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Key amino acids for differential coupling of α1-adrenergic receptor subtypes to Gs☆

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Abstract

We have established that differing effects of α1-adrenergic receptor (AR) subtypes on cell proliferation are due to differential coupling to the Gs/cAMP pathway; thus, both α1A- and α1B-ARs couple to Gs, while α1D-AR does not. To identify the region responsible for this difference in subtype-specific Gs coupling, we constructed a series of chimeric and a set of point-mutated human α1A- and α1D-ARs, and examined their signaling ability. Here, we show that the amino acid residues Thr 136 and Val138 in the intracellular loop II of the human α1A-AR are intimately involved with Gs coupling.

Keywords: α1A-AR; α1D-AR; Chimeric-receptor; Cell proliferation; Gs; cAMP production; Ca^{2+}

α1-adrenergic receptors (ARs) are members of the G protein-coupled receptor superfamily, which mediate the effects of the sympathetic nervous system, including smooth muscle contraction, cell proliferation and hypertrophy, and hepatic glucose metabolism [1–6]. The α1-ARs mediate their physiological effects by activating phospholipase C, resulting in production of inositol 1,4,5-triphosphate, mobilization of intracellular calcium and diacylglycerol, and finally activation of protein kinase C [7,8]. More recently, it has been demonstrated that the α1-ARs can also influence a variety of other effectors, such as the mitogen-activated protein kinase (MAPK) pathway, cAMP metabolism, and activation of phospholipases D and A2 in different cells [9–14]. α1-ARs comprise a heterogeneous family and molecular cloning studies have identified three distinct cDNAs encoding α1-AR subtypes (α1a, α1b, and α1d) [15–18]. Although the pharmacological properties of three distinct α1-AR subtypes (α1A-AR, α1B-AR, and α1D-AR) are well documented, little is known regarding differences in their physiological roles and signaling pathways.

We have previously reported that α1B-AR couples not only to the Gq/Ca^{2+} signaling pathway, but also the Gs/cAMP pathway via direct interaction with Gs [19]. Furthermore, we have recently observed that the activation of α1A- or α1B-AR inhibits serum-promoted cell proliferation, whereas the activation of α1D-AR has a potent growth-promoting effect. This subtype-dependent anti-proliferative effect might be caused by cAMP-dependent inhibition of serum-promoted down-regulation of $p27^{kip1}$ (submitted for publication).

In the present study, we have constructed a series of human chimeric α1A and α1D-ARs (α1A/α1D-CRs) to identify the region responsible for the difference between α1A-AR and α1D-AR in serum-stimulated cell proliferation and signaling pathways.

☆ *Abbreviations:* AR, α1-adrenergic receptor; CHO, Chinese hamster ovary; CHOα1A, CHO cells stably expressing cloned α1A-AR; CHOα1B, CHO cells stably expressing cloned α1B-AR; CHOα1D, CHO cells stably expressing cloned α1D-AR; NA, noradrenaline; α1A/α1D-CR, chimeric α1A and α1D-AR; CHOα1A/α1D-CR, CHO cells stably expressing α1A/α1D-CR; HEK, human embryonic kidney; TMD, transmembrane domain; ICL, intracellular loop; MAPK, mitogen-activated protein kinase; FCS, fetal calf serum.

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Materials and methods

Materials. The following drugs and reagents were used: 2-[2-(4-hydroxy-3-[^{125}I]iodo-phenyl)ethylaminomethyl]-α-tetralone ([^{125}I]HEAT) (specific activity, 2200 Ci mmol^{-1}; New England Nuclear, Boston, MA, USA); [methyl-^{3}H]thymidine (specific activity, 2 Ci mmol^{-1}; New England Nuclear, Boston, MA, USA); phentolamine mesylate (Novartis Pharmaceutical, Summit, NJ, USA); (−)-noradrenaline bitartrate, phenylephrine, DL-propranolol hydrochloride, and 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical, St. Louis, MO, USA); and Ham's F-12 medium, G418 (Gibco Life Technologies, Gaithersburg, MD, USA). All other chemicals were of reagent grade.

