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Biochemical Pharmacology 63 (2002) 977–984

Biochemical Pharmacology

Stimulation of catecholamine biosynthesis *via* the protein kinase C pathway by endothelin-1 in PC12 rat pheochromocytoma cells

Kazuhiro Takekoshi*, Kiyoaki Ishii, Shunsuke Shibuya, Yasushi Kawakami, Kazumasa Isobe, Toshiaki Nakai

Department of Clinical Pathology, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki 305-8575, Japan

Received 19 December 2000; accepted 4 June 2001

Abstract

It has been reported that endothelins (ETs) stimulate catecholamine release from chromaffin cells. However, it is not known whether ETs also affect catecholamine biosynthesis. Thus, using a rat pheochromocytoma cell line, PC12, we examined the effects of ETs on catecholamine biosynthesis. The mRNA level and activity of tyrosine hydroxylase (TH), a rate-limiting enzyme in catecholamine biosynthesis, were increased significantly by endothelin-1 (ET-1) (100 nM). These stimulatory effects were inhibited completely by a blocker for the A-type endothelin receptor, BQ-123 [cyclo(D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl)] (1 μM), but not by a blocker for the B-type endothelin receptor, BQ-788 (*N-cis* 2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine (1 μM). Also, Ro-32-0432 (3-[8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido-[1,2-*a*]indol-10-yl]-4-(1-methyl-3-indolyl)-*H*-pyrrole-2,5-dione hydrochloride) (100 nM), a protein kinase C inhibitor, completely inhibited ET-1-induced increases in TH activity and mRNA level. Furthermore, ET-1 (100 nM) significantly stimulated protein kinase C activity, as well as inositol 1,4,5-triphosphate production; these stimulatory effects were abolished by BQ-123 but not by BQ-788. Moreover, ET-1 (100 nM) significantly increased both the TH-protein level and the intracellular catecholamine content. By contrast to ET-1, endothelin-3 did not affect catecholamine synthesis. These results indicate that ET-1, but not ET-3, stimulates catecholamine synthesis through the PKC pathway in PC12 cells. Also, the use of selective ET receptor antagonists suggests that the effects of ET-1 on catecholamine biosynthesis are mediated through ET_A.

Keywords: Endothelins (ETs); Tyrosine hydroxylase (TH); PC12; Catecholamine synthesis; PKC

1. Introduction

ETs are potent vasoconstricting agents initially reported by Yanagisawa *et al.* [1]. The ETs consist of at least three isoforms, termed ET-1, ET-2, and ET-3 [1,2]. The biological actions of these ETs are mediated by two types of receptor, A-type endothelin receptor (ET_A) and B-type endothelin receptor (ET_B), both of which have seven transmembrane domains and belong to the G-protein-coupled receptor family [3,4]. ET_A is selective for ET-1 and ET-2, whereas ET_B can bind to ET-1, ET-2, and endothelin-3 (ET-3) with equal potencies, and these effects are linked to the activation of protein kinase C (PKC) and inositol 1,4,5-triphosphate (IP_3) production following phospholipase C (PLC) activation [3]. Indeed, mounting evidence suggests that most of the pharmacologic effects of the ETs in mesangial cells, vascular smooth muscle, and fibroblasts are mediated by the activation of PLC [5–9]. The wide distribution of the ETs and their receptors suggests that these peptides may play diverse roles in a variety of tissues.

The expression of ETs and ET receptors has been demonstrated in adrenal chromaffin cells, suggesting that these peptides may modulate the function of chromaffin cells in an autocrine and/or paracrine fashion [10–15]. Indeed, recent studies have shown that ETs stimulate catecholamine release from chromaffin cells [11,13,15].

In adrenal medullary cells, TH is a rate-limiting enzyme in the biosynthesis of catecholamines. TH activity can be regulated by both short- and long-term mechanisms. Short-term regulation of enzyme activity occurs at the post-transcriptional level. Central to this regulation is the phosphorylation of TH, which results in activation

* Corresponding author. Tel.: +81-298-53-3054; fax: +81-298-53-3039.
E-mail address: k-takemd@md.tsukuba.ac.jp (K. Takekoshi).

Abbreviations: ETs, endothelins; ET_A, A-type receptor; ET_B, B-type receptor; PLC, phospholipase C; TH, tyrosine hydroxylase; PKC, protein kinase C; cAMP, cyclic AMP; IP_3, inositol 1,4,5-triphosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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PII: S0006-2952(01)00862-0

of the enzyme [16]. Indeed, TH is phosphorylated and activated by a variety of protein kinases including PKC [16–20]. Long-term regulation is exerted at the TH protein synthesis level following TH gene transcription [21]. Furthermore, several protein kinases, including PKC, are also involved in the mechanisms regulating TH gene transcription.

