Full-length article

Ca^{2+} participates in α_{1B}-adrenoceptor-mediated cAMP response in HEK293 cells[1]

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Key words

alpha-1 adrenergic receptors; HEK293 cells; cyclic AMP; signal transduction; phospholipase C; protein kinase C; protein-tyrosine kinase; calcium

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Abstract

Aim: To investigate the α_{1B}-adrenoceptor (α_{1B}-AR)-mediated cAMP response and underlying mechanisms in HEK293 cells. **Methods:** Full-length cDNA encoding α_{1B}-AR was transfected into HEK293 cells using the calcium phosphate precipitation method, and α_{1B}-AR expression and cAMP accumulation were determined by using the saturation radioligand binding assay and ion-exchange chromatography, respectively. **Results:** Under agonist stimulation, α_{1B}-AR mediated cAMP synthesis in HEK293 cells, and blockade by PLC-PKC or tyrosine kinase did not reduce cAMP accumulation induced by NE. Pretreatment with pertussis toxin (PTX) had little effect on basal cAMP accumulation as well as norepinephrine (NE)-stimulated cAMP accumulation. In addition, pretreatment with cholera toxin (CTX) neither mimicked nor blocked the effect induced by NE. The extracellular Ca^{2+} chelator egtazic acid (EGTA), nonselective Ca^{2+} channel blocker $CdCl_2$ and calmodulin (CaM) inhibitor W-7 significantly reduced NE-induced cAMP accumulation from 1.59%±0.47% to 1.00%±0.31%, 0.78%±0.23%, and 0.90%±0.40%, respectively. **Conclusion:** By coupling with a PTX-insensitive G protein, α_{1B}-AR promotes Ca^{2+} influx via receptor-dependent Ca^{2+} channels, then Ca^{2+} is linked to CaM to form a Ca^{2+}-CaM complex, which stimulates adenylyl cyclase (AC), thereby increasing the cAMP production in HEK293 cell lines.

Introduction

It is well known that the classic signaling pathway of α_1-adrenoceptor (AR) is to couple with the $G_{q/11}$ protein and then stimulate phosphatidylinositol turnover[1,2]. However, it has been recently discovered that α_1-AR can also stimulate adenosine 3':5'-cyclic monophosphate (cAMP) accumulation in several cell lines, tissues and organs[3–12]. Our previous study showed that each of the three α_1-AR subtypes was able to mediate cAMP generation in human embryonic kidney 293 (HEK293) cells. As for the signal transduction pathway by which α_1-AR mediates cAMP production, several studies have shown that cAMP production is secondary to the α_1-AR-induced phospholipase C (PLC)-phosphokinase C (PKC) stimulation[10–12], which cross-talked with AC[13]. Horie *et al*[14] found that in a Chinese hamster ovary (CHO) cell line transfected with α_{1B}-AR, none of the PKC inhibitor, the Ca^{2+} ionophore, or the pertussis toxin (PTX) was able to inhibit NE-induced cAMP accumulation, whereas anti-$G_{s\alpha}$ antiserum inhibited the response, which suggests that α_{1B}-AR activates AC and increases intracellular cAMP by directly coupling with $G_{s\alpha}$. In fact the signal transduction pathway involved in α_{1B}-AR-mediated cAMP generation remains unclear. Therefore, we transfected HEK293 cells (human embryonic kidney 293 cell line, which does not express any other adrenoceptors except native β_2-AR) with full-length cDNA encoding α_{1B}-AR and selected for subcloning cell lines stably expressing α_{1B}-AR. This was a good model to investigate the regulating effects of the PLC-PKC pathway, the tyrosine kinase pathway, the Ca^{2+} signal system, and G proteins on cAMP production.

Materials and methods

Norepinephrine (NE), phenylephrine (PE), methoxamine (ME), prazosin (PRZ), propranolol (Prop), Triton X-100,

cAMP, 3-isobutyl-1-methyl-xanthine (IBMX), pyruvic acid, hygromycin B, cyclopiazonic acid (CPA), phorbol 12-myristate 13-acetate (PMA), genistein, tyrphostin A25, egtazic acid (EGTA), nifedipine (Nif), $CdCl_2$, PTX, and cholera toxin (CTX) were from Sigma Chemical Co (St Louis, USA); BAPTA/AM, Ro-31-8220, calphostin C, and W-7 were products of Calbiochem-Novabiochem International (San Diego, USA). BE2254 {[2-β(4-hydroxyphenyl)-ethylaminomethyl]-tetralone} was from Beiersdorf Co (Hamburg, Germany); [^{3}H]adenine was from Amersham Biosciences (Piscataway, United States); 2,5-diphenyl-oxazole (PPO) was bought from Farco-Pharma (Koln, Germany); fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were from Hyclone China (Beijing, China); HEK293 (human embryonic kidney 293) cells and full-length cDNA of hamster α_{1B}-AR (pREP4) were kindly provided by Prof Kenneth P MINNEMAN (Emory University, USA).

