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Epinephrine Increases DNA Synthesis via ERK1/2s Through cAMP, Ca^{2+}/PKC, and PI3K/Akt Signaling Pathways in Mouse Embryonic Stem Cells

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Abstract Epinephrine is a catecholamine that plays important roles in regulating a wide variety of physiological systems by acting through the adrenergic receptors (ARs). The cellular responses to AR stimulation are mediated through various signaling pathways. Therefore, this study examined the effects of epinephrine on DNA synthesis and related signaling molecules in mouse embryonic stem cells (ESCs). Epinephrine increased DNA synthesis in a dose- and time-dependent manner, as determined by the level of [^{3}H]-thymidine incorporation. AR subtypes ($\alpha1_A$, $\alpha2_A$, $\beta1$, $\beta2$, and $\beta3$) were expressed in mouse ESCs and their expression levels were increased by epinephrine. In this experiment, epinephrine increased cAMP levels, intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), and translocation of protein kinase C (PKC) from the cytosol to the membrane compartment. In addition, we observed Akt phosphorylation in response to epinephrine; this was stimulated by phosphorylation of the epidermal growth factor receptor (EGFR). Epinephrine also induced phosphorylation of ERK1/2 (p44/42 MAPKs), while inhibition of PKC or Akt blocked this phosphorylation. Epinephrine increased the mRNA levels of proto-oncogenes (c-fos, c-jun, c-myc), while inhibition of ERK1/2 decreased these mRNA levels. In experiments aimed at examining the involvement of cell cycle regulatory proteins, epinephrine increased the levels of cyclin E/cyclin-dependent kinase 2 (CDK2) and cyclin D1/cyclin-dependent kinase 4 (CDK4). In conclusion, epinephrine stimulates DNA synthesis via ERK1/2 through cAMP, Ca^{2+}/PKC, and PI3K/Akt signaling pathways in mouse ESCs. J. Cell. Biochem. 104: 1407–1420, 2008.

Key words: mouse ES cell; epinephrine; cell proliferation

Catecholamines function as neurotransmitters and hormones in animal tissues. The early embryo itself may produce catecholamines, as has been suggested for mice, rats, and frogs [Burden and Lawrence, 1973; Devic et al., 1997; Fujinaga and Scott, 1997; Herlenius and Lagercrantz, 2001]. The previous findings suggest that the enzymes responsible for norepinephrine synthesis are moderately detectable at embryonic Day 8.5 (8.5 d.p.c.), and catecholamines are first detectable at 9.5 and 10.5 d.p.c. [Thomas et al., 1995; Zhou et al., 1995]. There is evidence suggesting the role of endogenous catecholamines in basic developmental processes such as embryogenesis and morphogenesis where they may control cell proliferation, differentiation, and migration [Herlenius and Lagercrantz, 2001; Anitole-Misleh and Brown, 2004]. In vivo experiments also suggest that initiation of this proliferative activity might be mediated through adrenergic receptors (ARs). In contrast, little is known about the localization and physiological roles of AR subtypes in early embryonic development. The $\alpha1$-ARs mediate the effects of the sympathetic nervous system, particularly those effects related to the regulation of cellular hypertrophy and proliferation. There is substantial evidence that stimulation of $\alpha1$-AR by catecholamines generally enhances the expression of growth-related genes and the cell growth in a variety of cells including cardiac myocytes, vascular smooth muscle cells, hepatocytes, and adipocytes [Cruise et al., 1985; Thonberg et al., 1994; Spector et al., 1997]. The $\beta2$-AR protein is

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produced in oocytes and preimplantation embryos, which supports the hypothesis that functional signaling from the β2-AR exists in these cells. In blastocysts, immunofluorescent labeling of β2-AR is stronger in the inner cell mass than in trophectoderm cells, indicating that the β2-AR has a stronger impact in pluripotent cells that are destined to constitute the embryo proper than in more differentiated cells that later form the extraembryonal tissues [Cikos et al., 2005]. Indeed, it is possible that particular AR subtypes play important roles during development because a lack of norepinephrine synthesis leads to embryonic death.

It is generally accepted that activation of the α-AR stimulates phospholipase C (PLC), leading to increased hydrolysis of 4,5-bisphosphate to inositol-1,4,5-triphosphate and 1,2-diacyl glycerol (DAG). Both of these products play important roles as second messengers that increase the intracellular calcium concentration and activate various protein kinase C (PKC) isoforms. In addition, α1-AR stimulation couples the receptor to $G\alpha_s$ protein. This coupling to $G\alpha_s$ is functional and activates the cAMP/PKA signaling cascade, which leads to phosphorylation and I_{to} (transient outward K^+ current) reduction. This appears to be the first example, in a physiological preparation, in which α1-AR is linked to the stimulation of cAMP production and subsequent activation of a PKA-dependent response [Buxton and Brunton, 1986; García-Sáinz et al., 1999; Gallego et al., 2005]. Once activated, the β-ARs rapidly stimulate adenylate cyclase, resulting in an increase in the second messenger, cAMP, which in turn activates PKA [Crespo et al., 1995; Schmitt and Stork, 2000]. Aside from activating "classical" signaling pathways, ARs can also activate mitogen-activated protein kinases (MAPKs) and influence fundamental cellular processes [Communal et al., 2000; Kim et al., 2002; Shizukuda and Buttrick, 2002]. A detailed examination of the molecular mechanisms of MAPKs activation showed that the G-protein coupled receptors (GPCRs) transactivate the growth factor receptors, including epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), and platelet-derived growth factor (PDGF) receptors, and that these tyrosine kinase receptors might serve as a scaffolding structure or as an adaptor to which other signaling proteins can be recruited [Fischer et al., 2003; Shah and Catt, 2003]. These activated receptor tyrosine kinases phosphorylate several cellular signaling proteins and form receptor complexes that consist of Shc, Grb 2, and Sos, which in turn trigger activation of the MAPK cascade. Furthermore, PI3K, which in turn activates the downstream signaling kinase Akt and is essential for cell survival [Brazil et al., 2004; Shah and Catt, 2004; Wetzker and Rommel, 2004], has been shown to participate in MAPK activation through G_i/G_o-coupled receptors [Shah et al., 2006a; Camina et al., 2007]. However, there are very few reports involving epinephrine and the molecular events involved in the initiation of DNA synthesis in mouse embryonic stem cells (ESCs).

ESCs are pluripotent cell lines that are derived from the blastocyst stage of early mammalian embryos [Cedar et al., 2006]. These unique cells are characterized by their capacity for prolonged undifferentiated proliferation in tissue culture, during which time they maintain the potential to differentiate into derivatives of all three germ layers [Chambers, 2004]. Therefore, mouse ESCs are a versatile biological system that has ushered in major advances in cellular and developmental biology. Thus, mouse ESCs are a powerful tool with which to examine the effect of epinephrine on the proliferation of the inner cell mass in early embryos [Heo and Han, 2006; Heo et al., 2006; Heo et al., 2007]. In this study, we investigated the proliferation of mouse ESCs using [^{3}H]-thymidine incorporation in order to determine the role of epinephrine and the related signaling cascades.

MATERIALS AND METHODS

Materials

Mouse ESCs were obtained from the American Type Culture Collection (ES-E14TG2a). Fetal bovine serum (FBS) was purchased from BioWhittaker (Walkersville, MD, USA). Epinephrine, phenoxybenzamine, propranolol, phenylephrine, dobutamine, SQ22536, forskolin, PKA inhibitor, PD98059, bisindolylmaleimide I, staurosporine, MMP inhibitor, Akt inhibitor, LY294002, fluorescence isothiocyanate-conjugated (FITC-conjugated) goat-anti mouse IgM, and β-actin were acquired from Sigma Chemical Company (St. Louis, MO, USA). [^{3}H]-Thymidine and the [^{3}H]-cyclic AMP assay kit (code TRK 432) were purchased

from Amersham International (England, UK). Fluo 3-AM was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Anti-PKC, -cyclin D1, -cyclin E, -cyclin-dependent kinase 2 (CDK 2), -cyclin-dependent kinase 4 (CDK 4), and -phospho-epidermal growth factor receptor (EGFR) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-ERK1/2 and phospho-Akt (Thr^{308}) antibodies were purchased from New England Biolabs (Herts, UK). Goat anti-rabbit IgG was acquired from Jackson Immunoresearch (West Grove, PA, USA). Liquiscint was purchased from National Diagnostics (Parsippany, NY, USA). All other reagents were of the highest purity commercially available.