To date, it has not been determined whether ETs affect catecholamine biosynthesis in chromaffin cells. Thus, we investigated the effects of ET-1 and ET-3 on catecholamine biosynthesis in a rat pheochromocytoma cell line, PC12.

2. Materials and methods

2.1. Reagents

Unless otherwise noted, all reagents were purchased from Wako Seiyaku. ET-1 and ET-3 were purchased from the Peptide Institute. The ET_A and ET_B blockers, BQ-123 [cyclo-(D-α-aspartyl-L-prolyl-D-valyl-L-leucyl-D-tryptophyl)] and BQ-788 (*N-cis*2,6-dimethylpiperidinocarbonyl-L-γ-methyl-leucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine), were purchased from Research Biochemicals International, and the PKC inhibitor, Ro-32-0432 (3-[8-[(dimethylamino)-methyl]-6,7,8,9-tetrahydropyrido-[1,2-*a*]indol-10-yl]-4-(1-methyl-3-indolyl)-*H*-pyrrole-2,5-dione hydrochloride), was purchased from Calbiochem. U-73122 (1-[6-((17β-3-methoxyestra-1,3,5 [10]-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione) was purchased from the Sigma. The concentrations of ETs used in our experiments (1 nM–1 μM) were chosen according to Lee *et al.* [15].

2.2. Cell culture

The PC12 cell line (RCB009) was obtained from the RIKEN Cell Bank. Cells were grown in 75 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) containing 10% inactivated horse serum (Gibco BRL) and 10% fetal bovine serum (Gibco BRL) in a humidified atmosphere of 5% CO_2/95% O_2 at 37°. The culture medium was changed three times per week. Cells were removed from the flasks for subculture and for plating into assay dishes using a Ca^{2+}/Mg^{2+}-free solution containing 172 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, and 5.6 mM glucose, at pH 7.4. After about 2 min in this solution, the cells were detached by tapping the side of the flask. The cells (1×10^6) were plated onto 35 mm polystyrene dishes and cultured with 2 mL of DMEM for 2 days as described above, and then used for experiments in a serum-starved condition.

2.3. TH enzyme activity

TH enzyme activity was measured using a method previously reported by Kumai *et al.* [22]. Experiments were initiated by replacing the cell culture medium with HEPES-buffered Krebs solution containing various concentrations of test substance, and incubating the cells for 10 min at 37°. Then the cells were homogenized in 0.25 M sucrose (50 vol.%) using a glass tissue grinder. The standard incubation medium consisted of the following components in a total volume of 250 μL: 100 μL of tissue homogenate, 40 μL of 1 M sodium acetate buffer (pH 6.0), 40 μL of 1 mM L-tyrosine or D-tyrosine, 20 μL of 1 M 6-methyl-5,6,7,8-tetra-hydropterine in 1 M 2-mercaptoethanol, 20 μL of 20 mM catalase, and 30 μL water. The medium was incubated at 37° for 30 min, and then the reaction was stopped with 1 M perchloric acid, containing dihydroxy benzylamine as an internal standard, and 0.2 M EDTA in an ice bath. Then 1 M potassium carbonate and 0.2 M Tris–HCl (pH 8.5) containing 1% EDTA were added. 3-(3,4-Dihydroxyphenyl)-alanine (DOPA) was extracted using the aluminum oxide method. Forty microliters of extracted medium was mixed with 0.1 N NaOH and TSK-GEL ODS-120T (TOSOH) and analyzed by HPLC. The mobile phase consisted of the following components: 50 mM sodium acetate, 20 mM citric acid, 12.5 mM sodium octyl sulfate, 1 mM di-*n*-butylamine, and 0.134 mM EDTA. All separations were performed isocratically at a flow rate of 0.6 mL/min at 28°. The detector potential was maintained at +0.65 V. The TH enzyme activity was calculated as the amount of DOPA formed from tyrosine per milligram of protein per minute.

2.4. Northern blot analysis

For northern blot analysis, total RNA was extracted from the samples using an ISOGEN kit (Nippon Gene). The RNA concentration was determined spectrophotometrically (at 260 nm). RNA (10 g sample) was fractionated by electrophoresis on 1% agarose/5% formaldehyde gels (80 V, 2 hr). After staining with ethidium bromide and a visual inspection of the UV fluorescence to confirm the presence of equal amounts of 18 and 28S ribosomal RNA in each lane, the RNA was transferred to a nitrocellulose membrane and hybridized to ^{32}P-labeled probes. The following probe was used: a 1.9 kbp EcoRI fragment of pTHT1 coding for TH. Plasmid pTHT1 contained the full-length cDNA for human TH type 1 cDNA. This plasmid was developed by T. Nagatsu and was provided by the RIKEN Gene Bank. The probe was labeled using a random primer extension labeling kit (New England Nuclear). Rat GAPDH cDNA was used as an internal standard (Clontech). Hybridization signals were scanned on an image analyzer (BAS2000, Fuji).

