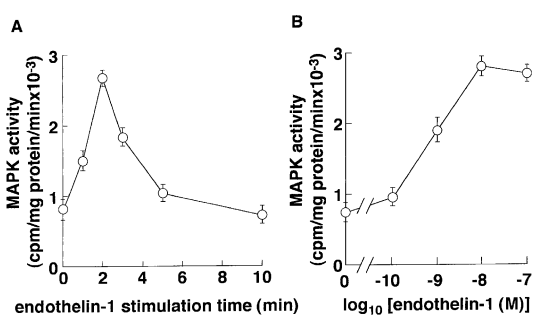

Fig. 5. (A) Time course of MAPK activity following stimulation with endothelin-1 in B103 cells. After cells had been cultured in serum-free media for 24 h, they were stimulated with endothelin-1 (10 nM) for the indicated times. (B) The effect of various concentrations of endothelin-1 on MAPK activity in B103 cells. After the cells had been deprived of sera for 24 h, they were stimulated with increasing concentrations of endothelin-1 for 2 min. MAPK activity was determined as described in the Materials and methods. The data presented are the means ± S.E.M. of three determinations, each done in triplicate.

Table 1
Effects of endothelin-1 on C6 glioma cell proliferation or apoptosis

Viable cell number	DNA synthesis	Apoptotic cell number
98.8 ± 6.2	101.6 ± 5.2	99.8 ± 5.7

Effect of 10 nM endothelin-1 on the number of viable cells, [^{3}H]thymidine incorporation and the number of apoptotic cells, expressed as percentages of values in the absence of ET-1.
Values are expressed as the means ± S.E.M. of five experiments.

4. Discussion

The results from the [^{125}I]endothelin-1 binding assay show that B103 cells express endothelin receptors. Data for $[Ca^{2+}]_i$ obtained by using endothelin A receptor- or endothelin B receptor-selective antagonists show that B103 cells predominantly express the endothelin B receptor or an endothelin B-like receptor (Fig. 3). As endothelin-1 has been identified as a growth factor in the CNS (Cazaubon et al., 1993), we examined the effect of endothelin-1 on B103 cells.

First, we attempted to identify the G-proteins coupled with the endothelin receptor, because regulation of adenylyl cyclase and phosphoinositide turnover are major cellular signal transduction pathways. Endothelin-1 failed to stimulate cAMP formation, while it did inhibit forskolin-induced cAMP formation (Fig. 1). Endothelin-1 stimulated the accumulation of [^{3}H]inositol phosphates in a concentration-dependent manner (Fig. 2). These results indicate that endothelin receptors in B103 cells couple with G_i and G_q but not with G_s. The IC_{50} value of endothelin-1 for forskolin-induced cAMP formation was lower than the EC_{50} value of endothelin-1 for inositol phosphates accumulation (Figs. 1 and 2). These differences are also observed in Chinese hamster ovary cells stably expressing endothelin B receptor (Okamoto et al., 1997). Further study is necessary to clarify the cause of these differences.

It is generally accepted that Ca^{2+} metabolism plays an important role in the control of cellular proliferation in central nervous system-derived cells (Lee et al., 1994; Jensen et al., 1995; Chattopadhyay et al., 1999; Kawanabe et al., 2001a). In B103 cells, endothelin-1 evoked a transient increase in $[Ca^{2+}]_i$, but no sustained increase in $[Ca^{2+}]_i$ was detected (Fig. 3). Because the transient increase in $[Ca^{2+}]_i$ persisted after removal of extracellular Ca^{2+} (Fig. 3B), it is likely the result of Ca^{2+} mobilization from intracellular stores, as noted previously (Van Renterghem et al., 1988; Kasuya et al., 1989). Moreover, the observation that endothelin-1 failed to induce a sustained increase in $[Ca^{2+}]_i$ (Fig. 3A) indicates that B103 cells have no Ca^{2+} channels activated by endothelin-1. To

our knowledge, this is the first cell line not to have Ca^{2+} channels activated by endothelin-1.

Thapsigargin evoked an increase in $[Ca^{2+}]_i$ consisting of two components: a rapid, initial transient phase and a sustained phase (Fig. 4A). The sustained phase was abolished by removal of extracellular Ca^{2+} (Fig. 4B). These results indicate that the sustained phase is due to transmembrane Ca^{2+} influx. Thapsigargin, a potent intracellular Ca^{2+} pump inhibitor, blocks Ca^{2+} accumulation and allows Ca^{2+} release from Ca^{2+} stores (Takemura and Putney, 1989). Thapsigargin-induced Ca^{2+} pool emptying mediates Ca^{2+} entry via the SOCC (Takemura and Putney, 1989); SK&F 96365 blocks this Ca^{2+} influx in A7r5 cells (Iwamuro et al., 1999). However, the thapsigargin-induced sustained increase in $[Ca^{2+}]_i$ was resistant to SK&F 96365 in B103 cells (Fig. 4A). These results indicate that the thapsigargin-activated Ca^{2+} channels in B103 cells are different from those in A7r5 cells (Iwamuro et al., 1999).This is the first report showing the existence of SK&F 96365-resistant SOCCs. Moreover, these Ca^{2+} channels were not activated by endothelin-1 because thapsigargin induced a sustained increase in $[Ca^{2+}]_i$ even after the cells were incubated with endothelin-1 (Fig. 4C). Thus, endothelin-1 failed to activate SOCCs in B103 cells.

We then examined whether endothelin-1 had mitogenic effects in B103 cells. It is generally accepted that MAPK is a major signaling pathway mediating the endothelin-1-induced mitogenic response (Malarkey et al., 1995; Iwasaki et al., 1999; Suzuki et al., 1999). Endothelin-1 also stimulated MAPK in B103 cells (Fig. 5). However, based on the data from the MTT and the [^{3}H]thymidine incorporation assays, endothelin-1 failed to induce a mitogenic response in B103 cells (Table 1). Moreover, endothelin-1 failed to induce apoptosis (Table 1). Endothelin-1-induced extracellular Ca^{2+} influx plays a role in endothelin-1-induced cell proliferation (Shichiri et al., 1991; Chattopadhyay et al., 1999; Kawanabe et al., 2001a,b). The absence of a mitogenic response to endothelin-1 in B103 cells may be because there was no endothelin-1-induced sustained increase in $[Ca^{2+}]_i$. In other words, these results are consistent with the concept that the endothelin-1-induced sustained increase in $[Ca^{2+}]_i$, but not the transient increase, plays a critical role in endothelin-1-induced cell proliferation.

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