ESC Culture

The mouse ESCs were cultured in DMEM (Gibco-BRL, Gaithersburg, MD) supplemented with 3.7 g/L sodium bicarbonate, 1% penicillin and streptomycin, 1.7 mM L-glutamine, 0.1 mM β-mercaptoethanol, 5 ng/ml mouse leukemia inhibitory factor, and 15% FBS, with or without a feeder layer, and cultured for 5 days in standard medium plus LIF. The cells were grown on gelatinized 12-well plates or 60-mm culture dishes in an incubator maintained at 37°C in an atmosphere containing 5% CO_2 and air. The medium was changed to serum-free DMEM with LIF before the experiments.

Alkaline Phosphatase Staining

Mouse ESCs were washed twice with phosphate-buffered saline (PBS) and fixed for approximately 15 min with 4% formaldehyde (in PBS) at room temperature. After washing with PBS, 2 ml alkaline phosphatase substrate solution (200 mg/ml naphthol AS-MX phosphate, 2% N,N-dimethylformamide, 0.1 mol/L Tris, and 1 mg/ml Fast Red TR salt) was added and incubated for approximately 10–15 min at room temperature; stained cells were washed with PBS and photographed.

[^{3}H]-Thymidine Incorporation

The experiments to examine the level of [^{3}H]-thymidine incorporation were carried out as described by Brett et al. [1993]. Most ESCs can be arrested in the G0/G1 phase through serum deprivation [Zhang et al., 2005]. Furthermore, synchronized ESCs can successfully re-enter the normal cell cycle after being resupplied with serum. In this study, the cells were cultured in a single well until they reached 50% confluence. They were then washed twice with PBS and maintained in serum-free DMEM containing all supplements. After 24 h incubation, the cells were washed twice with PBS and then incubated with fresh serum-free DMEM containing all supplements and the indicated agents. After the indicated incubation period, 1 μCi of [methyl-^{3}H]-thymidine (specific activity: 74 GBq/mmol, 2.0 Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) was added to the cultures. Incubation with [^{3}H]-thymidine continued for 1 h at 37°C. The cells were washed twice with PBS, fixed in 10% trichloroacetic acid (TCA) at 23°C for 15 min, and then washed twice with 5% TCA. The acid-insoluble material was dissolved in 0.2 N NaOH for 12 h at 23°C. Aliquots were then removed and the radioactivity was determined using a liquid scintillation counter (LS 6500, Beckman Instruments, Fullerton, CA). All values are reported as the mean ± the standard error (SE) of triplicate experiments. The values were converted from absolute counts to a percentage of the control in order to compare experiments.

RNA Isolation, RT-PCR and Real-Time RT-PCR

Total RNA was extracted from mouse ESCs using STAT-60, which is a monophasic solution of phenol and guanidine isothiocyanate that was purchased from Tel-Test, Inc. (Friendwood, TX, USA). Reverse transcription was performed on 3 μg of RNA using a reverse transcription system (AccuPower® RT PreMix, Korea) with oligo-dT18 primers. Subsequently, 5 μl of the RT products were amplified using a PCR kit (AccuPower® PCR PreMix) (Table I): denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 45 s, 55°C for 30 s, and 72°C for 30 s and then 5 min of extension at 72°C. PCR for β-actin was also performed as a control for RNA quantity. Real-time RT-PCR of the RNA targets was performed and quantified using the QuantiTect SYBR Green RT-PCR kit (QIAGEN, CA, USA) in a Rotor-Gene 2000 real-time thermal cycling system (Corbett Research, NSW, Australia). The reaction mixture (20 μl) contained 200 ng of total RNA, 0.5 μM of each primer, an appropriate amount of enzymes, and the fluorescent dyes. The Rotor-Gene 2000 cycler was programmed as follows: 30 min at 55°C for reverse transcription; 15 min at 95°C for DNA polymerase activation; 15 s at 95°C for denaturing; and 45 cycles of 15 s at 94°C, 20 s at 55°C, and 20 s at

TABLE I. PCR Primers Used in This Study

Primer	sequence sense/antisense (5′–3′)
Oct-4	CGTGAGACTTTGCAGCCTGA
	GGCGATGTAAGTGATCTGCTG
FOXD3	TCTTACATCGCGCTCATCAC
	TCTTGACGAAGCAGTCGTTG
SOX2	GTGGAAACTTTTGTCCGAGAC
	TGGAGTGGGAGGAAGAGGT-AAC
AR$\alpha 1_A$	GGACAAGTCAGACTCAGAGCAAGTGACG
	TATAGCCCAGGGCATGCTTGGAAGAC
AR$\alpha 2_A$	TGTTCGTGGTGTGTTGGTTT
	CGGAAGTCGTGGTTGAAGAT
ARβ1	CCGCTGCTACCACGACCCCAAG
	AGCCAGTTGAAGAAGAGCAAGAGGCG
ARβ2	GGTTATCGTCCTGGCCATCGTGTTTG
	TGGTTCGTGAAGAAGTCACAGCAAGTCTC
ARβ3	ACAGGAATGCCACTCCAATC
	TTAGCCACAACGAACACTCG
c-fos	CGTTGCAGACTGAGATTGCC
	ACCGGACAGGTCCACATCTG
c-jun	AACTCGGACCTTCTCACGTCG
	TGCTGAGGTTGGCGTAGACC
c-myc	TCCATTCCGAGGCCACAGCAAG
	TCAGCTCGTTCCTCCTCTGACG

Primer pairs were used to detect and confirm the expression of undifferentiation markers, ARs, and proto-oncogenes in mouse ESCs.

72°C. The data was collected during the extension step (30 s at 72°C). The PCR reaction was followed by melting curve analysis to confirm the specificity and identity of the RT-PCR products; this analysis can distinguish between specific PCR products and non-specific PCR products resulting from primer–dimer formation. The temperature of the PCR products was increased from 65 to 99°C at a rate of 1°C/5 s, and the resulting data was analyzed using software provided by the manufacturer.

cAMP Assay

The cells were incubated with 10^{-6} M epinephrine for 1 h at 37°C in a humidified 5% CO_2–95% air environment. To determine the intracellular cAMP levels, cell samples were prepared by homogenization in DMEM containing 4 mM EDTA using a Polytron PT 1200 (Brinkman Instruments, Westbury, NY), followed by incubation for 5 min at 100°C. After centrifugation at 900*g* for 5 min, the supernatants were transferred into new tubes and stored at 4°C. The samples were then tested using a [^{3}H]-cAMP assay kit. The values were converted from absolute counts to a percentage of the control in order to allow comparison among experimental groups.

Measurement of $[Ca^{2+}]_i$

The changes in $[Ca^{2+}]_i$ were monitored using Fluo-3/AM, which was dissolved in dimethylsulfoxide and stored at −20°C. Mouse ESCs in 35-mm culture dishes were rinsed twice with Bath Solution (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM glucose, 5.5 mM HEPES, pH 7.4), incubated in Bath Solution containing 3 μM fluo-3/AM in an atmosphere of 5% CO_2–95% O_2 at 37°C for 30 min, rinsed twice with Bath Solution, mounted on a perfusion chamber, and scanned every second by confocal microscopy (×400) (Fluoview 300, Olympus, Tokyo, Japan). The fluorescence emission was read at 515 nm after excitation at 488 nm. All analyses of $[Ca^{2+}]_i$ were processed at the single cell level and are expressed as the relative fluorescence intensity (RFI).

Immunofluorescent Staining of PKC

Cells were fixed, incubated with monoclonal antibody against rabbit PKC (1:50, Santa Cruz Biotechnology, Inc.) for 1 h, and then incubated for 30 min with FITC-conjugated goat anti-rabbit IgM (1:100) secondary antibody. The fluorescence images were visualized using fluorescence microscopy (Fluoview 300, Olympus).

