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Endothelin-1 increases the levels of mRNA and protein of muscarinic acetylcholine receptors and c-*fos* mRNA in cerebellar granule cells

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Abstract

Endothelin-1 (ET-1) induced a time- and dose-dependent increase in the levels of mRNA of m_2- and m_3-muscarinic acetylcholine receptors (mAChRs) in cultured cerebellar granule cells. The levels of immunoprecipitable m_3-mAChR protein and total mAChR binding sites were also increased by ET-1 treatment. The up-regulation of m_2- and m_3-mAChR mRNA was blocked by phorbol ester pretreatment to inhibit ET-1-stimulated phosphoinositide hydrolysis and was preceded by an increase in c-*fos* mRNA levels. Treatments that prevented ET-1-induced c-*fos* mRNA increase also abolished the subsequent m_2- and m_3-mAChR mRNA up-regulation, suggesting that c-Fos protein is involved in the ET-1-induced mAChR expression.

Key words: Endothelin-1; Muscarinic acetylcholine receptor mRNA; c-*fos* mRNA; Northern blot hybridization; Immunoprecipitation; Cerebellar granule cells

1. Introduction

Endothelins (ET; ET-1, ET-2 and ET-3) are a family of vasoconstrictive isopeptides first found to be synthesized in the endothelium (for a review, see [1]). It is now clear the ET peptides have a wide range of biological actions in both vascular and non-vascular tissues including the CNS [1–3]. ET-1 is expressed in several brain areas and in neurons of spinal cord and dorsal root ganglia [2,3]. In cultured neurons and neurally related cell types, ET-1 acting on specific G protein-coupled receptors induces a variety of intracellular events including phosphoinositide hydrolysis [2,4–6], an increase in intracellular free Ca^{2+} concentration [6–8], the release of neurotransmitters [3,9], the proliferation of glial cells [5,10], and the expression of *fos* and *jun* proto-oncogenes [11,12].

Using cultured cerebellar granule cells as a neuronal model, we have previously shown that ET-1 stimulates phosphoinositide hydrolysis and the release of endogenous glutamate [4,9,13,14]. We have also reported that cerebellar granule cells express in vitro the mRNA for m_2- and m_3-muscarinic acetylcholine receptors (mAChRs) coupled to adenylate cyclase inhibition and phospholipase C activation, respectively [15,16]. In addition, the levels of m_2- and m_3-mAChR mRNA in granule cells are regulated by a number of drugs at both transcriptional and post-transcriptional levels [15–17]. In light of multiple intracellular actions of ET in the nervous system, we examined the influence of ET-1 stimulation on the levels of m_2- and m_3-mAChR mRNA in cerebellar granule cells.

2. Material and methods

2.1. Cell cultures

Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague–Dawley rats, as described previously [16]. Briefly, cerebella were minced and cells dissociated in 0.025% trypsin solution at 37°C for 15 min. The dissociation process was terminated by trituration in Krebs-Ringer bicarbonate buffer containing 0.05% soybean trypsin inhibitor and 0.01% DNase. Cells were then plated at a density of about 10^7 cells per 60-mm dish coated with poly-L-lysine and grown in basal Eagle's medium containing 10% fetal calf serum, 2 mM glutamine, 50 μM gentamicin and 25 mM KCl. Cytosine arabinoside (10 μM) was added 24 h after plating to inhibit the replication of non-neuronal cells. Cerebellar granule cell cultures were treated with indicated concentrations of ET-1 (Peptide Institute Inc., Osaka, Japan) after 8 days in vitro; at that time the cultures consisted of a highly homogenous population of differentiated neurons with a purity of greater than 90%.