2.5. Western blot analysis

Western blot analyses were performed as previously described [23]. In brief, the PC12 cells were solubilized with 0.1% SDS containing 1% Triton X-100, 1% sodium

deoxycholate, and 20 mM Tris–HCl, pH 7.4. The supernatants, containing 10 mg protein, were separated on 10% SDS-polyacrylamide gels, and then transferred to nitrocellulose using a Bio-Rad Transblot apparatus. After transfer, the nitrocellulose sheets were incubated for 1 hr with BLOTTO buffer (5% skimmed milk, 0.05% Triton X-100, 100 mM NaCl, 200 mM Tris–HCl, pH 7.4). The nitrocellulose membranes were washed three times for 10 min with TBST solution (0.05% Triton X-100, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl), and then for 1 hr with 1 mg/mL of the monoclonal antibody to TH (Boehringer). Next the nitrocellulose membranes were washed three times for 10 min with TBST solution, and incubated for 1 hr with horseradish peroxidase-labeled Protein A (Amersham). Finally, the blots were washed three times, incubated with ECL reagent (Amersham) for 1 min, and then exposed to Polaroid films (ISO 3000).

2.6. PKC activity

PKC activity was measured in cultured confluent cells as previously described [24]. Cell cultures (in 90 mm dishes) were incubated in control medium (5% glucose) or with ET-1 or ET-3 for 10 min. Then the cells were harvested, homogenized by sonification in 20 mM Tris–HCl (pH 7.5), and incubated for 30 min with 100 μL of reaction buffer solution containing a pseudosubstrate and various phospholipids from a commercially available kit (Pep Taq-Non-radioactive PKC activity kit, Promega). The reaction was terminated by heating, and the reaction mixture was separated into phosphorylated and non-phosphorylated substrates on a 0.9% agarose gel. For quantification, the gel bands were visualized by UV light, then excised, and the absorbance was measured at 570 nm. The results are expressed in OD units.

2.7. Measurement of the production of IP_3

Measurement of IP_3 production was carried out using a specific IP_3 binding assay kit (Pharmacia). Briefly, the cells were washed twice with EM (Eagle's minimal essential medium) containing 0.5% BSA, and then were stimulated with various test substances for 10 min. The reaction was quenched by removing the medium and mixing it rapidly with an equal volume of ice-cold 15% trichloroacetic acid. After sedimentation of the precipitates, the supernatants were extracted three times with 10 vol.% of H_2O_2-saturated diethyl ether, evaporated to dryness, and the pH adjusted to 7.5 with $NaHCO_3$. The amount of IP_3 in the sample was determined according to the manufacturer's protocol for the assay kit.

2.8. Measurement of cAMP production

cAMP production by the cells was determined as previously described [23]. Briefly, cells were washed twice with EM and preincubated in EM containing 0.2 mM 3-isobutyl-1-methylxanthine (IBMX) for 5 min. Experiments were initiated by replacing the medium with HEPES-buffered Krebs solution containing test substances and 0.2 mM IBMX, and incubating the cells for 10 min at 37°. The reaction was terminated by adding 100 μL of 1 N HCl, followed by incubation on ice for 30 min. The cAMP in the acid extract was then measured with a cAMP kit (Yamasa).

2.9. Determination of intracellular catecholamine levels

Cells were treated with ET-1 or ET-3 (1 nM–1 μM) for 24 hr. The catecholamine levels in the cells were then determined as previously described [23], using a catecholamine autoanalyzer (TOSOH, H8030) with a built-in high performance liquid chromatograph and a spectrofluorimeter.

2.10. Statistical analysis

All data are expressed as means ± SEM. The significance of differences in the data was determined by ANOVA, and P values less than 0.05 were considered significant.

3. Results

3.1. Effects of ETs (ET-1 and ET-3) on TH enzyme activity

As shown in Fig. 1A, 1 nM ET-1 had no significant effect on TH enzyme activity. At concentrations of 10 nM and above (100 nM and 1 μM) ET-1 significantly stimulated TH enzyme activity by 24, 35, and 34%, respectively. The increased TH enzyme activity induced by ET-1 (100 nM) was inhibited completely by a blocker for ET_A, BQ-123 (1 μM). In contrast, a blocker for ET_B, BQ-788 (1 μM) did not affect ET-1-induced enzyme activity (Fig. 1B). The PKC inhibitor Ro-32-0432 (100 nM) completely inhibited ET-1-induced TH enzyme activity (Fig. 1B). The concentration of Ro-32-0432 used in our experiments (100 nM) was the same as that used by Chabot-Fletcher and Breton [25]. In contrast to ET-1, ET-3 did not alter TH enzyme activity.