Preparation of Cytosolic and Total Membrane Fractions

The cytosolic and total membrane fractions were prepared by slight modification of the method reported by Mackman et al. [1991]. The medium was removed and replaced with serum-free DMEM, including all supplements and LIF, for 12 h prior to the experiments. After removing the medium, the cells were washed twice with ice-cold PBS, scraped, harvested by microcentrifugation, and resuspended in buffer A (137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, 2.5 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.5 mM sodium orthovanadate, pH 7.5). The resuspended cells were then mechanically lysed on ice by trituration with a 21.1-gauge needle. The cell lysates were initially centrifuged at 1,000*g* for 10 min at 4°C. The supernatants were collected and centrifuged at 100,000*g* for 1 h at 4°C to prepare the cytosolic and total particulate fractions. The supernatant (cytosolic fraction) was precipitated with 5 volumes of acetone, incubated on ice for 5 min, and centrifuged at 20,000*g* for 20 min at 4°C. The resulting pellet was resuspended in buffer A containing 1% (vol/vol) Triton X-100. The particulate fractions, which contain the membrane fraction, were washed

twice and resuspended in buffer A containing 1% (vol/vol) Triton X-100. Protein in each fraction was quantified using the Bradford method [1976].

Western Blot Analysis

The cells were harvested, washed twice with PBS, and lysed with buffer [20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride, 0.5 mM sodium orthovanadate] for 30 min on ice. The lysates were then cleared by centrifugation (10 min at 15,000 r.p.m., 4°C), and protein concentration was determined by the Bradford method [1976]. Equal amounts of protein (40 μg) were resolved by electrophoresis on 10% SDS–PAGE gels and transferred to nitrocellulose membranes. After washing with TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20], the blots were blocked with 5% skimmed milk for 1 h and then incubated with the appropriate primary antibody at the dilution recommended by the supplier. The membranes were then washed, and the primary antibodies were detected with goat anti-rabbit IgG or goat anti-mouse IgG conjugated to horseradish peroxidase. The bands were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, England, UK).

Statistical Analysis

The results are expressed as the mean ± SE. All experiments were analyzed by ANOVA, and some experiments were compared with the control using a Bonferroni–Dunn test. A P-value <0.05 was considered significant.

RESULTS

Effect of Epinephrine on Cell Proliferation

The undifferentiated state of mouse ESCs used in this experiment was confirmed by examining the expression of undifferentiated stem cell markers, including alkaline phosphatase activity and Oct-4, FOXD 3, and SOX-2 expression levels. Light microscopic evaluation after alkaline phosphatase staining revealed that cultured mouse ESCs with or without epinephrine were stained violet (Fig. 1A). RT-PCR for Oct-4, FOXD 3, and SOX-2 was conducted to assess the undifferentiated state of mouse ESCs (Fig. 1B). The results of these experiments suggested that mouse ESCs maintained an undifferentiated state under the experimental conditions used in this study.

The effect of epinephrine on [^{3}H]-thymidine incorporation was examined by treating mouse ESCs with a fixed dose of epinephrine (10^{-6} M) for various times (0–24 h) or with various doses of epinephrine (0–10^{-4} M) for 8 h. As shown in Figure 2A, increase of [^{3}H]-thymidine incorporation was first observed at 4 h. This effect peaked at 8 h and then gradually decreased over an 8 h period. The level of [^{3}H]-thymidine incorporation was also increased at 10^{-7} and 10^{-6} M epinephrine, with the maximal stimulatory effect observed at 10^{-6} M (Fig. 2B). Finally, a significant increase in the number of cells was observed after incubation with epinephrine (10^{-6} M) for 8 h (Fig. 2C). To determine whether ARs mediated the effect of epinephrine on cell proliferation, mouse ESCs were pretreated with AR antagonists. As shown

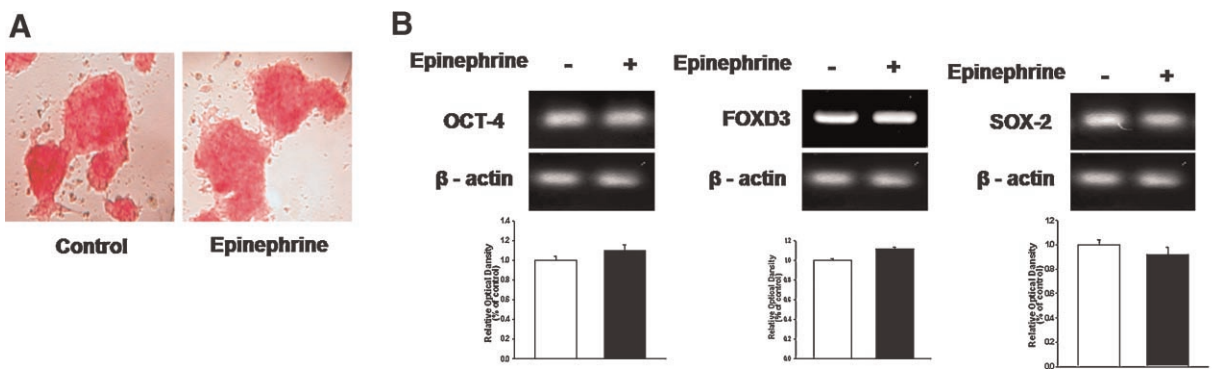

Fig. 1. Effect of epinephrine on the characteristics of mouse ESCs. **A**: Alkaline phosphatase enzyme activity was measured in cells in the presence or absence of epinephrine (10^{-6} M) for 8 h, as described in the Section "Materials and Methods." **B**: Oct-4 (519 bp), FOXD3 (171 bp), SOX-2 (550 bp), and β-actin (350 bp) mRNA expression levels in the presence or absence of epinephrine for 8 h. Each example shown is a representative of four independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