Construction of human α1A/α1D-CRs and amino acid-substituted α1A-AR mutants. The cDNAs encoding human α1A/α1D-CRs were constructed by digestion at common restriction sites (the *Sna*BI and *Afl*II sites created by oligonucleotide-directed mutagenesis within the wild-type human α1a- and α1d-AR cDNAs, Fig. 1) and then exchanging the corresponding regions of each. The restriction sites and their positions in the deduced amino acid sequence of the α1a/α1d-ARs, respectively, are as follows: *Sna*BI, at Y208/Y278 in the transmembrane domain (TMD) V; *Afl*II, at Leu262/Leu338 in the intracellular loop (ICL) III (Fig. 1). The structures of the chimeras are shown in Fig. 2. The positions of the junctions for individual human α1A/α1D-CRs and their component amino acids are as follows: AAD, α1a 1–262/α1d 339–569; ADA, α1a 1–208/α1d 279–338/α1a 263–438; ADD, α1a 1–208/α1d 279–569; DDA, α1d 1–338/α1a 263–438; DAD, α1d 1–278/α1a 209–262/α1d 339–569; DAA, α1d 1–278/α1A 209–438. Amino acid-substituted mutants of α1A-AR were constructed by PCR using site-directed mutagenesis. Comparing amino acid sequences of human α1A-AR, α1B-AR, and α1D-AR showed that homology was highly conserved. In the region extending from the amino-terminal extracellular tail to TMD V of the human α1D-AR, we found 31 amino acids that were different from those common to α1A-AR and α1B-AR (shaded amino acids in Fig. 1). In the ICLII of α1D-AR, only two amino acids, Ala-206 and Met-208, were replaced by Thr and Val in that region of α1A-AR and α1B-AR (Thr-136 and Val-138) (Fig. 1). Either Thr-136 or Val-138 or both in the α1A-AR was changed to the amino acid corresponding to the α1D-AR sequence. Mutations generated were confirmed by sequencing. The wild-type human α1A-AR, α1D-AR and all of the mutant receptors were inserted into *Eco*RI and *Xho*I sites of the mammalian expression vector pMEK19.

Cell transfection. Cell transfection was performed as described previously [20]. For stable expression of the human α1A-AR,α1D-AR and all α1A/α1D-CRs, CHO-K1 cells were seeded at a density of 2×10^4 cells into 35-mm tissue culture dishes. On the next day, F-12 medium was removed and 1 ml of serum-free F-12 medium containing 13.8 μg of LipofectAMINE reagent (Gibco Life Technologies, Gaithersburg, MD, USA) and 9.2 μg of the recombinant expression plasmid was added to the cells. After 24 h, 1 ml F-12 medium containing 20% fetal calf serum (FCS) was added. The cells were passaged at a low density 72 h later. Single colonies resistant to the antibiotic G418 (600 μg/ml) were isolated and maintained in F-12 medium with 10% fetal calf serum (FCS) and G418 (200 μg/ml) for four weeks.

In some experiments, the human α1A-AR, α1D-AR and the amino acid-substituted mutants were transiently expressed in HEK293 cells by electroporation (240 V, 975 μF, Bio-Rad gene Pulser II electroporator, Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. Cells were harvested 48–72 h after transfection.

Membrane preparations. The transfected cells were collected and homogenized by a Branson sonicator (model SONIFIER 250, Branson, Danbury, CT, USA; setting 5 for 8 s) in 2 ml ice-cold buffer A (250 mM sucrose, 5 mM Tris–HCl, and 1 mM $MgCl_2$, pH 7.4). The mixture was then centrifuged at 1000*g* at 4 °C for 10 min to remove nuclei. The supernatant was centrifuged at 35,000*g* for 20 min at 4 °C and the pellet was resuspended in the binding buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, and 10 mM EGTA, pH 7.4) [20]. The protein concentration was measured using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Radioligand binding assay. Radioligand binding studies were performed as described previously [18,20]. Briefly, membrane aliquots (∼ 0.1–0.5 μg protein) were incubated for 60 min at 25 °C with [^{125}I]HEAT, and either with or without a competing drug, in a final volume of 150 μl of the binding buffer. After dilution with ice-cold buffer, samples were immediately filtered through Whatman GF/C glass fiber filters with a Brandel cell harvester (Model-30, Gaithersburg, MD, USA). Each filter was collected and the radioactivity was measured. Binding assays were performed in duplicate at all times, and specific [^{125}I]HEAT binding was experimentally determined from the difference between counts in the presence or absence of 10 μM phentolamine. Data were analyzed by the iterative nonlinear regression program, LIGAND [21].