3.2. Effects of ET-1 and ET-3 on TH mRNA expression

As shown in panels A and B of Fig. 2, ET-1 (100 nM) significantly increased TH mRNA levels by 53%. This stimulatory effect was abolished completely by BQ-123 (1 μM) but not by BQ-788 (1 μM). Ro-32-0432 (100 nM) completely inhibited ET-1-induced TH enzyme activity. ET-3 did not alter TH mRNA levels.

3.3. Effects of ET-1 and ET-3 on TH protein level and intracellular catecholamine levels

To confirm the increase of catecholamine synthesis following TH mRNA induction, the effects of ET-1 or

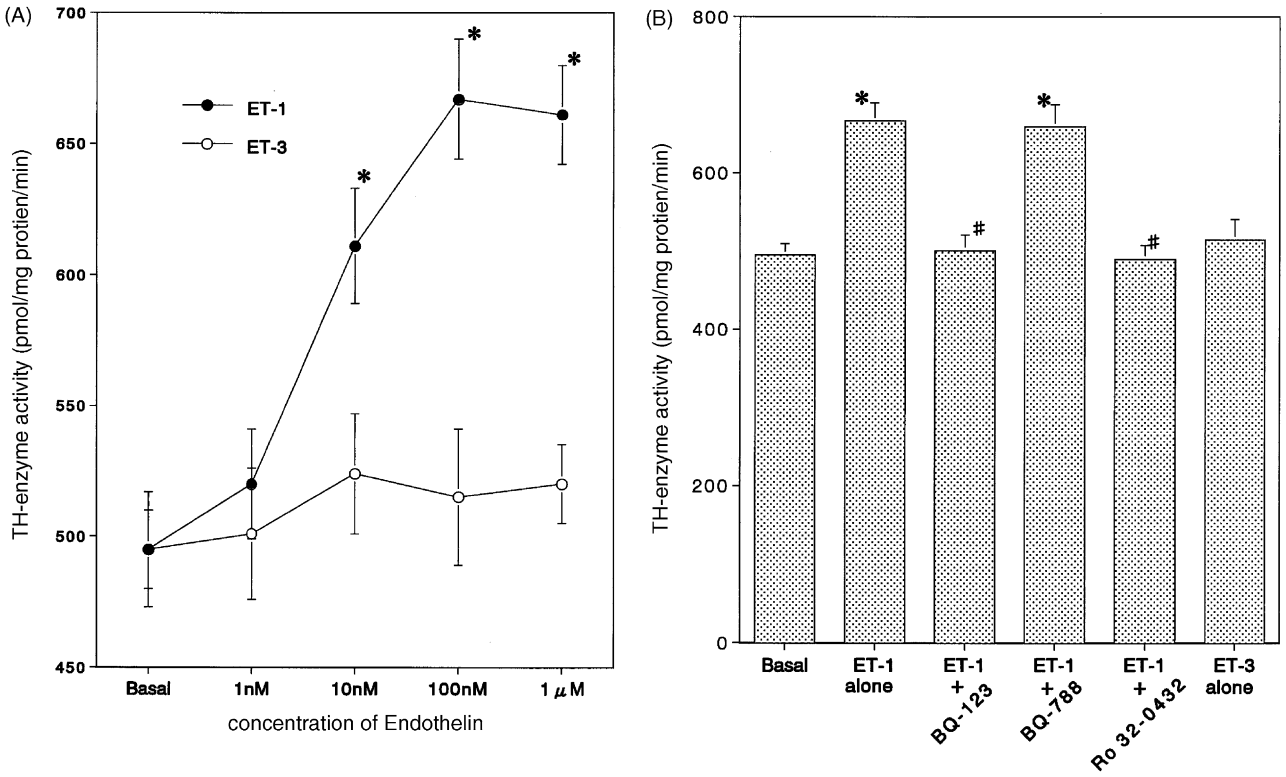

Fig. 1. Effects of ET-1 and ET-3 on TH enzyme activity. (A) PC12 cells were incubated for 10 min with various concentrations (1 nM–1 μM) of ET-1 or ET-3. (B) PC12 cells were incubated for 10 min with either ET-1 or ET-3 (both 100 nM) alone or in the presence of BQ-123 (1 μM), BQ-788 (1 μM), or Ro-32-0432 (100 nM), as indicated. TH enzyme activity was then measured as described in Section 2. Values are means ± SEM ($N = 4$). Key: (*) significantly different ($P < 0.05$) from basal value, and (#) significantly different ($P < 0.05$) from the value induced by ET-1 (100 nM) alone.