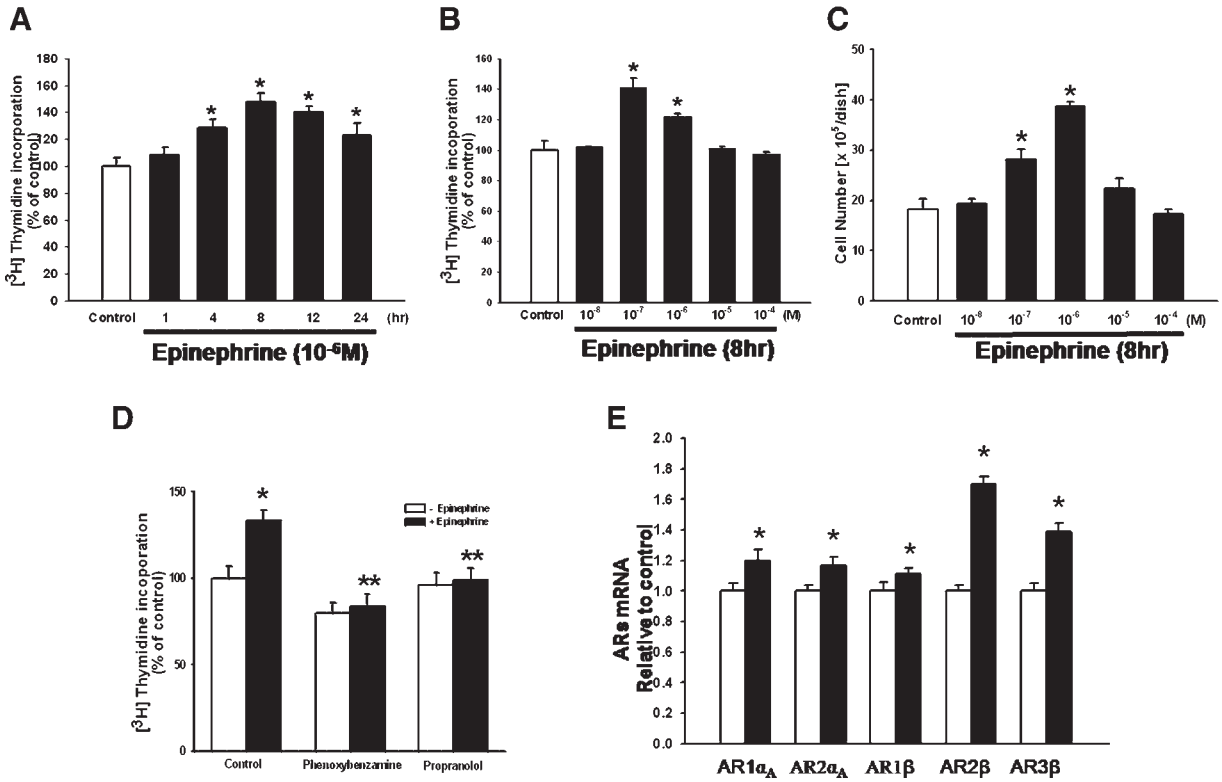

Fig. 2. Effects of epinephrine on [^{3}H]-thymidine incorporation and expression of ARs in mouse ESCs. **A**: Mouse ESCs were incubated in the presence of epinephrine (10^{-6} M) for various times (0–24 h) under serum-free conditions and subsequently pulsed with 1 μCi of [^{3}H]-thymidine for 1 h before counting. **B**: Mouse ESCs were incubated with various concentrations of epinephrine (0–10^{-4} M) for 8 h and pulsed with 1 μCi of [^{3}H]-thymidine for 1 h. **C**: Mouse ESCs were treated with epinephrine (0–10^{-4} M) for 8 h, and the number of cells was counted using a hemocytometer. **D**: Mouse ESCs were pretreated with phenoxybenzamine (10^{-6} M) and propranolol (10^{-6} M) for 30 min, incubated with epinephrine (10^{-6} M) for 8 h and pulsed with 1 μCi of [^{3}H]-thymidine for 1 h. Values represent the mean ± SE of four independent experiments with triplicate dishes. *$P < 0.05$ versus control; **$P < 0.05$ versus epinephrine alone. **E**: The α1A-, α2A-, β1-, β2-, and β3-AR mRNA expression levels in the absence or presence of epinephrine (10^{-6} M) for 30 min by real-time RT-PCR. The example shown is representative of three independent experiments. Abbreviations: AR, adrenergic receptor.

in Figure 2D, phenoxybenzamine (non-selective α-AR antagonist) and propranolol (non-selective β-AR antagonist, 10^{-6} M) inhibited the epinephrine-induced increase in [^{3}H]-thymidine incorporation. Gene expression of AR subtypes in mouse ESCs was examined by real-time RT-PCR. As shown in Figure 2E, $\alpha 1_A$, $\alpha 2_A$, β1, β2, and β3 ARs were expressed in mouse ESCs, and their expression levels were increased by epinephrine. Among them, β2-AR was significantly increased by epinephrine.

Involvement of cAMP, $[Ca^{2+}]_i$, PKC, EGFR, and Akt Activation in Epinephrine-Induced DNA Synthesis

The effects of epinephrine on cAMP, $[Ca^{2+}]_i$, PKC, and EGFR activation were examined to determine which of these were downstream of the ARs. As shown in Figure 3A, epinephrine increased the cAMP level, and this increase was inhibited by phenoxybenzamine and propranolol. Forskolin (cAMP activator, 10^{-6} M) was used as positive control. Epinephrine-induced increase in [^{3}H]-thymidine incorporation was also inhibited by SQ22536 (adenylate cyclase inhibitor) and PKA inhibitor (10^{-6} M) (Fig. 3B).

Next, the effect of epinephrine on $[Ca^{2+}]_i$ was examined. As shown in Figure 4A, epinephrine increased $[Ca^{2+}]_i$, and this effect was partially inhibited by phenoxybenzamine and propranolol (Fig. 4B and C). However, the epinephrine-induced increase in $[Ca^{2+}]_i$ was completely blocked by pretreatment with phenoxybenzamine plus propranolol (Fig. 4D). Phenylephrine (α1-AR agonist) and dobutamine (β1-AR agonist) (10^{-6} M) also partially induced increase in $[Ca^{2+}]_i$ (Fig. 4E and F). As shown in Figure 5A, immunofluorescent staining was used to examine the effect of epinephrine on the translocation of PKC. PKC was translocated by

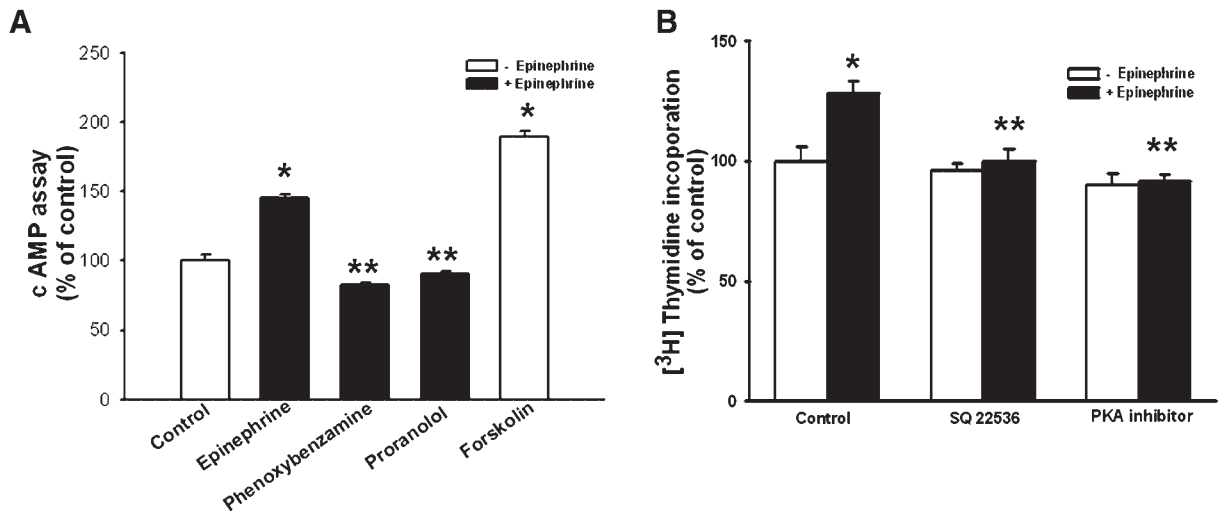

Fig. 3. Effect of epinephrine on cAMP levels. **A**: Mouse ESCs were pretreated with phenoxybenzamine and propranolol (10^{-6} M) for 30 min and then incubated with epinephrine (10^{-6} M) for 1 h before the cAMP assay. **B**: Mouse ESCs were pretreated with SQ22536 or PKA inhibitor (10^{-6} M) for 30 min, then incubated with epinephrine (10^{-6} M) for 8 h, and pulsed with 1 μCi of [^{3}H]-thymidine for 1 h. The values represent the mean ± SE of four independent experiments with triplicate dishes. *$P < 0.05$ versus control; **$P < 0.05$ versus epinephrine alone.

epinephrine, but epinephrine did not affect PKC translocation after pretreatment with phenoxybenzamine and propranolol for 30 min. These results were consistent with the results of Western blot analysis (Fig. 5B). Consequently, epinephrine-induced increase of [^{3}H]-thymidine incorporation was inhibited by bisindolylmaleimide I and staurosporine (PKC inhibitor, 10^{-7}M) (Fig. 5C).

Epinephrine-induced increases of MMP2 and MMP9 were blocked by phenoxybenzamine or propranolol (10^{-6} M) (Fig. 6A and B). In addition, epinephrine induced the activation of EGFR, which was maximally activated at 30 min after epinephrine treatment (Fig. 6C). Epinephrine-induced activation of EGFR was inhibited by pretreatment with phenoxybenzamine, propranolol (10^{-6} M), or MMP inhibitor (10^{-7} M) (Fig. 6D and E). Epinephrine also induced an increase in Akt phosphorylation that peaked at 30 min; this phosphorylation was inhibited by AG1478 (EFGR inhibitor, 10^{-5} M) (Fig. 6F and G).

