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Effects of Endothelin on Adrenomedullin Secretion and Expression of Adrenomedullin Receptors in Rat Cardiomyocytes

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Both endothelin (ET) and adrenomedullin (AM), produced by cardiac myocytes, are thought to be locally-acting hormones in the heart. Recently, calcitonin receptor-like receptor (CRLR) and receptor activity modifying proteins (RAMPs) have been shown to function together to serve as AM receptors stimulating cAMP production. In the present study, we examined the effects of ET on AM secretion, intracellular cAMP response to AM, and gene expressions of CRLR and RAMPs in cultured cardiac myocytes. Synthetic ET-1 dose-dependently increased AM secretion from the cardiomyocytes. AM increased the intracellular cAMP level in a dose-dependent manner and the cAMP accumulation by AM was significantly amplified by 24 h preincubation with ET-1. 10 nmol/L ET-1 significantly increased the CRLR mRNA level without any effect on RAMP1 mRNA. 1 μmol/L ET-1 significantly reduced the RAMP2 mRNA level, but ET-1 dose-dependently increased the RAMP3 mRNA level in the cardiac myocytes. These findings suggest that ET-1 not only stimulates AM secretion, but also modulates intracellular cAMP responses to AM probably by altering the expressions of CRLR and RAMPs in rat cardiomyocytes.

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Key Words: **adrenomedullin; endothelin; calcitonin receptor-like receptor; receptor activity modifying protein; cAMP; cardiomyocyte.**

Adrenomedullin (AM) is a potent vasodilator peptide composed of 52 amino acid residues, and belongs to the calcitonin gene-related peptide (CGRP) superfamily (1). Although AM was isolated originally from human pheochromocytoma, AM peptide and mRNA were found to be present in the cardiac atrium and ventricle (2, 3). Both AM content and AM mRNA in hypertrophied or failing cardiac ventricles increased compared with controls in rats (4–6), and Jougasaki *et al.* revealed a markedly increased AM immunoreactivity in failing human ventricles (7). Recently, we have reported that AM is synthesized and released from cultured cardiac myocytes and fibroblasts of rats (8, 9). Taken together with the presence of AM binding sites in the heart (10), these findings imply roles of AM as an endogenous autocrine or paracrine regulator of cardiac function.

Although many of the AM actions have been shown to be mediated by intracellular cAMP, the subtype for AM receptors has been a controversial field. McLatchie *et al.* have recently reported that the calcitonin receptor-like receptor (CRLR) could function as either a CGRP receptor or an AM receptor, depending on the expression of receptor activity modifying proteins (RAMPs) (11). They suggested that the RAMP1/CRLR complex serves as a CGRP receptor, whereas both the RAMP2/CRLR and RAMP3/CRLR serve as AM receptors. The latter was also confirmed in human endothelial and vascular smooth muscle cells, where expression of both CRLR and RAMP2 are required for constituting the functional AM receptors to elevate the intracellular cAMP level (12). It has been shown that the RAMP family genes are expressed in the majority of human and rat tissues (11, 13), but little is known about the regulation of these AM receptor components.

Endothelin-1 (ET-1) is a 21-residue vasoconstrictor peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells (14). Similar to AM, the gene for this bioactive peptide is expressed not only in the vascular endothelium but also in the myocardium. In fact, Yamazaki *et al.* found that ET-1 is constitutively secreted from cultured cardiomyocytes (15). Additionally, ET-1 has been shown to have a positive inotropic action on the myocardium (16) and to induce myocardial cell hypertrophy (17). These findings suggest a possible role of ET-1 as the local modulator of cardiac hypertrophy or function. Recently, we