2.2. Northern blot hybridization

Total RNA was isolated from the cell lysate by centrifugation through 5.7 M cesium chloride to pellet the RNA. An amount of 8–10 μg of total RNA was electrophoresed in each lane on agarose-formaldehyde gel followed by blotting to nitrocellulose membrane, as described previously [16]. Hybridization was performed using the RNA blot incubated at 42°C overnight with a ^{32}P-labeled m_2-mAChR probe (0.5 kb *Ava*I fragment of human Hm2p9), an m_3-mAChR probe (0.7 kb *Stu*I-*Nhe*I fragment of rat Rm3p8), a murine c-*fos* cDNA probe, or a chicken β-actin probe. High stringency washes were performed at 48°C for 15 min in the case of m_2-mAChR and 56°C for m_3-mAChR, c-*fos* and β-actin. Details of the hybridization conditions, quantifications of the autoradiographs, and sources of various cDNA probes were as described [16].

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Abbreviations: ET-1, endothelin-1; mAChR, muscarinic acetylcholine receptor; PDBu, phorbol dibutyrate.

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2.3. Immunoprecipitation assays

Procedures for immunoprecipitation of [^{3}H]QNB-labeled mAChRs solubilized from membranes were essentially as described by Wall et al. [18] with slight modifications [16]. Briefly, membranes prepared from cerebellar granule cells were labeled with 2 nM [^{3}H]QNB and the labeled mAChRs were solubilized with 1% digitonin and 0.2% cholic acid. The solubilized receptors were incubated with m_2- or m_3-mAChR subtype-specific antisera kindly supplied by Dr. Barry B. Wolfe (Department of Pharmacology, Georgetown University School of Medicine, Washington, DC) for 48 h at 4°C. Bound [^{3}H]QNB was separated from free [^{3}H]QNB by Sephadex G-50 column chromatography, and the labeled receptor-antibody complex was isolated by incubation with Pansorbin.