Involvement of ERK1/2 Activation and Cell Cycle Regulatory Proteins in Epinephrine-Induced DNA Synthesis

ERK1/2 activation in response to epinephrine was examined to investigate involvement of ERK1/2 in epinephrine-induced DNA synthesis. ERK1/2 phosphorylation peaked at 10 min after epinephrine treatment (Fig. 7A), and pretreatment with phenoxybenzamine and propranolol inhibited the epinephrine-induced ERK1/2 phosphorylation (Fig. 7B). In addition, the epinephrine-induced increase in [^{3}H]-thymidine incorporation was inhibited by PD98059 (ERK1/2 inhibitor, 10^{-5} M) (Fig. 7C). In order to determine whether PKC and Akt were involved in ERK1/2 activation, Western blot analysis was carried out. Bisindolylmaleimide I (10^{-7} M) and Akt inhibitor (10^{-6} M) inhibited epinephrine-induced phosphorylation of ERK1/2 (Fig. 7D and E). However, SQ22536 (10^{-6} M) did not block ERK1/2 phosphorylation (Fig. 7F).

In experiments to examine the effect of epinephrine on cell cycle regulatory proteins, epinephrine increased the levels of cyclin E, CDK 2, cyclin D1, and CDK 4 expression. These increases were blocked by pretreatment with phenoxybenzamine, propranolol, SQ22536, bisindolylmaleimide I, PD98059, LY294002 (PI3K inhibitor, 10^{-6} M), and Akt inhibitor (Fig. 8A–C). Moreover, epinephrine increased the mRNA expression of the proto-oncogenes, c-fos, c-jun, and c-myc, and this expression was blocked by PD 98059 (Fig. 8D–F).

DISCUSSION

The cellular responses to epinephrine alter the proliferation status through receptor-mediated

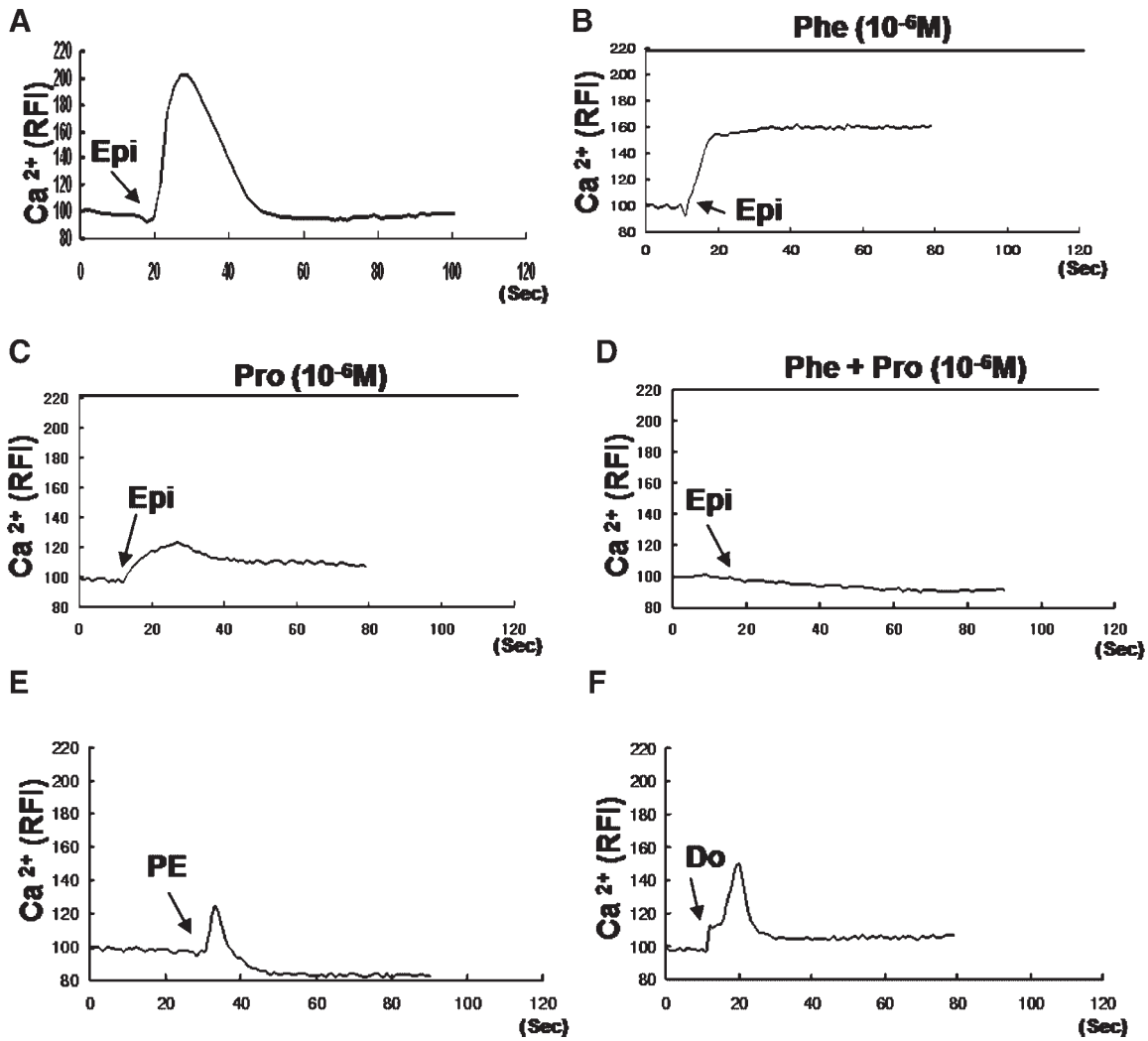

Fig. 4. Effect of epinephrine on $[Ca^{2+}]_i$. **A**: Mouse ESCs were loaded with 2 μM fluo-3-AM in serum-free medium for 30 min and treated with epinephrine (10^{-6} M). Mouse ESCs were pretreated with phenoxybenzamine (**B**), propranolol (**C**) or a combination of phenoxybenzamine and propranolol (**D**), phenylephrine (**E**), and dobutamine (10^{-6} M) (**F**) for 30 min, respectively. The epinephrine-induced Ca^{2+} influx was then measured using confocal microscopy. Each example shown is a representative of five independent experiments. Abbreviations: Epi, epinephrine; Phe, phenoxybenzamine; Pro, propranolol; PE, phenylephrine; Do, dobutamine; sec, seconds; RFI, relative fluorescence intensity.

intracellular signaling cascades. Epinephrine-induced stimulation of ESCs proliferation is thought to occur mainly through α- and β-ARs. This study directly demonstrated, for the first time, that mouse ESCs express some α- and β-AR subtypes. Moreover, epinephrine stimulates the key intracellular signaling pathways in these cells and induces cell proliferation. However, little is known about the role of epinephrine in mouse ESCs growth. A search of public databases containing gene sequences from mouse preimplantation embryos revealed that the β2-AR may be expressed at the blastocyst stage [Cikos et al., 2005]. In the present study, the expression of α1A-, α2A-, β1-, β2-, and β3-AR in mouse ESCs was confirmed. Also, the expression levels were increased after epinephrine treatment. Therefore, we surmised that α1A-, α2A-, β1-, β2-, and β3-AR play an important role in mouse ESCs. This study demonstrated that α- and β-ARs are involved in the proliferation of mouse ESCs. Indeed, 8 h treatment of mouse ESCs with epinephrine stimulates DNA synthesis, and this stimulation was blocked by the non-selective α- and β-AR antagonists, phenoxybenzamine, and propranolol, respectively. As expected, the increase in DNA synthesis was associated with an increase