Transfection of HEK293 cells with cDNA encoding α_{1B}-AR using the calcium phosphate precipitation method HEK293 cells were cultured in DMEM containing 10% FBS at 5% CO_2 at 37 ºC. The cells were transfected with pREP4/α_{1B}-AR by calcium phosphate precipitation at 70% confluence and selected with hygromycin B (0.05 g/L). Three days later the cells were diluted and planted in a 96-well dish, with 0-5 cells in each well. After 2 to 3 cloning sessions, a cell line stably expressing α_{1B}-AR was obtained. The cells were continuously cultured and kept in DMEM containing selective antibiotics.

Determination of α_{1B}-AR expression by a saturation radioligand binding assay The cells were grown in 75-mL flasks and harvested in PBS. After centrifugation at 3000×*g*, 4ºC for 10 min then at 21 000×*g*, 4ºC, for 20 min, the pellet was resuspended with 30 mL PBS and kept on ice. The ligand-binding properties of the receptors were determined in a series of radioligand binding studies using the α_1-AR antagonist radioligand [^{125}I]BE2254, which was radioiodinated to theoretical specific activity as described by Engel and Hoyer[19]. Saturation reactions (total volume 250 μL) containing 100 μL or 50 μL PBS with 1% bovine serum albumin (BSA), 50 μL of [^{125}I] BE2254 at different concentrations (15 000-500 000), and 100 μL cell membranes. Nonspecific binding was determined in the presence of 50 μmol/L phentolamine. Reactions were allowed to proceed for 20 min at 37 ºC. Reactions were terminated by adding 7 mL ice-cold Tris-HCl buffer (pH 7.4) and the mixture was filtered onto glass fiber filters. Filters were washed twice with 7 mL ice-cold Tris-HCl buffer and then dried. Bound radioactivity was measured using an auto-gamma counter. Binding data were analyzed by using nonlinear regression and Scatchard analysis (GraphPad Prizm Software) on a computer and thus the dissociation constant (K_D) between the receptor and antagonist and maximal bound capacity (B_{max}) could be obtained. Protein content was determined by using the Coomassie protein quantitation method.

cAMP determination in intact cells using ion-exchange chromatography HEK293 cells expressing α_{1B}-AR were cultured in 24-well dishes at 37 ºC, 5% CO_2, with 2.5×10^5 cells per mL medium. When the cells were fully confluent, the medium was changed, and [^{3}H]adenine 18.5 MBq (0.5 mCi) was added into each well. After incorporation for 4 h at 37 ºC in 5% CO_2 , the medium was discarded and the cells were washed twice with pre-warmed Krebs' solution. After addition of antagonists in 1 mL Krebs' solution containing 200 μmol/L IBMX and incubation for 30 min, the cells were incubated for a further 20 min with different concentrations of agonists. The reaction was terminated by adding 100 μL of 77% trichloracetic acid, followed by a centrifugation at 3000×*g*, 4 ºC, for 20 min. Then 50 μL supernatant was transferred into 3 mL scintillation liquid to measure radioactivity as total activity (cpm). The remaining supernatant was applied to Dowex columns and aluminal columns. After being washed with distilled water, the aluminal columns were eluted with 2 mL Tris-HCl (pH 8.0), and the radioactivities of the eluates were measured as newly-produced cAMP. cAMP accumulation is equal to proportion of total radioactivity represented by newly-produced cAMP as a percentage. The formula is as follows:

$$\text{cAMP accumulation} = \frac{\text{Radioactivity of newly-produced cAMP (cpm)} \times 100\ \%}{\text{Total radioactivity (cpm)} \times 22}$$

Twenty-two is the volume constant.

Statistical analysis Results are expressed as mean±SD. To compare mean values between two groups, Student's *t*-test was used; ANOVA was used for comparison among three or more groups. $P<0.05$ was considered statistically significant.