ET-3 at the TH protein level were examined. PC12 cells were treated with ET-1 or ET-3 (100 nM each) for 24 hr. Then TH protein levels were examined by western blot analyses. ET-1 (100 nM) significantly stimulated the TH protein level by 20% (Fig. 3A and B). We also examined intracellular catecholamine levels. PC12 cells were treated with ET-1 or ET-3 (1 nM–1 μM) for 24 hr. Then the intracellular catecholamine levels were examined by HPLC. Since the major catecholamine synthesized in PC12 cells is dopamine and the production of noradrenaline and adrenaline is negligible, only findings on dopamine levels are presented in Table 1. At concentrations of 10 nM and above, ET-1 significantly increased intracellular catecholamine levels (Table 1). Regardless of the concentration used, ET-3 did not affect TH protein or intracellular catecholamine levels.

3.4. Effects of ET-1 and ET-3 on PKC activity

We examined the effects of ET-1 and ET-3 on PKC activity. As shown in Fig. 4, ET-1 (100 nM) significantly increased PKC activity by about 1.5-fold, whereas ET-3 had no effect. This stimulatory effect of ET-1 on PKC was abolished completely by BQ-123 but not by BQ-788. In addition, the PKC inhibitor Ro-32-0432 (100 nM) completely inhibited ET-1-induced PKC activity. Furthermore, U-73122 (10 μM), a PLC inhibitor, abolished ET-1-induced PKC activity. The concentration of U-73122 used in our experiments (10 μM) was that used by Zheng *et al.* [26].

3.5. Effects of ET-1 and ET-3 on the production of IP_3

Because ETs were found to increase IP_3 in several cell types [10–15], we examined the effects of ETs on IP_3

Table 1
Effect of ET-1 and ET-3 on intracellular catecholamine content

Concentration	Dopamine (ng/mg protein)	
	ET-1	ET-3
Basal	1505 ± 28	1515 ± 30
1 nm	1519 ± 31	1502 ± 38
10 nM	1582 ± 18*	1525 ± 32
100 nM	1640 ± 25*	1520 ± 28
1 μM	1628 ± 32*	1528 ± 33

PC12 cells were incubated for 10 min with various concentrations (1 nM–1 μM) of either ET-1 or ET-3 for 24 hr and then the intracellular catecholamine levels (dopamine) were measured as described in Section 2. As the major catecholamine synthesized in PC12 cells is dopamine and the production of noradrenaline and adrenaline is negligible, only the findings on dopamine levels are presented. Values represent means ± SEM ($N = 4$–6).
* Significantly different ($P < 0.05$) from the basal value.

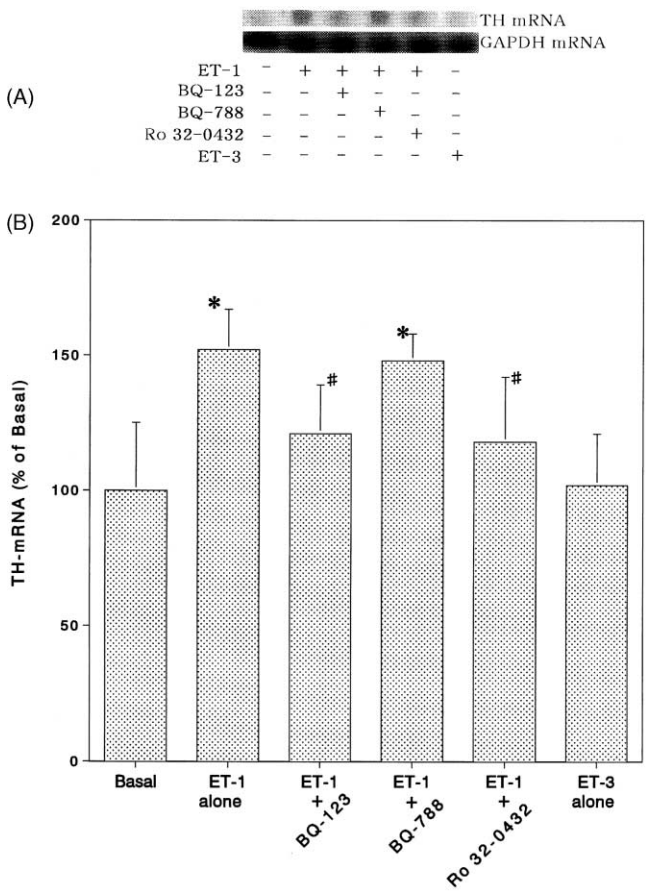

Fig. 2. Effects of ET-1 and ET-3 on TH mRNA expression. (A) PC12 cells were incubated for 8 hr with ET-1 or ET-3 (both 100 nM) in the presence of BQ-123 (1 μM), BQ-788 (1 μM), or Ro-32-0432 (100 nM), as indicated. Total cellular RNA (10 μg/lane) from PC12 cells was characterized by northern blot analysis as described in Section 2. The lower blot shows the control mRNA (GAPDH) containing equivalent amounts of mRNA. (B) The radioactivity associated with TH mRNA was quantitated by subtracting the photo-stimulated luminescence on the northern blots from the background. Values are the means ± SEM ($N = 4$) of the radioactivity associated with TH mRNA following various treatments with ET-1, ET-3, and inhibitors of ET_A (BQ-123), ET_B (BQ-788), and PKC (Ro-32-0432). Key: (*) significantly different ($P < 0.05$) from the basal value (100% = basal), and (#) significantly different ($P < 0.05$) from the value induced by ET-1 (100 nM) alone.