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TABLE 1

Oligonucleotide Sequences Used for Quantitative PCR

Target		Sequence
CRLR	forward primer (1666–1693)	5′-CTC-TAC-ATG-AAA-GCT-GTA-AGA-GCC-ACT-C-3′
	reverse primer (1807–1780)	5′-CCT-GAT-AGT-GCA-TGA-GAA-TGT-GCA-TGA-C-3′
	labeled probe (1734–1762)	5′-TCC-ATG-GCG-GCC-TGA-AGG-AAA-GGT-TGC-TG-3′
RAMP-1	forward primer (169–192)	5′-GGA-AGA-CTC-TGT-GGT-GTG-ACT-GG-3′
	reverse primer (334–311)	5′-GAC-TGG-GCA-CTT-GCT-GAA-GTA-GC-3′
	labeled probe (255–284)	5′-TGG-CTG-TTT-CTG-GCC-CAA-TCC-GGA-AGT-GGA-3′
RAMP-2	forward primer (389–414)	5′-GCA-GAA-AGT-ATC-ATC-CTT-GAG-GCT-C-3′
	reverse primer (544–519)	5′-CTC-CAC-ACC-ACA-AGC-GTA-ACG-AGG-A-3′
	labeled probe (436–464)	5′-GCT-CCT-TGG-TGC-AGC-CTA-CCT-TCT-CCG-AT-3′
RAMP-3	forward primer (188–212)	5′-GGA-GTT-CAT-CGT-GTA-CTA-CGA-AAG-C-3′
	reverse primer (305–279)	5′-CTG-CCT-ATG-GAT-ACC-CGT-GAT-AAA-GC-3′
	labeled probe (235–264)	5′-AGA-CAA-ACA-TCG-TGG-GCT-GCT-ACT-GGC-CCA-3′
GAPDH	forward primer (475–496)	5′-TCC-TGC-ACC-ACC-AAC-TGC-TTA-G-3′
	reverse primer (675–655)	5′-CAC-AGC-CTT-GGC-AGC-ACC-AGT-3′
	labeled probe (548–577)	5′-TGA-CCA-CAG-TCC-ATG-CCA-TCA-CTG-CCA-CTC-3′

have reported that AM secretion is stimulated by angiotensin II (Ang II) in cultured cardiac myocytes (8), although it is unclear whether ET-1 affects the AM production in the myocytes.

In the present study, we examined whether ET-1 stimulates AM production and whether ET-1 modulates intracellular cAMP response to AM by altering the gene expressions of CRLR and RAMPs in cultured cardiomyocytes.

MATERIALS AND METHODS

Chemicals. ET-1 and rat AM were purchased from Peptide Institute, Inc. (Osaka, Japan). Holo-transferrin (human), collagenase (type IV), trypsin, and insulin (bovine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell culture. Primary cultures of cardiomyocytes were prepared from the cardiac ventricles of 1- to 3-day-old Wistar rats. After digestion of the minced ventricles with 0.12% trypsin and 0.03% collagenase, the cells were collected and preincubated for 30 min at 37°C in a culture dish to obtain a medium enriched with cardiomyocytes. Cells not attached to the bottom of the culture dish were plated onto collagen-coated 24-well plates (Sumitomo Bakelite Co. Ltd., Tokyo, Japan) at 1.0×10^5 cells/cm^2 and cultured for 48 h with Dulbecco's modified Eagle medium (DMEM) containing 15 mmol/L *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (Hepes) (pH 7.4), 10% fetal bovine serum (FBS), 10 μg/mL insulin, 10 μg/mL holo-transferrin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphoterisin B, in humidified 5% CO_2/95% air at 37°C. Bromodeoxyuridine was added to the medium at 0.1 mmol/L during this 48 h incubation period to prevent proliferation of the remaining nonmyocytes. At 48 h after seeding, the culture medium was changed to a serum-free medium, a DMEM containing the above-listed chemicals without bromodeoxyuridine, and the cells were cultured for a further 24 h.

This experiment was conducted according to the regulations of the Animal Research Committee of Miyazaki Medical College (1999-039-3).

Assay of AM in conditioned medium. After the cardiomyocytes were incubated with serum-free medium for 24 h, the cells were cultured with serum-free media in the presence or absence of various concentrations of ET-1 for 24 h. After the incubation, the conditioned medium was collected from a 24-well culture plate, followed by acidification with acetic acid to a final concentration of 1.0 mol/L. The medium was then heated at 100°C for 10 min to inactivate protease and was applied to a Sep-Pak C18 cartridge (Millipore-Waters, Milford, MA). After the cartridge was washed with 10% CH_3CN in 0.1% trifluoroacetic acid, the adsorbed materials were eluted with 60% CH_3CN in 0.1% trifluoroacetic acid, lyophilized, and stored at −30°C. The lyophilized samples were dissolved in radioimmunoassay (RIA) buffer and were subjected to RIA for rat AM as described previously (3).