Results

Density of α_{1B}-AR HEK293 cells transfected with α_{1B}-AR were cultured under selective pressure from hygromycin B for several passages, thus we obtained a cell line stably expressing α_{1B}-AR. The density of α_{1B}-AR was 2004±138 pmol/g as determined by a radioligand binding assay (n=5, data not shown).

cAMP accumulation induced by stimulation of α_{1B}-AR In blank HEK293 cells , NE (100 nmol/L–30 μmol/L),

PE (100 nmol/L–300 μmol/L), or ME (1 μmol/L–1 mmol/L) did not cause cAMP accumulation in the presence of propranolol, an antagonist of β-AR (data not shown).

However, NE, PE, and ME all increased cAMP accumulation in HEK293 cells transfected with α_{1B}-AR in a dose-dependent manner, with R_{max}/pD_2 of (3.32%± 0.34%)/(6.15±0.33) (n=7), (2.43%±0.46%)/(5.37±0.55) (n=8), and (0.66%±0.17%)/(3.79±0.39) (n=6), respectively (Figure 1), all of which were antagonized by prazosin (100 nmol/L) (Data not shown).

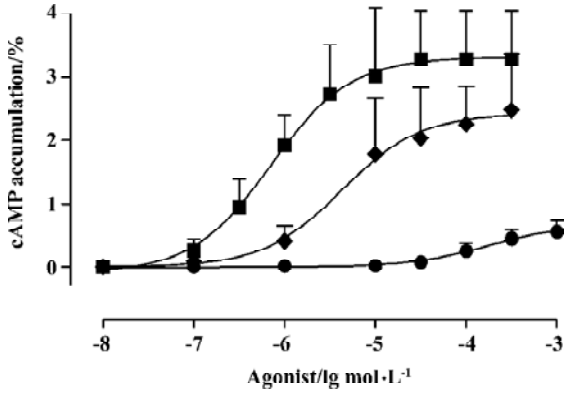

Figure 1. NE (■, n=7), PE (◆, n=8) and ME (●, n=6) induced cAMP synthesis in a dose-dependent manner in the presence of propranolol (1 μmol/L) in HEK293 cells transfected with α_{1B}-AR.

PLC-PKC pathway In the presence of propranolol (10 μmol/L) to block β_2-AR in HEK293 cells, NE (10 μmol/L) increased cAMP accumulation from a basal level of 0.28%± 0.07% to 4.93%±1.13% (n=11, P<0.01). The PLC inhibitor, U73122, had no effect on the NE-induced cAMP accumulation at 1 μmol/L (4.91%±1.43%, n=11) or 10 μmol/L (4.97%±1.15%, n=11, Fig 2A) for 1 h incubation. Neither U73122 itself (0.26%±0.09%, n=5) nor the vehicle alone (Me_2SO, 0.1% v/v final, 0.26%±0.05%, n=3) affected the basal level of cAMP (0.28%±0.07%, n=11). Similarly, when PKC inhibitors were added, neither Ro31-8220 (5.37%± 1.33%) and (4.99%±1.56%, n=8) nor calphostin C (5.14%± 1.09%) and 4.70%±1.28%, n=8) at 10 nmol/L and 100 nmol/L affected NE-induced cAMP production (4.93%± 1.13%, n=11, Figure 2B). The two inhibitors had no effect on the basal cAMP level (0.24%±0.07%, n=5, and 0.22%± 0.09%, n=5, respectively, *vs* 0.28%±0.07%, n=11). To further clarify the role of PKC in this response, we examined whether PKC activator had any effect on cAMP synthesis. Without any AR agonist or antagonist, pretreatment with 1, 10, and 100 nmol/L PMA did not increase cAMP synthesis,