[^{3}H]Thymidine incorporation. We measured [^{3}H]thymidine incorporation as previously reported [22].CHO cells stably expressing the cloned α1A-AR (CHOα1A), α1D-AR (CHOα1D), or the α1A/α1D-CRs (CHO α1A/α1D-CRs), were grown to confluence in 96-well plates and rendered quiescent by serum deprivation for 24 h. These cells were treated with 10 μM phenylephrine or left untreated; they were also pretreated or not pretreated with 10 μM phentolamine and then incubated with 10% FCS for 18 h. Next, cells were incubated for the following 12 h with [^{3}H]thymidine (1 μCi/ml). The cells were rinsed twice with ice-cold phosphate-buffered saline and precipitated with cold 5% trichloroacetic acid, then washed with cold ethanol, and solubilized with 0.5 N NaOH. DNA radioactivity was measured by scintillation counting.

Measurement of cAMP production. cAMP production in transiently transfected HEK293 cells was determined as described previously [19]. Briefly, these cells were seeded in 6-well plates at a density of 5×10^5 cells/well and cultured for 12–16 h. The cells were washed twice with phosphate-buffered saline and incubated at 37 °C for 30 min in a buffered salt solution (140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1.25 mM $CaCl_2$, 1 mM $NaHPO_4$, 5 mM HEPES, and 11 mM glucose, pH 7.4) and 1 mM IBMX. Propranolol (1 μM) was included to block endogenous β_1-ARs present in the cells [6]. The reaction was started by adding NA (10 μM) with or without phentolamine (10 μM). After incubation for 10 min at 37 °C, the medium was aspirated and the reaction was stopped with 100 mM HCl. Aliquots were taken and assayed for cAMP by radioimmunoassay (Yamasa cAMP Assay kit, Yamasa Shoyu, Chiba, Japan).

Results and discussion

Chimeric receptors

To determine the region responsible for the α1-AR subtype-specific differential effects on cell proliferation, we first constructed a series of α1A/α1D-CRs. The structures of these chimeras (AAD, ADA, ADD, DDA, DAD, and DAA) are shown in Fig. 2. They consist of three components that belong to either the wild-type human α1A-AR or α1D-AR. The first component is from the amino-terminal extracellular tail to TMD V. The second component is the ICL III and the third component is from TMD III to the intracellular carboxy-terminus. Three components of the wild-type human α1A-AR or α1D-AR were substituted by one another. Radioligand binding studies using [^{125}I]HEAT