Intracellular cAMP measurement. The cardiomyocytes were exposed to 10^{-8} mol/L ET-1 in serum-free medium for 24 h. Then the cells were washed twice with 1.0 ml/well of assay buffer (Eagle medium containing 0.05% bovine serum albumin and 25 mmol/L Hepes, pH 7.2), and were preincubated with 0.5 ml of assay buffer containing 0.5 mmol/L 3-isobutyl-1-methylxanthine at 37°C. After preincubation for 10 min, AM peptide was added at the indicated concentrations and the incubation was continued for a further 5 min. This incubation time was chosen based on the previous report in which intracellular cAMP concentration in cultured myocytes peaked at 5 to 10 min after adding synthetic AM (18). The reaction was terminated by aspirating the medium and adding 0.5 ml/well of 6% trichloroacetic acid. After scraping the incubated cells, the extracts were washed with water-saturated ethyl ether three times. The cell extracts were then lyophilized and stored at −30°C until assay. cAMP concentrations were measured using a specific RIA as described previously (1).

mRNA measurement by real-time quantitative polymerase chain reaction (PCR). Total RNA Isolation Reagent (Gibco-BRL, Grand Island, NY) was used to extract total RNA from the myocytes, which then underwent reverse-transcription by means of SuperScript reverse transcriptase (Gibco-BRL, Life Technologies, Inc., Gaithersburg, MD) into cDNA. To measure rat CRLR, RAMP1, 2, 3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, we used a novel quantitative PCR method, real time-quantitative PCR (Prism 7700 Sequence Detector, Applied Biosystems, Foster City, CA) as previously reported (19), with the oligonucleotide primers and probes listed in Table 1. The probes were labeled with 6-carboxyfluorescein for reporter fluorescence and with 6-carboxytetramethyl-rhodamine for quencher fluorescence, and cDNAs of CRLR, RAMP1, 2, 3 and GAPDH were amplified with the respective primers. DNA sequence analyses verified that cDNA products amplified by the PCR were identical to those of CRLR, RAMP1, 2 and 3 of rats (13, 20). The PCR products were used as standards and the mRNA levels were compared after they had been normalized relative to those of GAPDH.

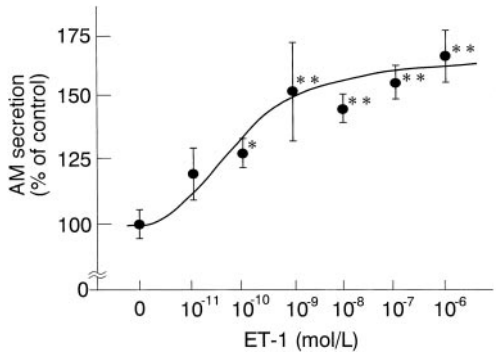

FIG. 1. Effect of ET-1 on AM secretion from cultured cardiac myocytes. Cardiac myocytes were cultured with DMEM containing 10% FBS and 0.1 mmol/L bromodeoxyuridine for 48 h. After the myocytes were washed twice with serum-free DMEM, they were incubated in serum-free media for 24 h. Then, the cells were incubated for a further 24 h with serum-free media in the absence or presence of the indicated doses of ET-1. AM concentrations in the conditioned media were measured as described under Materials and Methods. The basal AM secretion without ET-1 was 7.4 ± 0.1 fmol/10^5 cells/24 h. Values are the means ± SE of four to seven samples examined. $^{**}P < 0.01$, compared with control cells.