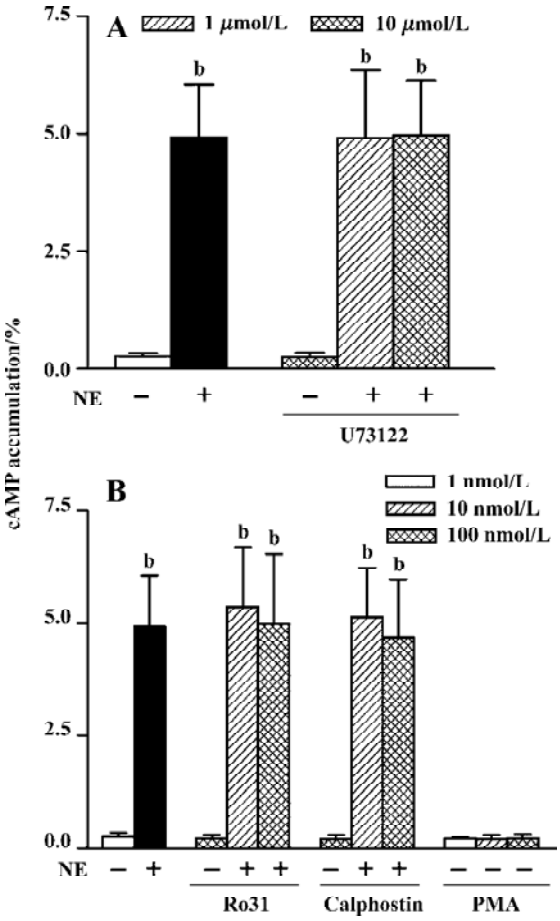

Figure 2. (A) NE-induced cAMP accumulation and the effect of U73122 (n=11) in the presence of 10 μmol/L propranolol in HEK293 cells expressing α_{1B}-AR. (B) NE-induced cAMP accumulation and the effects of Ro31 and calphostin C (n=8) in the presence of 10 μmol/L propranolol and cAMP accumulation in the presence of PMA at 1–100 nmol/L (n=7), respectively. Mean±SD. ^bP<0.05 *vs* basal accumulation.

the levels of which were (0.23%±0.03%, n=7), (0.22%± 0.08%, n=7), and (0.24%±0.08%, n=7), respectively, showing no significant difference compared with the basal level (0.28%±0.07%, n=11).

Tyrosine kinase signaling pathway The effects of two kinds of tyrosine kinase inhibitors, tyrphostin A25 and genistein, on NE-induced cAMP synthesis were examined. Tyrphostin A25 and genistein (0.30%±0.07% and 0.30%± 0.04%, n=5, respectively) or the vehicle alone (Me_2SO, 0.1% v/v final, 0.26%±0.03%, n=3) had no effect on the basal cAMP level (0.28%± 0.07%, n=11). After pretreating cells with tyrphostin A25 or genistein at 1 μmol/L and 10 μmol/L for 1 h, neither tyrphostin A25 (4.98%±1.33% and 4.75%± 1.07% at the two concentrations, respectively, n=7) nor genistein (4.97%±1.30% and 4.69%±0.62%, respectively,

n=7) influenced NE-induced cAMP synthesis (4.93%± 1.13%, *n*=11 in the presence of propranolol 10 μmol/L) (Figure 3).

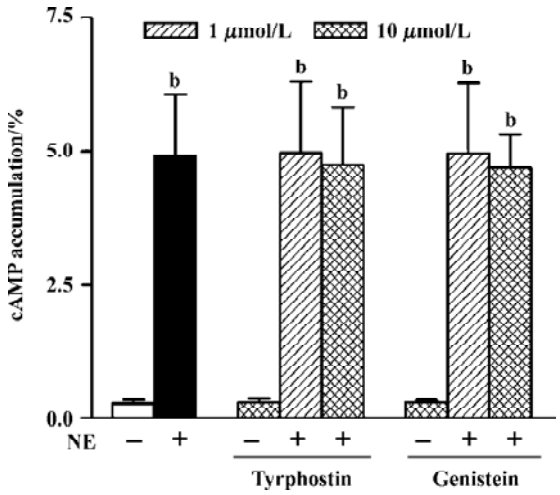

Figure 3. The effects of tyrphostin A25 and genistein (*n*=7) on NE-induced cAMP accumulation in the presence of 10 μmol/L propranolol in HEK293 cells transfected with α_{1B}-AR. Mean±SD. $^{b}P<0.05$ *vs* basal accumulation.

Regulation of cAMP response by G proteins After incubating cells with PTX 500 μg/L for 16 h, the effects of PTX on basal or NE-induced cAMP accumulation were examined. The results showed that PTX neither had any effect on basal cAMP (0.31%±0.07% *vs* 0.23%±0.07%, *n*=8) nor affected the NE-induced cAMP response (1.76%±0.50% *vs* 1.52%±0.44%, *n*=8, Figure 4). In order to examine whether α_1-AR mediates cAMP response by directly coupling with the G_s protein, we compared cAMP accumulation upon stimulation of the G_s protein after incubation with 500 μg/L CTX for 3 h with NE-induced cAMP accumulation. The results showed that cAMP accumulation induced by CTX alone was significantly higher than that induced by 10 μmol/L NE (2.50%±0.60% *vs* 1.52%±0.44%, *n*=8, *P*<0.05), and when CTX and NE were used simultaneously, cAMP accumulation (5.24%±1.37%, *n*=8) was significantly higher than their combined effect when used alone (*P*<0.05, Figure 4).