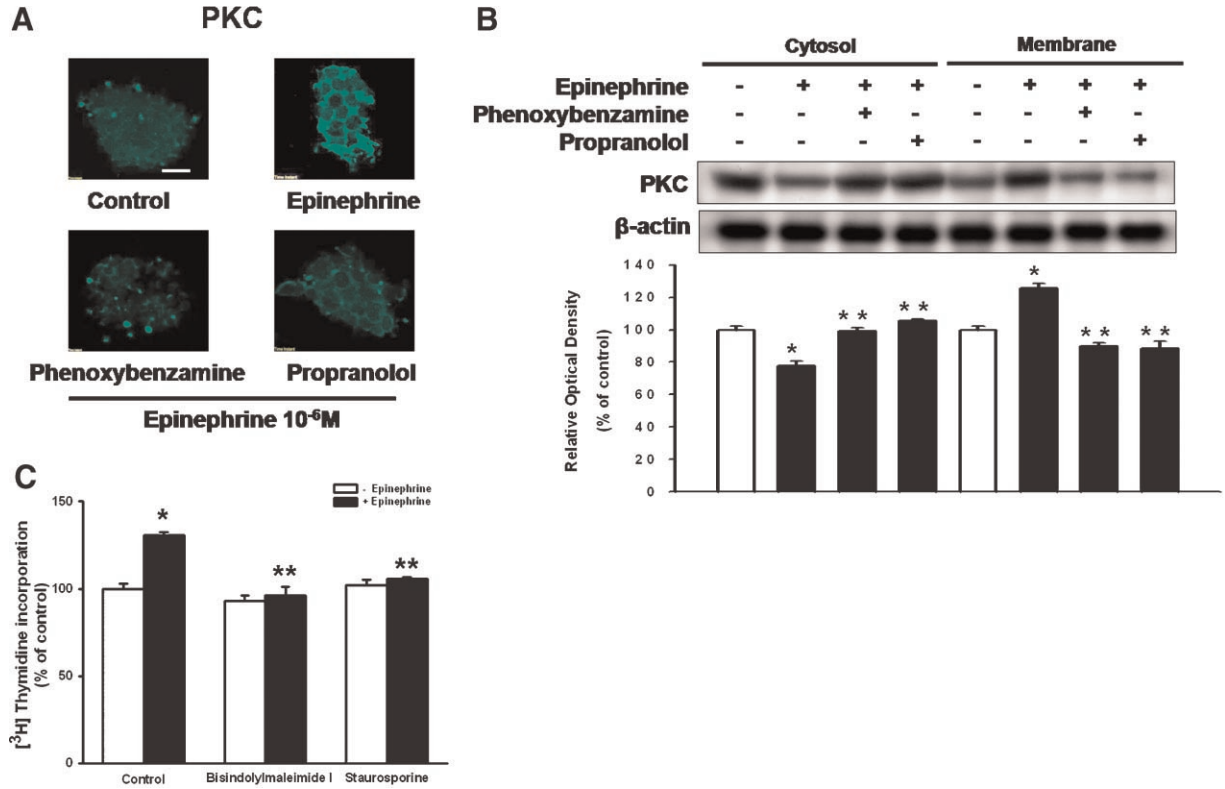

Fig. 5. Effect of epinephrine on PKC activation. **A**: Mouse ESCs were pretreated with phenoxybenzamine and propranolol (10^{-6} M) for 30 min and then incubated with epinephrine (10^{-6} M) for 30 min. PKC translocation was then detected by immunofluorescent staining. **B**: Mouse ESCs were pretreated with phenoxybenzamine or propranolol (10^{-6} M) for 30 min and then incubated with epinephrine (10^{-6} M) for 30 min. The translocation of PKC was detected by Western blotting. Each example shown is representative of four experiments. The **lower panels** of B denote the mean ± SE of four experiments for each condition as determined from densitometry relative to β-actin. $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus epinephrine alone. **C**: Mouse ESCs were pretreated with bisindolylmaleimide I or staurosporine (10^{-7} M) for 30 min, then incubated with epinephrine (10^{-6} M) for 8 h and pulsed with 1 μCi of [^{3}H] thymidine for 1 h. The values represent the mean ± SE of four independent experiments with triplicate dishes. $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus epinephrine alone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in the level of [^{3}H]-thymidine incorporation into mouse ESCs. This suggests that activation of α- and β-ARs by an appropriate concentration of epinephrine can stimulate the proliferation of mouse ESCs. In this study, a higher number of mouse ESCs was observed after exposure to 1 μM and 100 nM epinephrine than of control cells after 8 h. Since mitogenic effects were observed in the nanomolar range in vitro, which corresponds to the circulating levels in oviductal fluid, these results might indeed be physiologically relevant. In rabbit oviductal fluid, the concentration of epinephrine ranged from 0.56 to 3.6 μM; the concentrations of norepinephrine in bovine and rabbit oviductal fluid ranged from 4.9 nM to 6.6 μM and from 0.34 to 27.3 μM, respectively [Khatchadourian et al., 1987; Way et al., 2001].

In ESCs, AR activation stimulates the activation of G_s, adenylyl cyclase, which produces the classical second messenger cAMP, and PKA [Molenaar et al., 2007]. In this study, the epinephrine-induced increase in the level of [^{3}H]-thymidine incorporation was associated with a concomitant increase in intracellular cAMP, as cAMP elevating agents increased the basal level of [^{3}H]-thymidine incorporation. Therefore, the concomitant increase in cAMP can promote the epinephrine-induced stimulation of DNA synthesis, suggesting the recruitment of cAMP-sensitive growth-promoting signaling pathways. In a previous study, it was observed that $G\alpha_q$ or $G\alpha_{11}$ can mediate the activation of phospholipase C by any of the three α1 AR subtypes. In the brain and heart, PKA increases the activity of the L-type Ca^{2+} channel in response to β-AR stimulation [Maltsev et al., 1999; Hall et al., 2007]. The L-type Ca^{2+} channel forms a complex with the β-AR, the trimeric G_s protein, adenylyl cyclase, and