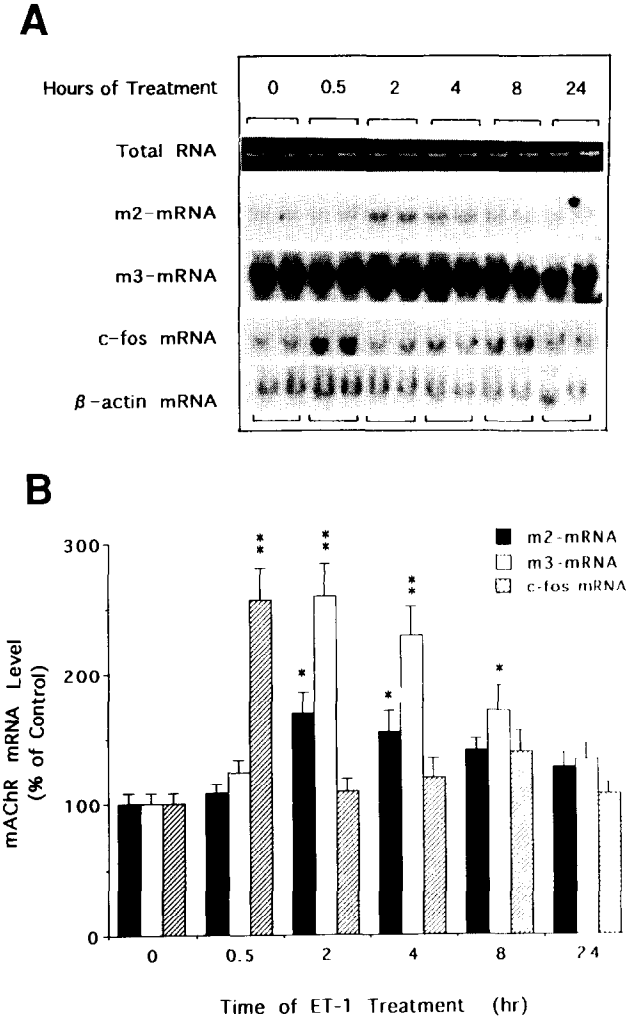


Fig. 1. Time-course of ET-1-induced increase in the levels of m_2-, m_3-mAChR mRNA, and c-*fos* mRNA. Cerebellar granule cells after 8 days in culture were treated with 10 nM ET-1 for the indicated times by adding the peptide sequentially and terminating all reactions simultaneously. (A) Autoradiographs of mRNA hybridization to cDNA of m_2-mAChR, m_3-mAChR, c-*fos*, and β-actin as indicated in each lane. The results were obtained from the same blot using different ^{32}P-labeled cDNA probes. (B) Densitometry of the autoradiographs. Levels of mRNA of m_2-mAChR, m_3-mAChR, and c-*fos* have been normalized to total cellular RNA present in each lane and expressed as values relative to their respective 0 time control. The data represent the means ± ranges of duplicate samples. $^*P < 0.05$; $^{**}P < 0.01$ when compared with the 0 time control (Student's *t*-test). Similar results have been obtained in three independent experiments.

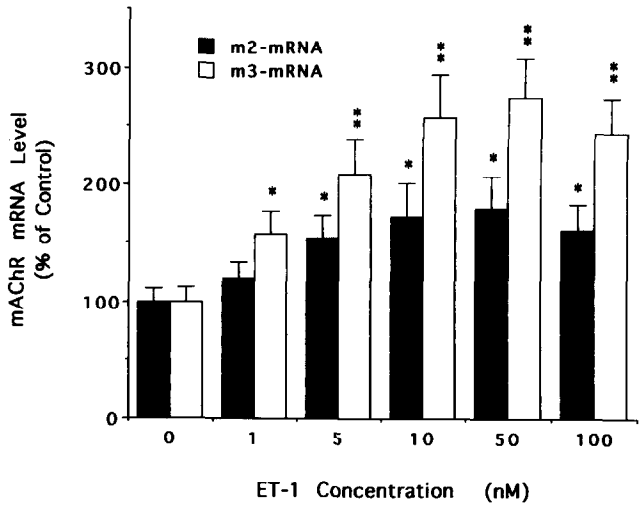


Fig. 2. Dose–effect relationship for ET-1-induced increase of m_2- and m_3-mAChR mRNA levels. Cells were treated with indicated concentrations of ET-1 for 2 h; mRNA levels for m_2-, m_3-mAChR, c-*fos* and β-actin were then determined. The results shown are quantified mRNA levels of m_2- and m_3-AChRs normalized to total cellular RNA and are expressed as the means ± ranges of values relative to their respective controls in duplicate experiments. $^*P < 0.05$; $^{**}P < 0.01$ when compared with the untreated control (Student's *t*-test).

3. Results and discussion

Stimulation of cerebellar granule cells with 10 nM ET-1 produced a dose-dependent increase in levels of m_2- and m_3-mAChR mRNA (Fig. 1). The increase of m_3-mAChR mRNA peaked at 2 h after stimulation, reaching a level approximately 260% of the 0 time control. The increase was still significant at 8 h (about 170% of the control) but returned to basal value at 24 h. The level of m_2-mAChR mRNA was increased in parallel with that of m_3-mAChR mRNA; however, the increase was of smaller magnitude and shorter lasting. We also found that c-*fos* mRNA was transiently increased by about 2.5-fold at 30 min after ET-1 stimulation. The levels of total RNA (which contained mostly ribosomal RNA) and β-actin mRNA remained unchanged throughout the 24-h treatment with ET-1. The ET-1-induced increase in m_2- and m_3-mAChR mRNA levels following a 2-h treatment was concentration-dependent with an EC_{50} value of approximately 5 nM (Fig. 2). At this time-point, levels of total RNA and mRNA of β-actin and c-*fos* were unchanged by ET-1 in the concentration range tested (1–100 nM) (data not shown). To assess whether levels of m_2- and m_3-mAChR protein were changed in conjunction with an increase in m_2- and m_3-mAChR mRNA, immunoprecipitations of m_2- and m_3-mAChR protein were performed using m_2- and m_3-mAChR specific antisera, respectively (Fig. 3). Following treatment with 10 nM ET-1 for 8 h, the level of m_3-mAChR protein was increased to about 165% of the control; the level of