Regulating effects of Ca^{2+} signaling system Because α_1-AR can induce the release of the IP_3-sensitive Ca^{2+} store, CPA, a Ca^{2+}-ATPase inhibitor, was used to block Ca^{2+} being taken in sarcoplasmic reticulum again so as to increase $[Ca^{2+}]_i$ and deplete the Ca^{2+} store. The results showed that CPA 10 μmol/L alone did not increase cAMP accumulation (0.20%±0.03% *vs* 0.22%±0.12%, *n*=8), neither did it have any effect on NE-induced cAMP production (1.63%±0.56%, *n*=8 *vs* 1.59%±0.47%, *n*=9, Figure 5A).

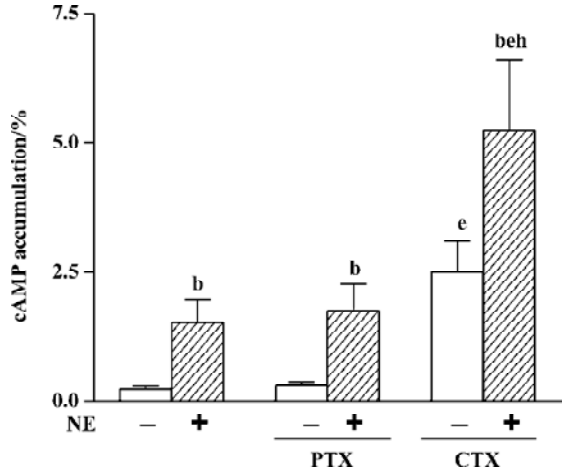

Figure 4. The effects of PTX 500 μg/L and CTX on basal and NE-induced cAMP accumulation (*n*=8) in the presence of propranolol 10 μmol/L in HEK293 cells transfected with α_{1B}-AR. Mean±SD. $^{b}P<0.05$ *vs* basal accumulation. $^{e}P<0.05$ *vs* NE-induced cAMP accumulation. $^{h}P<0.05$ *vs* CTX-induced cAMP accumulation.

In addition, the influence of the Ca^{2+} influx on cAMP synthesis was studied. It was found that NE-induced cAMP accumulation under Ca^{2+}-free condition was equal to that when Ca^{2+} was present (1.63%± 0.40% *vs* 1.59%±0.47%, *n*=9). However, when an extracellular Ca^{2+} chelator, EGTA (50 μmol/L), was added in Ca^{2+}-free Krebs buffer to pretreat the cells for 1 h, the NE-induced cAMP synthesis was obviously reduced (1.00%±0.31%, *n*=9, *P*<0.05, Figure 5B), whereas 10 μmol/L intracellular Ca^{2+} chelator, BAPTA/AM, had no effect on the NE-induced cAMP response under Ca^{2+}-free conditions (1.62%±0.58% *vs* 1.59±0.47%, *n*=9). There was no further inhibition on cAMP synthesis when BAPTA was used in combination with EGTA (1.03%±0.28% *vs* 1.00%±0.31%, *n*=9, Figure 5B).

To find out what type of Ca^{2+} channel admitted Ca^{2+}, we examined the effects of two kinds of Ca^{2+} channel blockers on cAMP response and found that nifedipine 10 μmol/L (L-type Ca^{2+} channel blocker) did not affect the NE-induced cAMP synthesis (1.47%±0.38% *vs* 1.59%±0.47%,*n*=9), whereas $CdCl_2$ 1 mmol/L (nonselective Ca^{2+} channel blocker) obviously reduced NE-induced cAMP accumulation (from 1.59%±0.47% to 0.78%±0.23%, *n*=9, *P*<0.05, Figure 5C). Moreover, when pretreating cells with W-7, a calmodulin (CaM) inhibitor, NE-induced cAMP synthesis decreased markedly (0.90%± 0.40%, *n*=8, *P*<0.05, Figure 5C), and the extent of decrease was similar to that induced by EGTA or $CdCl_2$ (*P*>0.05), while neither of them had any effect on the basal cAMP level.