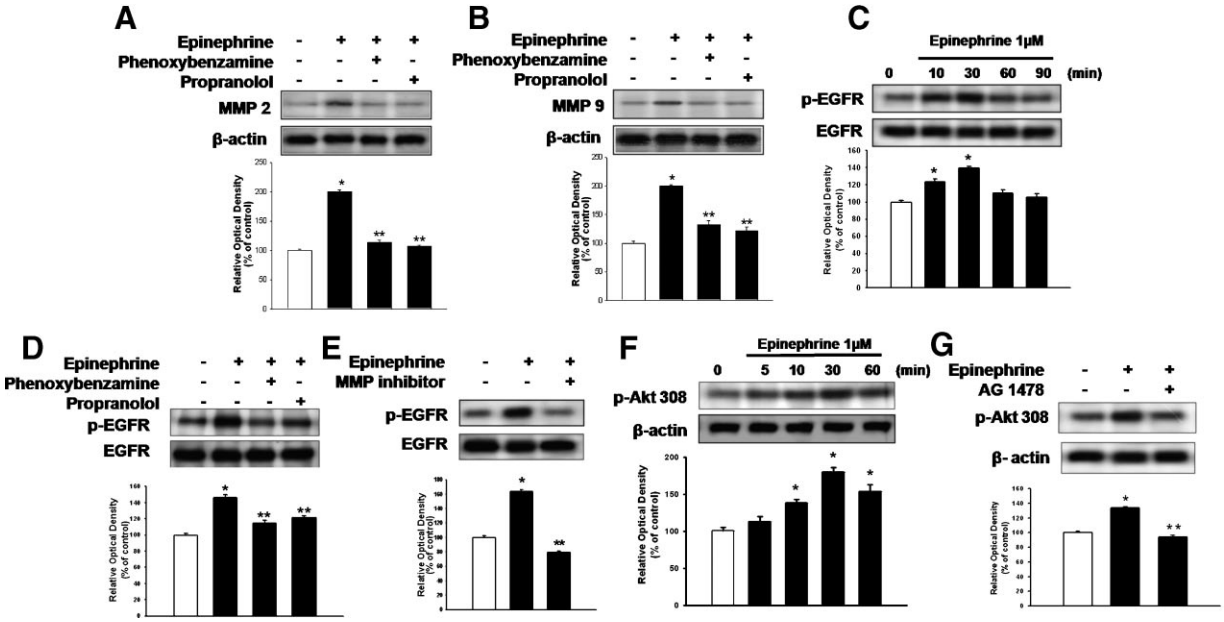

Fig. 6. Effect of epinephrine on MMP, EGFR, and Akt phosphorylation. Mouse ESCs were pretreated with phenoxybenzamine and propranolol (10^{-6} M) for 30 min and then incubated with epinephrine (10^{-6} M) for 8 h. MMP 2 (**A**) and MMP 9 (**B**) were detected. Mouse ESCs were treated with epinephrine (10^{-6} M) for various times (0–90 min), and the phosphorylation of EGFR (**C**) was detected. Mouse ESCs were pretreated with phenoxybenzamine, propranolol (10^{-6} M) (**D**), and MMP inhibitor (10^{-7} M) (**E**) for 30 min and then incubated with epinephrine (10^{-6} M) for 30 min. The phosphorylation of EGFR was detected by Western blotting. **F**: Mouse ESCs were treated with epinephrine (10^{-6} M) and incubated for various times (0–60 min); the level of Akt phosphorylation was then determined. **G**: Mouse ESCs were pretreated with AG 1478 (10^{-5} M) for 30 min and then incubated with epinephrine (10^{-6} M) for 30 min; the phosphorylation of Akt was then detected. The example shown is representative of four experiments. The **lower panels** of A, B, C, D, E, F, and G denote the mean ± SE of four experiments for each condition as determined from densitometry relative to total EGFR or β-actin. $*P < 0.05$ versus control; $**P < 0.05$ versus epinephrine alone. Abbreviations: EGFR, epidermal growth factor receptor.

PKA wherein highly localized signaling occurs [van der Heyden et al., 2005]. It is possible that a sustained increase in $[Ca^{2+}]_i$ or stimulation of PKC leads to the subsequent activation of mouse ESCs proliferation. Indeed, both conventional and unconventional PKC play a major role in AR-activated mouse ESCs proliferation.

In this study, epinephrine-induced phosphorylation of EGFR was totally abrogated by prior treatment of the cells with phenoxybenzamine and propranolol, indicative of a ligand-dependent mechanism for EGFR transactivation that involves the activity of matrix metalloproteinases. A previous study on AR phosphorylation induced by lysophosphatidic acid clearly also showed that the shedding of heparin-binding-EGF by metalloproteinases, the transactivation of EGFR and the activity of PI3K and PKC were involved [Wang et al., 1999; Prenzel et al., 2000; Casas-Gonzalez et al., 2003; Shah and Catt, 2004]. Therefore, these results indicate that such a mechanism is widespread. These findings and our present data strongly suggest that EGFR transactivation might be involved in the actions and feedback processes triggered by AR activation [Shah et al., 2006b]. In addition, these results suggest that the receptor phosphorylation induced by epinephrine is complex, likely involving molecular elements in addition to PI3K.

Stimulation of AR elevates cAMP and activates PKA, which in turn stimulates the activation of MAPKs. However, this effect may be cell-specific, e.g., an increase in cAMP may also lead to inhibition of MAPKs in oligodendrocyte progenitors [Palacios et al., 2005]. Moreover, β-ARs can either stimulate or inhibit MAPKs, which supports the role of the receptors in the regulation of fundamental cellular processes such as proliferation, differentiation, growth, migration, survival, and apoptosis [Mayor et al., 1998; Singh et al., 2001; Zhang et al., 2005; Johnson et al., 2006; Pullar and Isseroff, 2006; Shin et al., 2007]. In some of these studies, the ability of MAPKs signaling to either increase or decrease proliferation also appeared to be dependent on the amount of signal generated. In addition, these results suggest

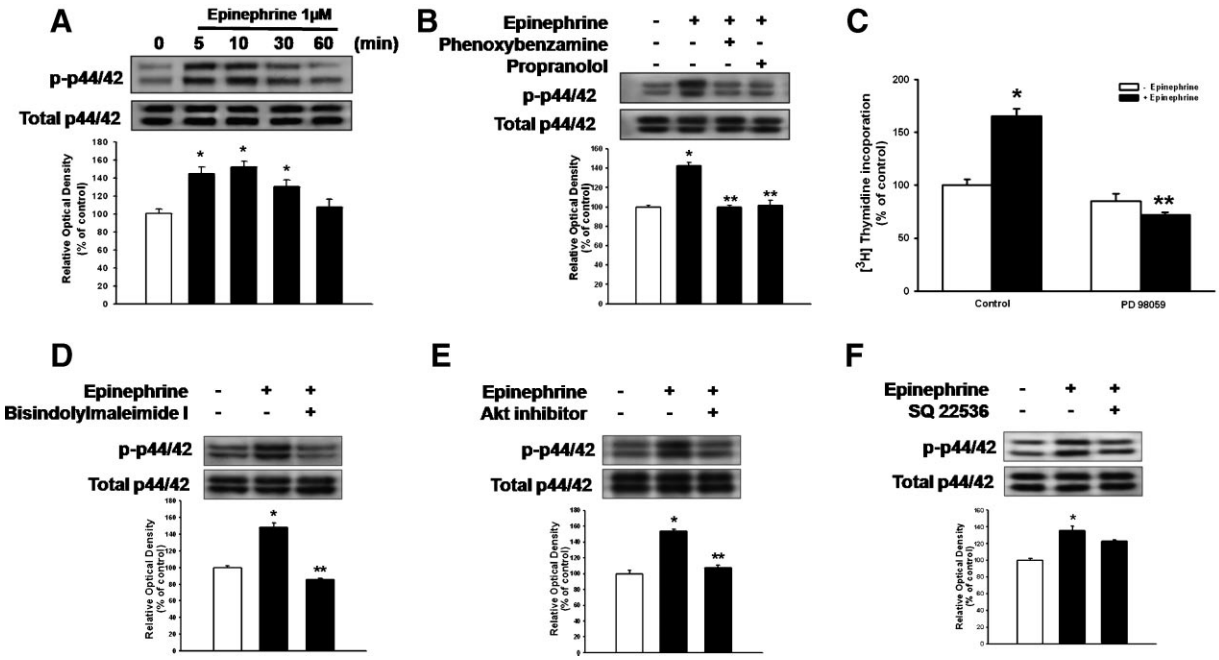

Fig. 7. Effect of epinephrine on ERK1/2 activation. **A**: Mouse ESCs were treated with epinephrine (10^{-6} M) for various times (0–60 min) and p44/42 phosphorylation was detected. Mouse ESCs were pretreated with phenoxybenzamine or propranolol (10^{-6} M) (**B**) for 30 min before incubating the cells with epinephrine (10^{-6} M) for 10 min, and the p44/42 phosphorylation was detected. The **lower panels** of A and B denote the mean ± SE of four experiments for each condition as determined from densitometry relative to Total p44/42. **C**: Mouse ESCs were pretreated with PD98059 for 30 min, incubated with epinephrine (10^{-6} M) for 8 h and pulsed with 1 μCi of [^{3}H]-thymidine for 1 h. The values represent the mean ± SE of four independent experiments with triplicate dishes. Mouse ESCs were pretreated with bisindolylmaleimide I (10^{-7} M) (**D**), Akt inhibitor (10^{-6} M) (**E**), and SQ22536 (10^{-6} M) (**F**) for 30 min and then incubated with epinephrine (10^{-6} M) for 30 min. The phosphorylation of p44/42 was then examined. The **lower panels** of D, E, and F denote the mean ± SE of three experiments for each condition as determined from densitometry relative to total p44/42. *$P < 0.05$ versus control; **$P < 0.05$ versus epinephrine alone.

that PKC is involved in the epinephrine-mediated activation of MAPKs. Therefore, the Western blotting results suggest that epinephrine increases the PKC activity in mouse ESCs. In addition, treatment of the cultures with bisindolylmaleimide I significantly reduced the effect of epinephrine on ERK1/2 activation. Consistent with these results, downregulation of PKC by chronic TPA treatment blocked the activation of ERK1/2 by epinephrine (data not shown). On the other hand, formation of β-arrestin–ERK complexes may provide a mechanism for controlling the substrate specificity of the kinase, thereby determining the consequences of GPCR-stimulated ERK activation. This complex can lead to signals that are important for the regulation of cellular growth or differentiation [Dhanasekaran et al., 1995; McDonald et al., 2000; Yang and Xia, 2006]. Moreover, the EGFR multi-signaling complex allows activation of multiple downstream pathways such as the PI3K-Akt and ERK1/2 pathways. Therefore, epinephrine may activate MAPKs through both AR-dependent signaling and EGFR-induced Akt activation.