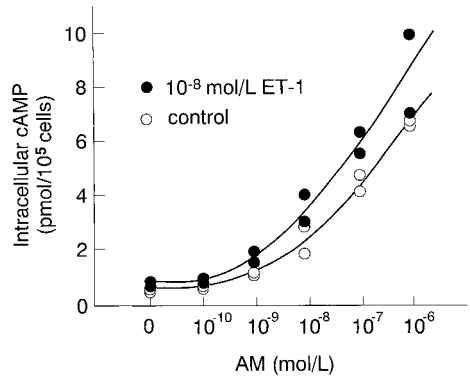

FIG. 2. Intracellular cAMP accumulation stimulated by various concentrations of AM in myocytes preincubated with or without ET-1. After incubation with serum-free media for 24 h, the myocytes were further incubated in the presence or absence of 10^{-8} mol/L ET-1 for 24 h. Then the cells were washed twice with assay buffer and subjected to the experiment for stimulation of intracellular cAMP by AM. The intracellular cAMP concentrations were measured as described under Materials and Methods. Each value represents the intracellular cAMP of an individual well.

Statistical analysis. Multiple comparisons were assessed with one-way ANOVA followed by Scheffe's test. All data were expressed as the means ± SE of the samples examined. $P < 0.05$ was considered significant. All the experiments were repeated at least twice using cells isolated separately from different groups of neonatal rats, and identical results were obtained by the repeated experiments.

RESULTS

Effect of ET-1 on AM Secretion from Cardiomyocytes

To examine the effect of ET-1 on AM secretion, the cardiomyocytes were cultured with serum-free media in the presence or absence of various concentrations of ET-1 for 24 h. As shown in Fig. 1, ET-1 dose-dependently increased the AM secretion from the myocytes.

Effect of ET-1 on Intracellular cAMP Accumulation by AM

To determine whether ET-1 affects intracellular cAMP response to AM, cultured cardiomyocytes were pretreated with ET-1 for 24 h. Figure 2 shows the stimulatory effects of AM on intracellular cAMP in the myocytes preincubated with or without 10^{-8} mol/L ET-1. AM increased the intracellular cAMP level in a dose-dependent manner, and interestingly, the cAMP accumulation by AM was amplified by 24 h preincubation with ET-1. As shown in Fig. 3, the cAMP responses to 10^{-8} and 10^{-6} mol/L AM were significantly augmented by 31% ($P < 0.05$) and 30% ($P < 0.01$), respectively, by the pretreatment with 10^{-8} mol/L ET-1.

Effects of ET-1 on CRLR and RAMPs mRNA Expressions

We measured the mRNA levels of CRLR and RAMPs in the myocytes following incubation with various concentrations of ET-1 by means of a quantitative PCR. ET-1 significantly ($P < 0.01$) increased the CRLR mRNA level at 10^{-8} mol/L, but not at 10^{-10} and 10^{-6} mol/L of concentration (Fig. 4). As shown in Fig. 5, ET-1 had no significant effect on the RAMP1 mRNA expression, while it significantly ($P < 0.01$) reduced RAMP2 mRNA at 10^{-6} mol/L. In contrast to the effect on RAMP2, ET-1 dose-dependently increased the

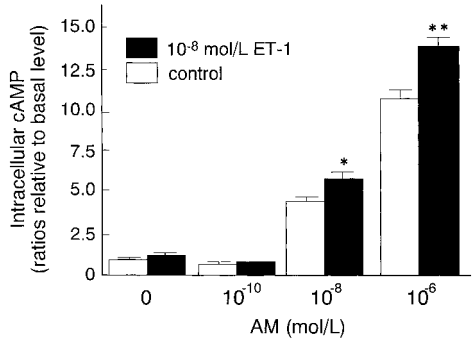

FIG. 3. Intracellular cAMP accumulation stimulated by various concentrations of AM in myocytes preincubated with or without ET-1. After incubation with serum-free media for 24 h, the myocytes were further incubated in the presence or absence of 10^{-8} mol/L ET-1 for 24 h. Then, the cells were washed twice with assay buffer and subjected to the experiment for stimulation of intracellular cAMP by AM. The intracellular cAMP concentrations were measured as described in Materials and Methods. Values represent the means ± SE of four wells examined. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with respective control.