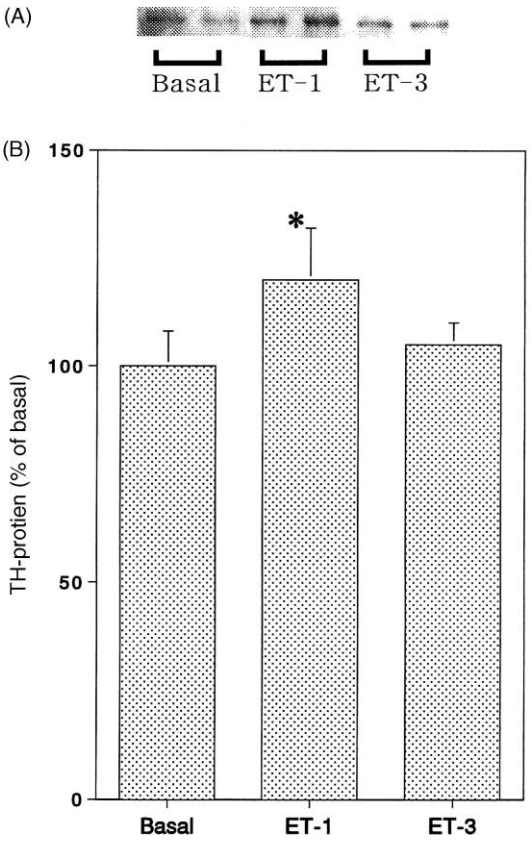

Fig. 3. Effects of ET-1 and ET-3 on TH protein level. (A) PC12 cells were incubated for 24 hr with ET-1 or ET-3 (both 100 nM), and then the TH protein levels were measured by western blot analysis as described in Section 2. (B) Values are means ± SEM ($N = 4$). Key: (*) significantly different ($P < 0.05$) from the basal value (100% = basal).

production in PC12 cells. As shown in Fig. 5, ET-1 (100 nM) significantly increased the level of IP_3 by approximately 1.6-fold. This stimulatory effect of ET-1 on IP_3 was abolished completely by BQ-123 but not by BQ-788. Unlike ET-1, ET-3 (100 nM) did not alter IP_3 production.

3.6. Effects of ET-1 and ET-3 on cAMP production

Since elevated cAMP levels play a key role in regulating TH enzyme activity and TH mRNA levels [21,27], we next examined the effects of ET-1 and ET-3 (1 nM–1 μM, each) on cAMP production. Neither ET-1 nor ET-3 had any effect on cAMP levels (Fig. 6).

4. Discussion

The present study shows that ET-1 stimulates parallel increases in both TH enzyme activity and TH mRNA levels, suggesting that both contribute to ET-1-induced catecholamine biosynthesis. Also, the ET-1-induced increases in TH enzyme activity and mRNA level were inhibited completely by BQ-123 but not by BQ-788, suggesting that the effects are mediated through the ET_A (Figs. 1 and 2).

In addition, we have shown that ET-1 markedly stimulated TH protein levels (Fig. 3). Therefore, it is likely that ET-1 stimulates TH gene expression, resulting in increased TH protein levels in PC12 cells. Furthermore, we demonstrated that ET-1 markedly stimulates intracellular catecholamine levels (Table 1), supporting the suggestion that ET-1 stimulates catecholamine biosynthesis as well as catecholamine release.

In the present study, we also showed that ET-1 significantly increased PKC activity in PC12 cells (Fig. 4). The increase in PKC activity stimulated by ET-1 was inhibited completely by Ro-32-0432, confirming that Ro-32-0432 is indeed a PKC inhibitor in our experimental system [25]. It should also be noted that the increases in TH enzyme