The downstream targets of epinephrine-mediated MAPKs activation are just beginning to be explored. The central players in the epinephrine-induced cell cycle control mechanisms are cyclin D1-CDK 4 and cyclin E-CDK 2, which allow the initiation of DNA synthesis. In this study, PD98059 abrogated the epinephrine-induced cyclin D1-CDK 4 and cyclin E-CDK 2 stimulation. In addition, there is evidence suggesting that activation of ERK1/2 by AR stimulation might regulate gene expression. Therefore, we examined the effect of epinephrine on the levels of proto-oncogene mRNA in cultures of mouse ESCs. Epinephrine induced c-fos, c-jun, and c-myc mRNA in a time- and concentration-dependent manner. To test the involvement of ERK1/2, the cultures were treated with PD98059, an ERK1/2 inhibitor, and proto-oncogene induction by epinephrine was determined. Proto-oncogene induction was blocked by PD98059, confirming that MAPKs

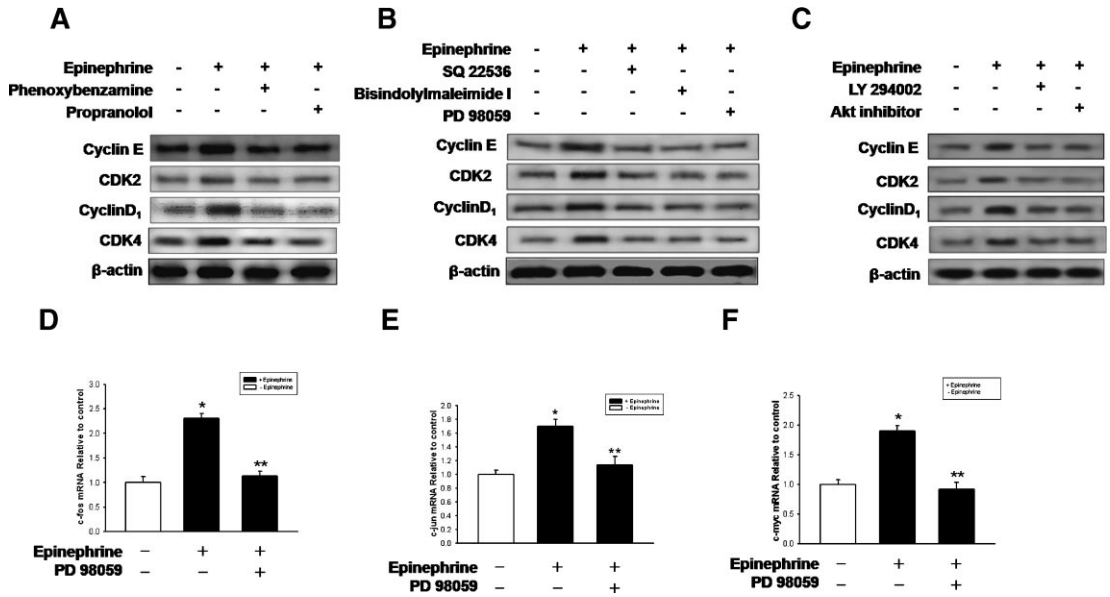

Fig. 8. Effect of epinephrine on cell cycle regulatory proteins and proto-oncogene expression. Mouse ESCs were pretreated with phenoxybenzamine and propranolol (10^{-6} M) (**A**), SQ22536 (10^{-6} M), bisindolylmaleimide I (10^{-7} M), and PD98059 (10^{-5} M) (**B**), LY294002 (10^{-6} M), and Akt inhibitor (10^{-6} M) (**C**) for 30 min and then incubated with epinephrine (10^{-6} M) for 8 h. The total lysates were then subjected to SDS–polyacrylamide gel electrophoresis (PAGE) and blotted with cyclin D1, cyclin E, CDK2, and CDK4 antibodies. The example shown is representative of four experiments. Abbreviations: CDK, cyclin-dependent kinase. The mRNA expression of the proto-oncogenes were determined by real-time RT-PCR. Mouse ESCs were pretreated with PD98059 (10^{-5} M) for 30 min and then treated with epinephrine (10^{-6} M) for 30 min. **D**: c-fos. **E**: c-jun. **F**: c-myc. The example shown is representative of three experiments. $^{*}P < 0.05$ versus control; $^{**}P < 0.05$ versus epinephrine alone.

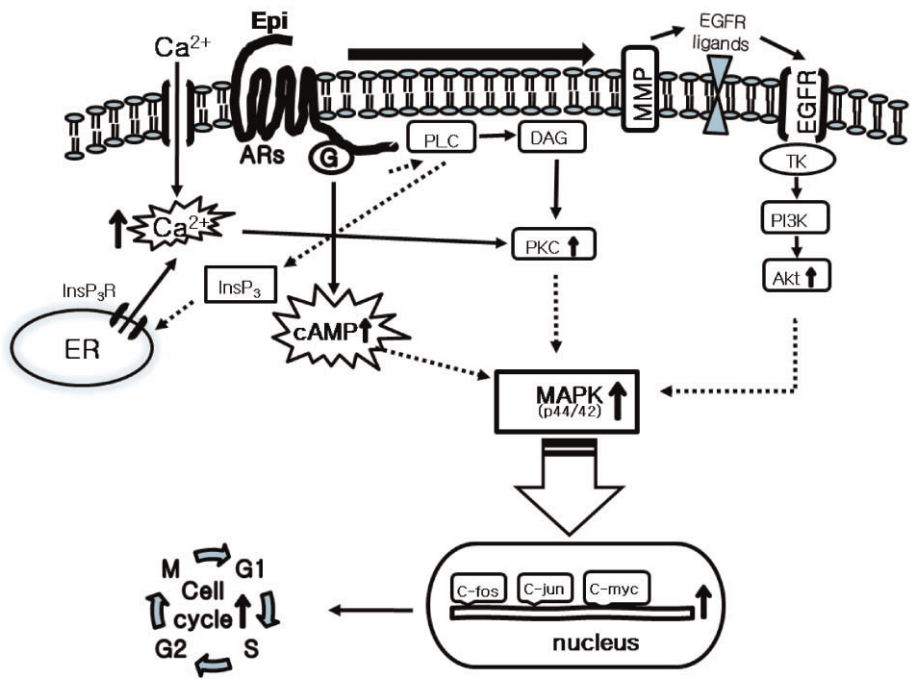

Fig. 9. The hypothesized model for the signaling pathways involved in epinephrine-induced ESC proliferation. Epinephrine activates ARs, which in turn increases cAMP and $[Ca^{2+}]_i$ and activates PKC. These signals also increase ERK1/2. In another pathway, epinephrine activates MMP to stimulate EGFR activation. Continuously, PI3K/Akt pathway is activated. Subsequently, ERK1/2 activation is increased, which increases proto-oncogene (c-fos, c-myc, c-jun) expression levels. Finally, cell cycle regulatory proteins, cyclin E/CDK2, and cyclin D1/CDK4 levels are increased. Abbreviations: AR, adrenergic receptor; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; EGFR, epidermal growth factor receptor; PI3K, phosphatidylinositol 3-kinase. The solid line is the proposed pathway and the dashed line is the suspected pathway.

activation is essential for the induction of proto-oncogenes in mouse ESCs. Therefore, our results indicate that epinephrine signaling pathways can provide a novel paradigm for ESCs proliferation (Fig. 9). Further study will be needed to determine the physiological response of mouse ESCs to AR stimulation and to reveal the signaling pathways involved. In summary, this study demonstrated that epinephrine increases DNA synthesis via ERK1/2 through cAMP, Ca^{2+}/PKC, and PI3K/Akt signaling pathways. This study suggests that AR signaling pathways may play an important role in the control of mouse ESCs growth.

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