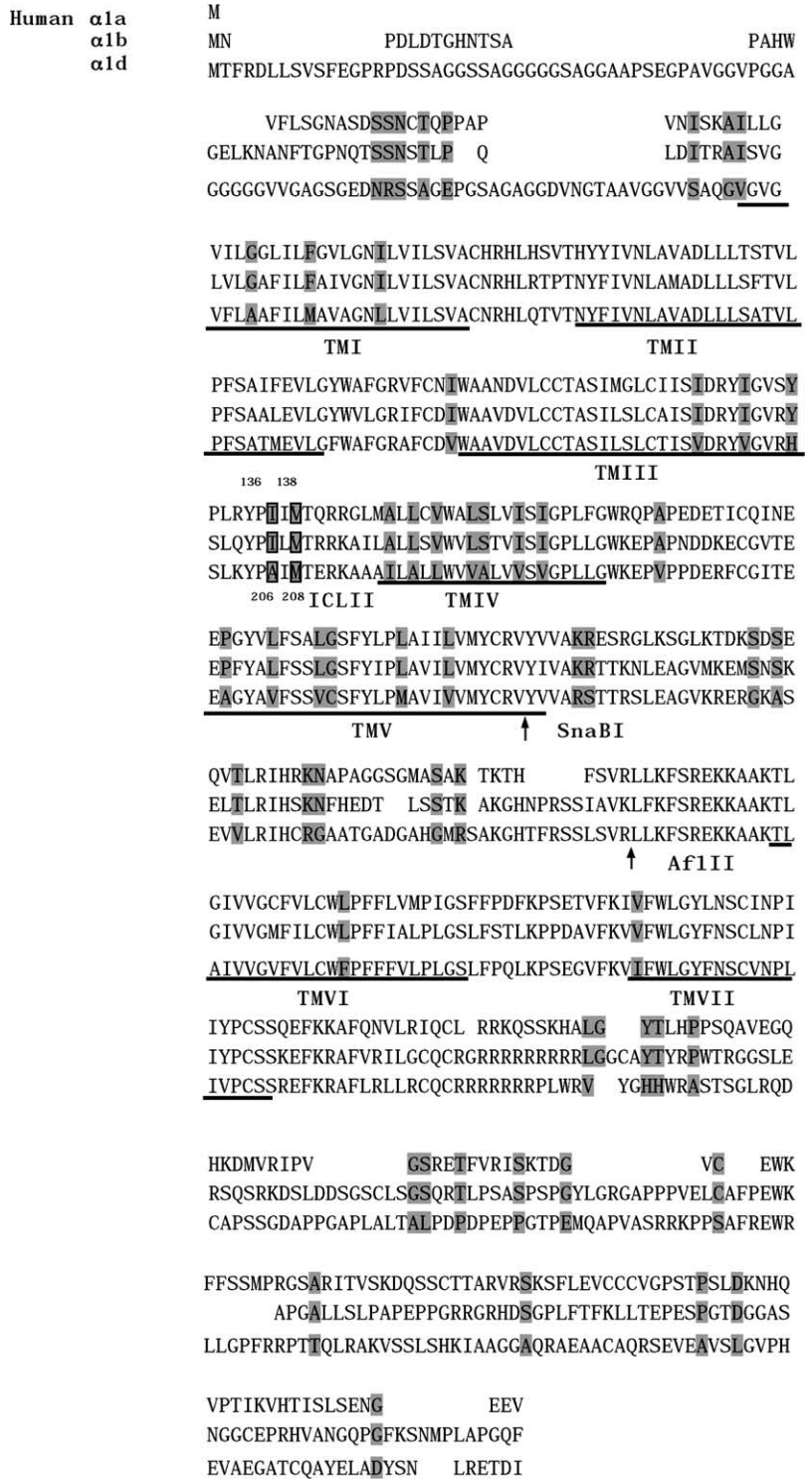

Fig. 1. Alignment of the wild-type human α1A-AR, α1B-AR, and α1D-AR amino acid sequences. Positions of the restriction sites used to construct chimeric receptors are shown by arrowheads with the enzymes (*Sna*BI, *Afl*II). The shaded amino acids indicate the observed differences in α1D-AR, whereas they are identical between the aligned human α1A-AR and α1B-AR sequences. The shaded box indicates two amino acids, Thr-136 and Val-138 in ICL II of α1A-AR, which differ from those of α1D-AR. These amino acids were changed individually to the corresponding amino acids of the α 1D-AR, Ala-206, and Met-208, respectively, by PCR using site-directed mutagenesis.

confirmed that all the cell lines examined are exhibiting similar receptor expression; CHOα1A ($K_d = 110$ pM, $B_{max} = 1.3$ pmol/mg protein), CHOα1D ($K_d = 300$ pM, $B_{max} = 1.1$ pmol/mg protein), and CHOα1A/α1D-CRs ($K_d = 180$–350 pM, $B_{max} = 0.50$–1.3 pmol/mg protein) (Table 1).

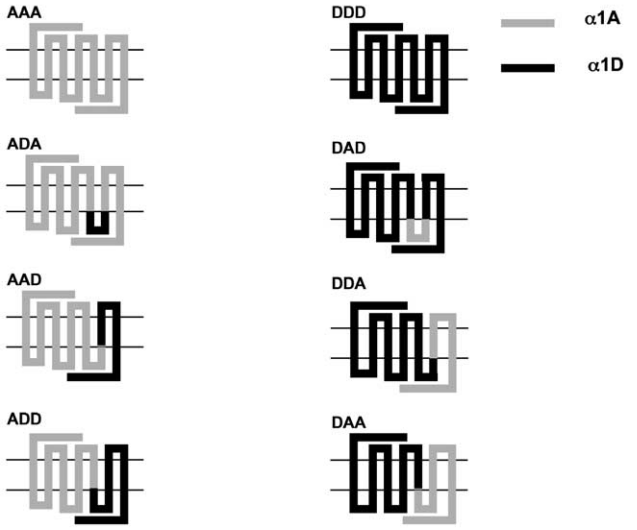

Fig. 2. Structure of chimeric human α1A/α1D-ARs(α 1A/α1D-CRs). Filled black bars, sequences of the wild-type human α1A-AR; filled blue bars, sequences of the wild-type human α1D-AR. The position of junctional amino acids in these chimeric receptors and the methods used for their construction are described in Materials and methods.