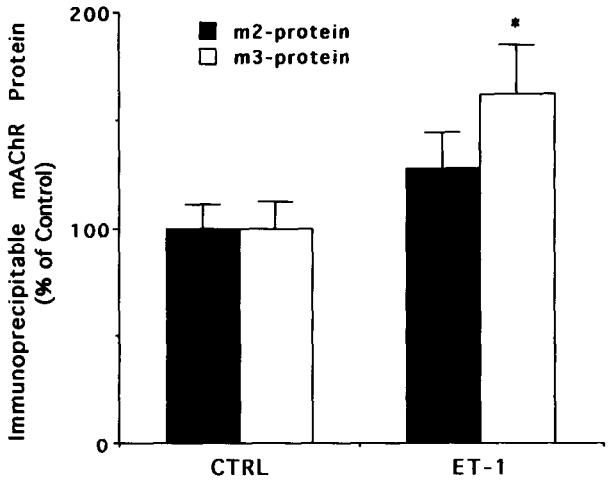


Fig. 3. Effects of ET-1 on immunoprecipitable m_2- and m_3-mAChR protein. Cells were treated with 10 nM ET-1 for 8 h and then lysed for preparing membranes. [^{3}H]QNB-labeled mAChRs were solubilized from membranes for immunoprecipitation using m_2- and m_3-mAChR-specific antisera. Immunoprecipitable levels of m_2- and m_3-mAChR protein after ET-1 treatment are expressed as percentage of untreated control and are the means ± S.E.M. of three independent experiments. *$P < 0.05$ when compared with the respective untreated control (Student's t-test).

m_2-mAChR protein showed an apparent increase but the change did not reach statistical significance.

To examine the involvement of second messenger production in ET-1-induced increase of mAChR mRNA levels, phorbol ester pretreatment was employed to inhibit the known ET-1-induced phosphoinositide turnover. Our results showed that a 15-min pretreatment with 500 nM phorbol dibutyrate (PDBu) attenuates ET-1-induced inositol phosphate accumulation in cerebellar granule cells by 65% [13]. Under these PDBu-pretreated conditions, the ET-1-induced increase of m_2- and m_3-mAChR mRNA levels was completely abolished (Fig. 4), suggesting an essential role of phosphoinositide hydrolysis in this ET-1-induced process. It should be mentioned that ET-1 enhances the release from cerebellar granule cells of [^{3}H]D-aspartate, which labels the glutamate uptake pool [13]. This response is potentiated rather than inhibited by short-term PDBu pretreatment [13], thus indicating that the ET-1-induced mAChR mRNA up-regulation is unreleased to the action of released glutamate on glutamate receptors. In support of this conclusion, we found that the ET-1-induced mAChR mRNA up-regulation was unaffected by MK-801, an antagonist of the *N*-methyl-D-aspartate subtype of glutamate receptors (data not shown).

To assess the potential role of c-*fos* mRNA increase in the subsequent ET-1-induced mAChR mRNA up-regulation, we employed treatment with 2-aminopurine or cycloheximide which has been shown to inhibit the induction of c-*fos* mRNA and protein [19,20]. Confirming these reports, we found that in cerebellar granule cells pretreated with 2-aminopurine (100 μM) or cycloheximide (35 μM), the induction of c-*fos* mRNA at 30 min after ET-1 stimulation was completely abolished (Fig. 5A). Under these treatment conditions, the ET-1 induced delayed increase of m_2- and m_3-mAChR mRNA and total mAChR binding sites were also completely blocked (Fig. 5B). 2-Aminopurine and cycloheximide alone did not affect basal levels of c-*fos* mRNA or mAChR mRNA and binding sites. Interestingly, 2-aminopurine in combination with ET-1 decreased c-*fos* mRNA as well as mAChR mRNA and binding sites to levels less than those of the untreated control. Taken together, the close correlation between changes in c-*fos* mRNA and mAChR expression strongly suggests that c-*fos* protein induction is implicated in the transcriptional regulation of m_2- and m_3-mAChR mRNA.