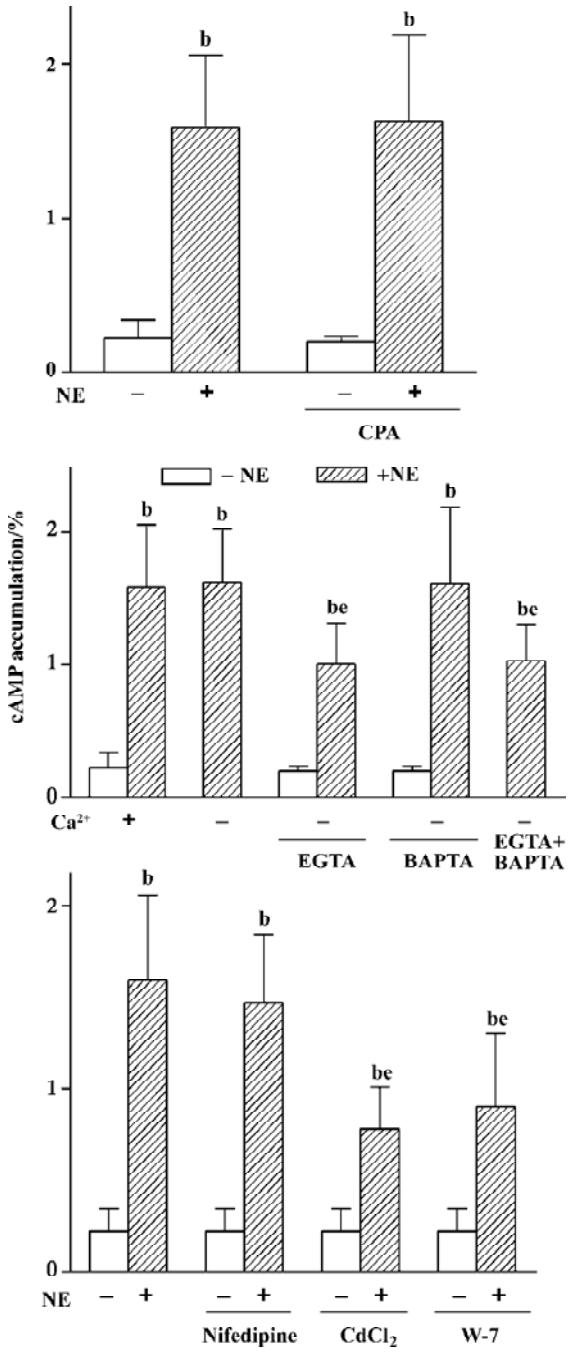

Figure 5. (A) NE-induced cAMP accumulation (n=9) and the effect of CPA 10 μmol/L (n=8) in the presence of propranolol 10 μmol/L in HEK293 cells transfected with α_{1B}-AR. (B) NE-induced cAMP response in Krebs' buffer and in Ca^{2+}-free Krebs' buffer, and the effects of EGTA 50 μmol/L, BAPTA 10 μmol/L , and EGTA plus BAPTA (n=9) on NE-induced cAMP response under Ca^{2+}-free conditions in the presence of propranolol 10 μmol/L. (C) NE-induced cAMP response and the effects of nifedipine 10 μmol/L (n=9), $CdCl_2$ 1 mmol/L (n=9), and W-7 500 μmol/L (n=8) in the presence of propranolol 10 μmol/L in HEK293 cells transfected with α_{1B}-AR. Mean±SD. $^bP<0.05$ *vs* basal accumulation. $^eP<0.05$ *vs* NE-induced cAMP accumulation.

Discussion

Several studies have demonstrated that α_{1B}-AR mediates the cAMP response in HEK293 cells. However, the signaling pathway through which α_1-AR mediates the cAMP response is unknown. Many studies have indicated that besides its classical signaling pathway, α_1-AR can stimulate many other signal transduction pathways as well, such as the tyrosine kinase pathway[15-18], the phospholipase A_2-arachidonic acid (PLA_2-AA) signaling system[20], etc. Moreover, α_1-AR also associates with other signaling pathways through its classical signal transduction pathway and consequently produces cross-talk. cAMP is a second messenger, a product of ATP catalyzed by adenylyl cyclase (AC), and a substrate of phosphodiesterase (PDE). There are at least 10 isozymes of AC. Besides the G_s and G_i proteins, many factors have been found to regulate their activities[21,22]. In the present study, we investigated the underlying mechanism involved in α_1-AR-mediated cAMP synthesis using HEK293 cells transfected with α_{1B}-AR.

PLC-PKC pathway α_1-AR preferentially activates its classical signaling pathway. It stimulates PLC by coupling with the $G_{q/11}$ protein, then PLC hydrolyzes PIP_2 to produce IP_3 and DAG, which induces Ca^{2+} release and stimulates PKC, thereby producing biological effects. It has been reported that in some cell lines transfected with α_1-AR subtypes, α_1-AR first stimulates PI turnover to activate PKC, and then stimulates AC. This means that α_1-AR-mediated cAMP response is secondary to the PLC-PKC pathway[10-12]. However, our results indicated that neither the PLC antagonist, U73122, nor the PKC antagonists, Ro31 and calphostin C, inhibited α_1-AR-mediated cAMP synthesis, and the PKC activator, PMA, did not increase cAMP accumulation. Furthermore, there was no influence on cAMP synthesis when using the Ca^{2+}-ATPase inhibitor, CPA, to block Ca^{2+} restoration and thus deplete the Ca^{2+} pool. All of the above suggests the taches lying in downstream of PLC in the classical pathway are not connected with α_1-AR-mediated cAMP response.