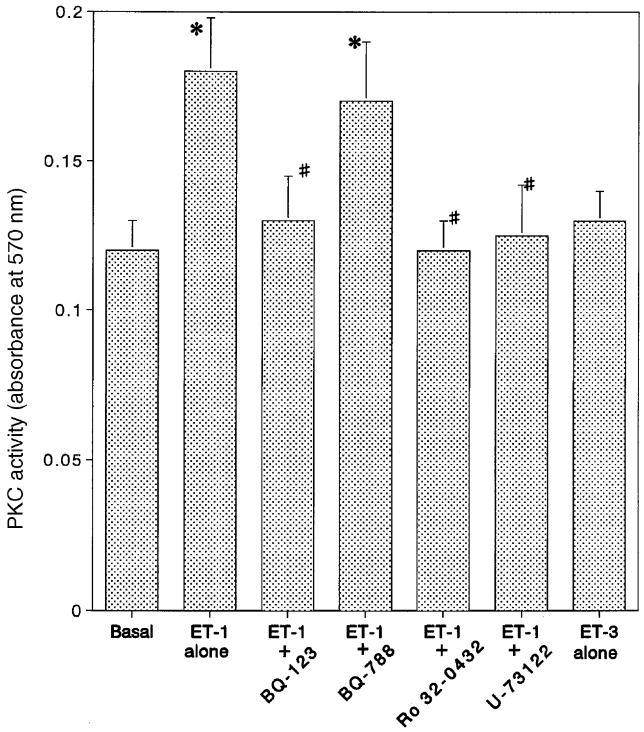

Fig. 4. Effects of ET-1 and ET-3 on PKC activity. PC12 cells were incubated for 10 min with either ET-1 or ET-3 (100 nM) alone or in the presence of BQ-123 (1 μM), BQ-788 (1 μM), Ro-32-0432 (100 nM), or U-73122 (10 μM), as indicated. Then PKC activity was measured as described in Section 2. Values are means ± SEM ($N = 4$–6). Key: (*) significantly different ($P < 0.05$) from the basal value, and (#) significantly different ($P < 0.05$) from the value induced by ET-1 (100 nM) alone.

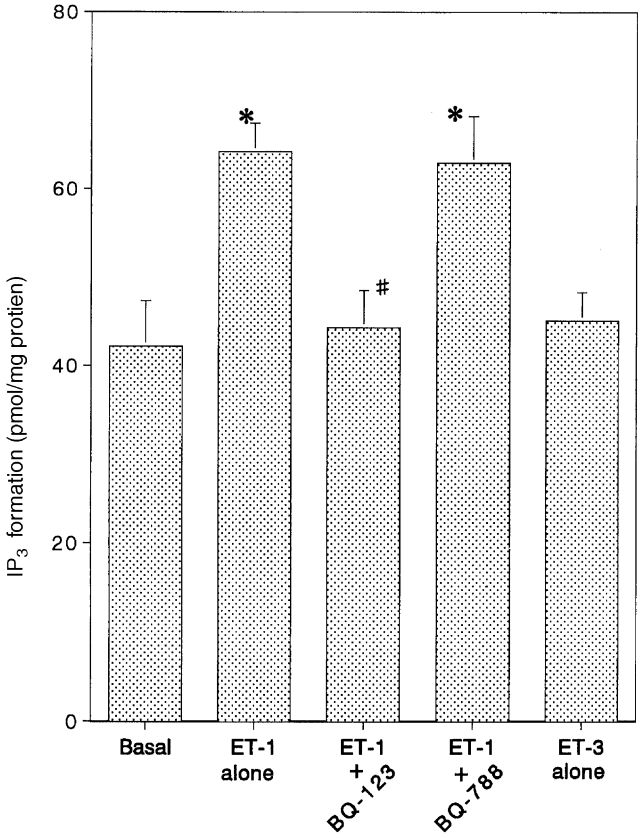

Fig. 5. Effects of ET-1 and ET-3 on IP_3 production. PC12 cells were incubated for 10 min with either ET-1 or ET-3 (100 nM) alone or in the presence of BQ-123 (1 μM) or BQ-788 (1 μM), as indicated. IP_3 was measured as described in Section 2. Values are means ± SEM ($N = 4$). Key: (*) significantly different ($P < 0.05$) from the basal value, and (#) significantly different ($P < 0.05$) from the value induced by ET-1 (100 nM) alone.

activity and mRNA levels evoked by ET-1 stimulation were inhibited by 100 nM Ro-32-0432 (Figs. 1B and 2). This would suggest that the stimulatory effects of ET-1 on TH enzyme activity and mRNA levels in chromaffin cells are mediated, at least in part, by the PKC pathway. Moreover, we have shown that the increase in PKC evoked by ET-1 was abolished by BQ-123 but not by BQ-788, indicating that the effect on PKC was mediated through the ET_A (Fig. 4). Consistent with the stimulatory effect of ET-1 on PKC activity, it should be noted that ET-1 also significantly increased the level of IP_3 through an ET_A receptor-mediated mechanism (Fig. 5).