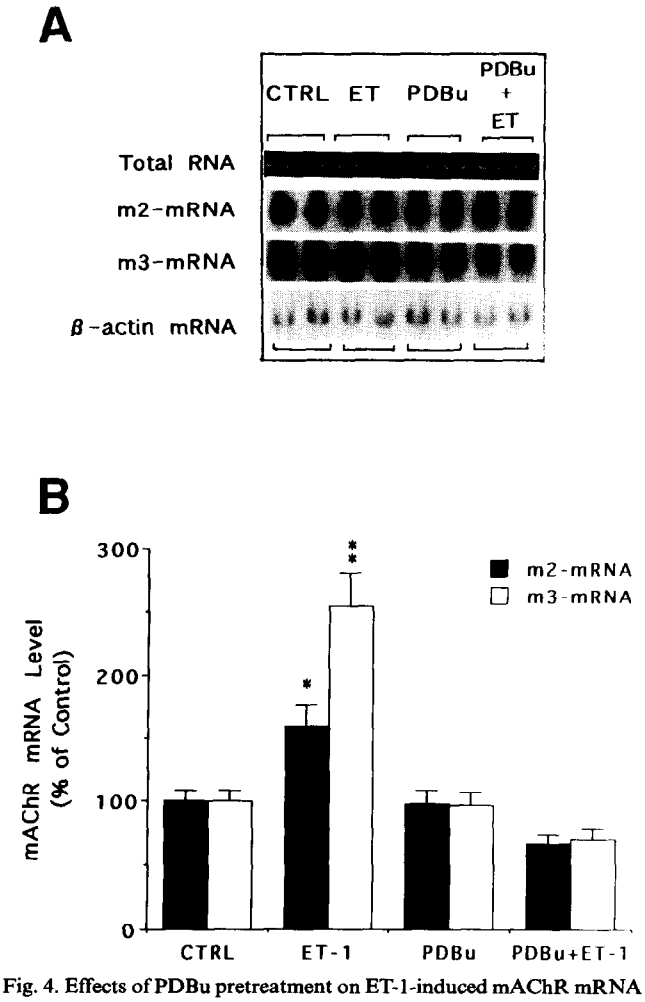


Fig. 4. Effects of PDBu pretreatment on ET-1-induced mAChR mRNA up-regulation. Cells were pretreated with 500 nM PDBu for 15 min and then stimulated with 10 nM ET-1 for 2 h. (A) Autoradiographs of m_2- and m_3-mAChR mRNA as well as β-actin mRNA. (B) Densitometry of the autoradiographs. Levels of m_2- and m_3-mAChR mRNA have been normalized to total cellular RNA in each lane and are expressed as the means ± ranges of values relative to the untreated control. *$P < 0.05$; **$P < 0.01$ when compared with the untreated control (Student's t-test).