Tyrosine kinase signal transduction pathway The tyrosine kinase signal transduction pathway relates closely to the classical signaling pathway of α_1-AR. Some studies have shown that tyrosine kinase probably participates in α_1-AR-mediated biological effects[15-18]. In our previous study, we found that the major functional α_1-AR subtype of the vascular bed in rat hind legs was α_{1A}-AR, and that tyrosine kinase antagonists could dose-dependently reduce the vascular contractive response to NE. Moreover, tyrosine kinase antagonists can inhibit α_1-AR-mediated increase in intracellular Ca^{2+} in HEK293 cells[23], showing that α_1-AR can

activate tyrosine kinase. It is also known that tyrosine kinase can indirectly stimulate AC through the PLC-γ-IP_3/DAG pathway, which results in intracellular Ca^{2+} mobilization and stimulation of PKC[22]. Since we have confirmed that α_1-AR does not mediate cAMP synthesis through the PLC-PKC pathway in HEK293 cells, the possibility mentioned above can be excluded. Although it is known that tyrosine kinase can indirectly stimulate AC through the PLC-γ-IP_3/DAG pathway[21], this would not happen in HEK293 cells because our study indicated that the PLC-PKC pathway was not involved in α_1-AR-mediated cAMP accumulation. However, whether tyorsine kinase plays a role through other pathways or taches is unclear. In our present study, two kinds of tyrosine kinase inhibitors, different in structure and mechanism, exerted no influence on NE-induced cAMP synthesis, suggesting that tyrosine kinase is not involved in α_{1B}-AR-mediated cAMP response in HEK293 cells.

Regulation of cAMP response by G proteins α_1-AR is a typical G protein coupled receptor, whose classical signaling pathway is to couple with the $G_{q/11}$ protein. But it has been found that α_1-AR also couples with G_s and G_i proteins[14,24]. Overexpressed α_1-AR in the heart of transgenic mice can couple with the G_i protein and inhibit AC[24]. Horie *et al*[14] found that stimulation of transfected α_1-AR directly activated G_s and increased cAMP accumulation. These phenomena occur when the density of α_1-AR is much higher than normal. Some researchers[14,25] consider that receptor-G promiscuity happens when receptors are overexpressed, ie, receptors not only couple with their traditional G proteins, but also couple with irrelevant G proteins under normal conditions, and then produce new biological effects. This phenomenon is called receptor-G protein promiscuity, which would occur when receptors are overexpressed[14,25]. Since our experiments were performed under similar conditions, this leads to the following question: did promiscuity occur between α_1-AR and other G proteins? Firstly, the results showed α_{1B}-AR-mediated cAMP response was not affected by PTX, thereby excluding the possibility that G_i protein or $G_{\beta\gamma}$, which is sensitive to PTX[27] and activates ACII and IV[21,22,28], which is involved in the response. Secondly, cAMP accumulation induced by CTX alone was markedly higher than that induced by NE, and when CTX and NE worked together cAMP accumulation was far higher than the sum of cAMP accumulation when each of them worked alone. The response induced by NE 10 μmol/L almost reached a maximum, and since there is no receptor reserve in cloned HEK293 cells[25], if α_{1B}-AR directly coupled with the G_s protein while the number of G_s were relatively insufficient or equally by the number of α_{1B}-AR, cAMP accumulation induced by NE should equal that induced by CTX, and should not increase further when NE was added together with CTX. However, it did not occur. Thus there are two possibilities, one is that α_{1B}-AR directly couples with the G_s protein but that the amount of G_s protein far exceeds what α_{1B}-AR requires; another is that α_{1B}-AR itself does not couple with the G_s protein, which is more likely, because in our study, stimulation of α_{1B}-AR and the G_s protein at the same time induced a synergistic effect. In addition, the G_α, $G_{\beta\gamma}$ subunit should not be ignored, because it has been shown to activate ACII and IV[21,22,28]. However, because $G_{\beta\gamma}$ is PTX-sensitive[27], and PTX did not inhibit NE-induced cAMP sythesis in our study, we conclude that $G_{\beta\gamma}$ does not participate in the response.