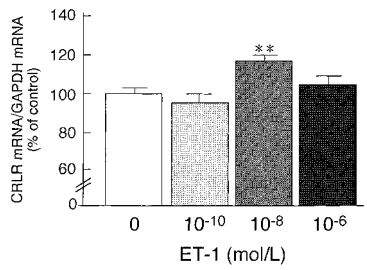

FIG. 4. Effect of ET-1 on CRLR mRNA expression in cardiac myocytes. After incubation with serum-free media for 24 h, the cultured cardiomyocytes were treated with the indicated concentrations of ET-1 for 24 h. CRLR mRNA levels were measured as described in Materials and Methods, and the basal level for CRLR mRNA was at 1.8×10^{-20} mol/μg total RNA in the untreated myocytes. Data are shown as the means ± SE of eight to twelve samples examined. $^{**}P < 0.01$, compared with control cells.

RAMP3 mRNA expression in the myocytes following the incubation for 24 h.

DISCUSSION

AM has been shown to be expressed in various organs and tissues including cardiac atria and ventricles (2, 3, 7, 10). We reported that AM is produced in cultured cardiac myocytes and secreted AM acts to inhibit hypertrophy of these cells in an autocrine or a paracrine fashion (8). Similarly, ET-1 was shown to be produced in the myocytes (15), but contrary to AM, it stimulated cardiac hypertrophy presumably by the activation of protein kinase C (PKC) and the Ca^{2+}-signaling system (17, 21). In the present study, we found that AM production is stimulated dose-dependently by ET-1 in the cardiac myocytes. Since the AM production was found to increase following the activation of PKC and the Ca^{2+}-signaling system in the myocytes (19), ET-1-stimulated AM secretion seems to be mediated by these intracellular signaling pathways, and this finding is also in accord with our hypothesis that AM may participate in a mechanism acting against cardiac hypertrophy as an autocrine or paracrine hormone (8). In fact, according to our unpublished observation, ET-1-stimulated protein synthesis in the myocyte was effectively inhibited in the presence of synthetic AM.

Many of the AM actions have been shown to be mediated by intracellular cAMP: for instance, AM was found to stimulate cAMP production in cultured vascular endothelial and smooth muscle cells (22, 23). The role of intracellular cAMP in the development of cardiac hypertrophy has not been fully explored at present, though we showed that AM stimulates intracellular cAMP accumulation in the cultured cardiac myocytes in the present study. Parkes *et al.* found that AM has a direct, positive inotropic action on the heart when infused intravenously to conscious sheep (24). In addition, Ihara *et al.* showed a positive inotropic effect of AM on the papillary muscle of the rat cardiac ventricle by an *ex vivo* experiment (25). They also reported that this AM effect was augmented by 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, and was attenuated by inhibitors of protein kinase A, suggesting an inotropic action via an increased intracellular cAMP level. Produced by cardiac myocytes, ET-1 has been shown to possess a positive iontropic effect on the heart (16). In the present study, ET-1 was found not only to stimulate the AM secretion, but also to enhance the cAMP response to AM in the cultured cardiac myocytes. It has been reported that both the