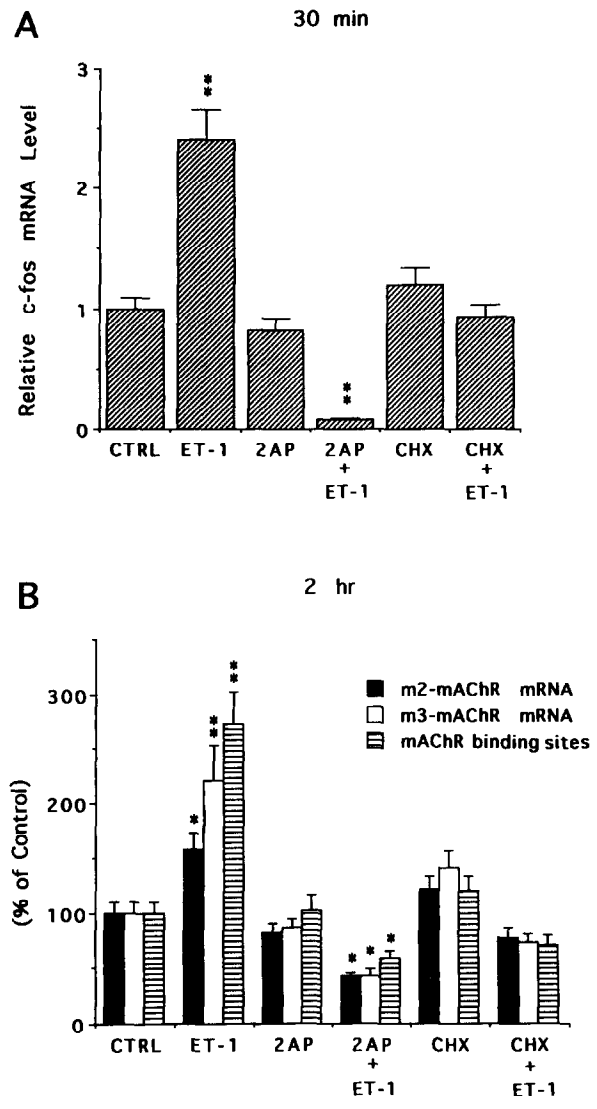


Fig. 5. Effects of 2-aminopurine and cycloheximide on c-*fos* mRNA level and levels of m_2- and m_3-mAChR mRNA and binding sites. Cells were treated with 100 μM 2-aminopurine (2-AP) or 35 μM cycloheximide (CHX) for 30 min prior to stimulation with 10 nM for 30 min in (A) or 2 h in (B). Levels of c-*fos* mRNA were determined at 30 min, while levels of m_2- and m_3-mAChR mRNA as well as total mAChR binding sites were determined at 2 h. mRNA levels were normalized to total RNA levels in each lane. The levels of total RNA or β-actin mRNA were not significantly changed in our experimental conditions. mAChR binding sites were quantified by binding of 0.5 nM [^{3}H]QNB to intact cells grown in a 35-mm dish containing 3×10^6 cells, as described previously [16]. The data are the means ± ranges of values relative to their untreated control in duplicate experiments. Comparable results have been obtained in three independent experiments. $^*P < 0.05$; $^{**}P < 0.01$ when compared with the untreated control (Student's *t*-test). The 100% value for the mAChR binding site was in the range of 262–279 fmol/dish of 3×10^6 cells.

In addition to stimulation of phosphoinositide turnover, forming inositol triphosphate and diacylglycerol, ET-1 has been shown to induce receptor-gated Ca^{2+} influx in a variety of cell types [1–3, 6–8]. The induction of c-*fos* by ET-1 is therefore likely to occur via the promoter regulation involving the CaM kinase-Ca^{2+}/CRE or protein kinase C-SRE pathway (for a review, see [21,22]). In mesangial cells, ET-1 induces c-*fos* and *jun* gene expression, which in turn binds to the AP-1 *cis*-element to increase transcriptional activity [23]. Experiments are in progress to assess whether an AP-1 site-mediating mechanism is involved in the ET-1-induced expression of mAChRs in cerebellar granule cells. The enhanced expression of m_3-mAChR mRNA and protein by ET-1 may be physiologically significant. ET-1 is endogenously present in the CNS including the cerebellum [2,3] and m_3-mAChR is the major mAChR subtype expressed in cerebellar granule cells [15,16]. Besides its robust effect in stimulating phosphoinositide hydrolysis [15], m_3-mAChR mediates Ca^{2+} influx independent of inositol phosphates and intracellular Ca^{2+} release [24]. Thus, ET-1-induced expression of m_3-mAChR protein may result in long-lasting changes in neuronal Ca^{2+} homeostasis and excitability.

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