Regulating effects of Ca^{2+} signaling system α_1-AR can induce the mobilization of intracellular Ca^{2+} as well as the influx of extracellular Ca^{2+}. As the most ubiquitous and most active second messenger in cells, Ca^{2+} not only mediates many physiological effects directly, but also regulates many signaling pathways and molecules. It has been shown that the Ca^{2+}-CaM complex can stimulate ACI, III, and VIII[21,22]. For this reason the effect of Ca^{2+} on α_{1B}-AR-mediated cAMP response was investigated. Our results showed that the Ca^{2+}-ATPase inhibitor, CPA, had no effect on NE-induced cAMP accumulation, indicating that mobilization of intracellular Ca^{2+} was not involved in the response. NE-induced cAMP accumulation was not reduced under Ca^{2+}-free condition, but reduced after addition of the extracellular Ca^{2+} chelator, EGTA. We speculate that it is because even under Ca^{2+}-free conditions, there inevitably existed a little Ca^{2+} in the buffer, which is enough to meet with the needs of the α_{1B}-AR-mediated cAMP response. When extracellular Ca^{2+} is chelated by EGTA, Ca^{2+} cannot flow into cells, thereby reducing cAMP production. So cAMP production was reduced. All of the above shows that extracellular Ca^{2+} influx plays a role in the α_{1B}-AR-mediated cAMP response. It is well known that extracellular Ca^{2+} enters cells mainly via voltage-dependent Ca^{2+} channels (VDCC) or voltage-independent Ca^{2+} channels, which includes Ca^{2+} store depletion-dependent Ca^{2+} channels (SDDCC), receptor-dependent Ca^{2+} channels, and so on. Then arises the new question of what kind of channel is involved in this response. It has been proving that there is no VDCC in HEK293 cells[29], and in our study, the L-type Ca^{2+} channel inhibitor nifedipine did not affect NE-induced cAMP sythesis. The nonselective Ca^{2+} channel inhibitor $CdCl_2$ obviously reduced NE-induced cAMP accumulation, and the extent of reduction was the same as that with EGTA. If these facts are taken together with the fact that CPA-induced Ca^{2+} store depletion did not increase cAMP

accumulation, it seems that it was via receptor-dependent Ca^{2+} channels that extracellular Ca^{2+} entered the cells. CaM inhibitor W-7 significantly reduced NE-induced cAMP accumulation, with the extent of reduction almost equal to that induced by EGTA and $CdCl_2$, which indicates that it is Ca^{2+}-CaM that activates AC. Intracellular Ca^{2+} chelator BAPTA can chelate Ca^{2+} released from the Ca^{2+} store as well as Ca^{2+} entering cells from the outside, so theoretically it should have an effect similar to EGTA. In fact, it neither inhibited NE-induced cAMP synthesis nor enhanced the inhibitory effect of EGTA. As for the reason why intracellular Ca^{2+} chelator BAPTA could not inhibit NE-induced cAMP synthesis, we suppose either that the penetrability of BAPTA/AM into HEK293 cells was rather weak, or that the activity of esterase in HEK293 cells is so low that BAPTA/AM cannot be degraded to active BAPTA. Furthermore, our previous study showed that $[Ca^{2+}]_i$ increased to 4-5 times the basal level 8 s after activation of α_1-AR. Active BAPTA was perhaps not enough to chelate all intracellular Ca^{2+} completely and rapidly, so BAPTA/AM had little effect. However, the exact mechanism by which the system functions remains to be studied. In summary, the results suggest that stimulation of α_{1B}-AR triggers the receptor-dependent Ca^{2+} channel via an unknown pathway and lets extracellular Ca^{2+} in, then Ca^{2+} links CaM into a Ca^{2+}-CaM complex, which activates AC to increase cAMP synthesis. However none of EGTA, $CdCl_2$, and W-7 are able to completely inhibit NE-induced cAMP accumulation, suggesting that the Ca^{2+} influx is only partly involved in the response.

In conclusion, by coupling with a PTX-insensitive G protein, α_{1B}-AR promotes Ca^{2+} influx via receptor-dependent Ca^{2+} channels, then Ca^{2+} links to CaM to form a Ca^{2+}-CaM complex, which stimulates adenylyl cyclase (AC) and thereby increases the cAMP production in HEK293 cell lines. But Ca^{2+} only partly contributes to α_{1B}-AR-mediated cAMP accumulation. The other mechanisms remain to be investigated.

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