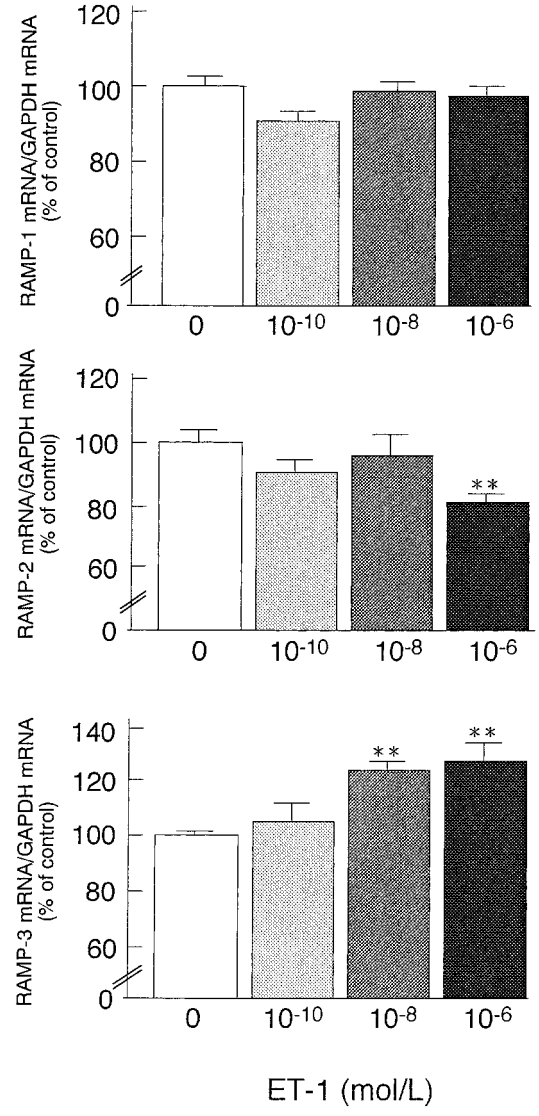

FIG. 5. Effect of ET-1 on RAMPs mRNA expressions in cardiac myocytes. After incubation with serum-free media for 24 h, the cultured cardiomyocytes were treated with the indicated concentrations of ET-1 for 24 h. The basal levels for RAMP-1, 2 and 3 mRNA were at 8.1, 7.1 and 2.0×10^{-20} mol/μg total RNA, respectively. Data are shown as the means ±SE of eight to twelve samples examined. $^{**}P < 0.01$, compared with control cells.

plasma and myocardial ET-1 concentrations are elevated in patients with congestive heart failure (26, 27), and Jougasaki *et al.* found an increased AM immunoreactivity in failing cardiac ventricles of humans (7). Based upon these findings, we may be able to raise a possibility for ET-1 modulating cardiac contractility partly by stimulating AM production or by enhancing intracellular cAMP accumulation by AM. Meanwhile, since the pharmacological doses of ET-1 and AM were used in the present study to significantly amplify the cAMP response, this hypothesis must be tested by future *in vivo* experiments.

Consisting of 52 amino acid residues, AM belongs to a calcitonin-gene-related peptide (CGRP) superfamily, while there has been substantial controversy over the subtypes of the AM or CGRP receptors that are linked to adenylate cyclase. Recently, McLatchie *et al.* have reported that the calcitonin receptor-like receptor (CRLR) functions as either a specific receptor for CGRP or AM, depending on the expression of the three types of receptor-activity-modifying proteins (RAMP1 to 3) (11). CRLR coexpressed with RAMP1 serves as a CGRP receptor, while it functions as an AM receptor when coexpressed with either RAMP2 or 3. Nagae *et al.* showed a more abundant mRNA expression of RAMP2 than RAMP1 and 3 in the rat heart (13), and Tomoda *et al.* reported that RAMP1 and 3 are expressed in cultured rat cardiac myocytes without a detectable level of RAMP2 mRNA using a conventional Northern blot method (20). In the present study, we found that genes of all the components of CRLR and RAMP1 to 3 are expressed in the myocytes by means of a novel quantitative PCR, a method that is more sensitive and accurate than the Northern blot analysis.

Frayon *et al.* found that glucocorticoid upregulates the CRLR and RAMP1 mRNA expression in cultured vascular smooth muscle cells (28), although there is currently very limited information about the regulation of the gene expressions of CRLR and RAMPs. To clarify the cellular mechanism for the amplified cAMP response to AM by ET-1, we measured the CRLR and RAMPs mRNA levels in the myocytes treated with ET-1 using quantitative PCR and compared with those in the untreated cells. As shown in Figs. 4 and 5, ET-1 reduced the RAMP2 expression, while it dose-dependently upregulated the RAMP3 mRNA level with increased CRLR mRNA at 10^{-8} mol/L in the myocytes. Since CRLR coexpressed with RAMP3 is shown to function as an AM receptor, the ET-1-induced RAMP3 expression accompanied with increased CRLR mRNA may account at least in part for the augmented intracellular cAMP response to AM in the cultured cardiac myocytes.

In summary, ET-1 stimulates AM production from cultured rat cardiomyocytes and augments intracellular cAMP accumulation with upregulation of the RAMP3 mRNA expression, suggesting the possibility of local interaction between AM and ET-1 in rat cardiac myocytes.

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