It is well established that PKC exerts stimulatory effects on both TH enzyme activity and TH mRNA [16–20]. Thus, it is likely that ET-1 stimulates PLC breakdown *via* ET_A, which leads to stimulation of the PKC pathway. This, in turn, results in the stimulation of TH enzyme activity and TH synthesis. In support of this, the PLC inhibitor U-73122 inhibited ET-1-induced PKC activity (Fig. 4).

The PKC family consists of three isoforms: classical (c)PKC, which is sensitive to Ca^{2+}, diacylglycerol (DAG), or phorbol esters; novel (n)PKC, which is regulated by DAG or phorbol esters but is independent of Ca^{2+}; and atypical (a)PKC, which is insensitive to Ca^{2+}, DAG, and phorbol esters [28,29]. Since IP_3 is known to mobilize intracellular Ca^{2+}, it can also be suggested that (c)PKC may be the prominent isoform involved in ET-1-induced TH synthesis.

However, ET-1 did not alter cAMP production (Fig. 6). This finding indicates that the cAMP-PKA pathway does not play a major role in the mechanism of ET-1-stimulated catecholamine synthesis.

We have demonstrated clearly in this study that ET-1 markedly increases IP_3 levels (and catecholamine production) through an ET_A-mediated mechanism in PC12 cells. In contrast to our findings, a previous study by Martin *et al.* [30] failed to demonstrate ET-1-induced IP_3 production in PC12 cells, which may reflect the different PC12 subclone (ATCC CRL 107) used.

In contrast to the stimulatory effects of ET-1, ET-3 had no significant effects on TH enzyme activity, TH mRNA and protein levels, and intracellular catecholamine content. In addition, ET-3 had no significant effects on PKC or IP_3 (Figs. 4 and 5). Recently, Lee *et al.* [15] demonstrated that ET-1, but not ET-3, increased catecholamine release, and $[Ca^{2+}]_i$ and $45Ca^{2+}$ uptake exclusively through the ET_A in cultured bovine adrenal chromaffin cells. Together with the current study, these results demonstrate that ET-3 does not play a major role in the regulation of catecholamine biosynthesis in chromaffin cells.

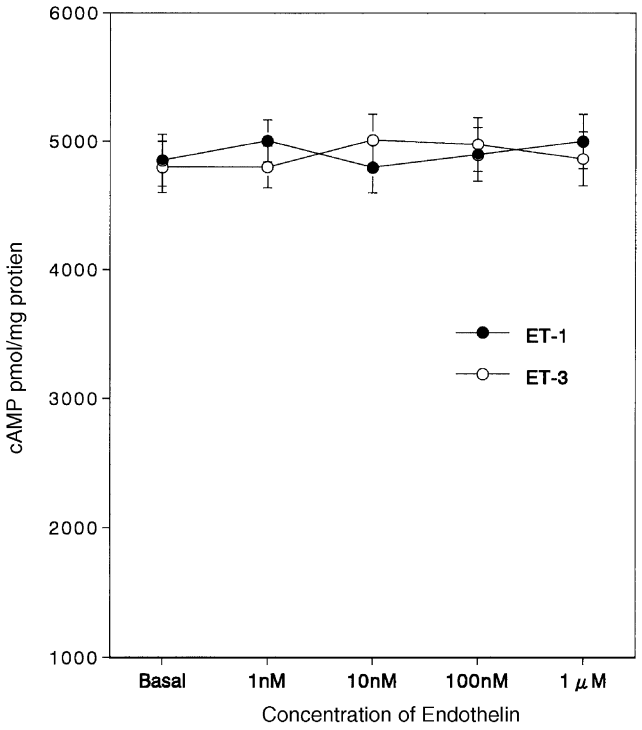

Fig. 6. Effects of ET-1 and ET-3 on cAMP production. PC12 cells were incubated for 30 min with various concentrations (1 nM–1 μM) of ET-1 or ET-3. Then intracellular cAMP was measured by enzyme immunoasay (EIA) as described in Section 2. Values are means ± SEM ($N = 4$–6).

Belloni *et al.* [11] have reported that the ET-1-induced release of catecholamines was mediated by both ET_A and ET_B in the rat adrenal gland, which disagrees with our study. The reason for this discrepancy is unclear, but it might reflect differences in the cells used (a cell line vs. freshly dispersed cells).

Although, several reports have proposed that ETs may regulate the function of the adrenal medulla [10–15,31], the physiological relevance of ET-1-mediated catecholamine biosynthesis (*via* PKC activation) is unclear. Furthermore, although PKC has been reported to stimulate both TH enzyme activity and TH mRNA *in vitro* [16–21], the precise role of PKC in the regulation of TH activity *in vivo* is unknown [32]. Further studies will be needed to clarify these points.

In summary, we have shown that ET-1, but not ET-3, stimulates catecholamine biosynthesis in PC12 cells *via* an ET_A-mediated mechanism utilizing the PKC signaling pathway.

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