Table 1
Ligand-binding characteristics of the wild-type α1A, α1D, and chimeric α1A and α1D adrenergic receptor

	B_{max} (pmol/mg protein)	K_d (nM)
CHOα1A	1.3 ± 0.2	110 ± 21
AAD	0.65 ± 0.1	200 ± 10
ADA	1.3 ± 0.15	350 ± 23
ADD	0.5 ± 0.05	180 ± 16
CHOα1D	1.1 ± 0.1	300 ± 26
DDA	0.56 ± 0.22	200 ± 32
DAD	0.63 ± 0.18	210 ± 18
DAA	0.84 ± 0.11	250 ± 36

The specific binding of various concentrations of [^{125}I]HEAT to membranes of stable clones expressing the wild-type α1A, α1D, and α1A/α1D-CRs were measured, and B_{max} and K_d values were estimated. Data are presented as means ± SEM from three independent experiments.

Cell proliferation

Proliferation of CHOα1A, CHOα1D, and CHOα1A/α 1D-CRs was measured by [^{3}H]thymidine incorporation after serum deprivation. No significant differences were observed between these cells regarding the basal level of [^{3}H]thymidine incorporation. When they were exposed to 10% serum, in CHOα1A, CHOα1D, and CHOα1A/α1D-CRs, a 3.2–5.8-fold increase in [^{3}H]thymidine incorporation compared to serum-deprived cells was observed (Fig. 3A). Activation of α1-AR by phenylephrine (10 μM) inhibited this serum-promoted [^{3}H]thymidine incorporation to 31–49% in CHOα1A, AAD, ADA, and ADD (Fig. 3A). All of these receptors contained the region stretching from the amino-terminal extracellular tail to TMD V of α1A-AR. Pretreatment

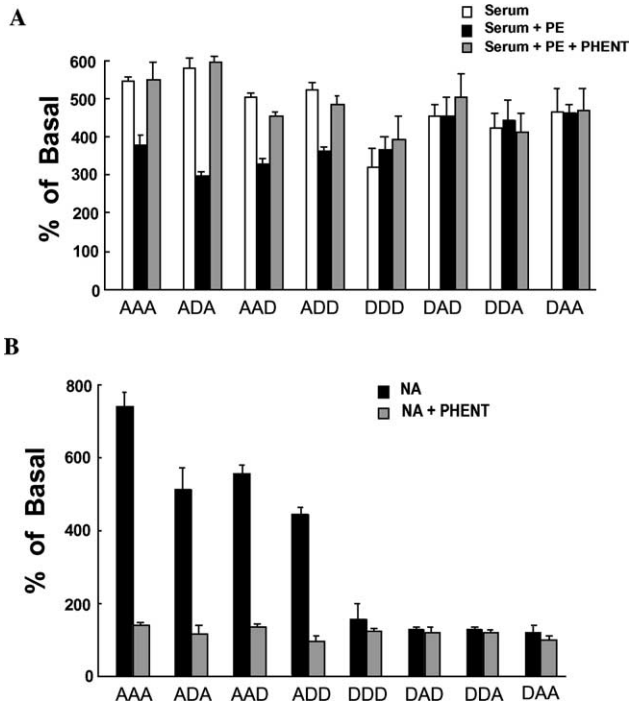

Fig. 3. (A) Effect of phenylephrine on serum-stimulated [^{3}H]thymidine incorporation. [^{3}H]Thymidine incorporation was measured in each CHO cell line stably expressing the wild-type α1A-AR, α1D-AR or the α1A/α1D-CRs. (B) Effect of α1-AR subtype activation on intracellular cAMP production in each CHO cell line stably expressing the wild-type α1A-AR, α1D-AR or the α1A/α1D-CRs. In all experiments, phentolamine (10 μM) treatment was started 30 min prior to stimulation by phenylephrine or noradrenaline. The results are expressed as means ± SEM of 3–4 different experiments each performed in duplicate. PE, phenylephrine; PHENT, phentolamine; NA, noradrenaline.

with the α-antagonist phentolamine (10 μM) almost completely blocked the phenylephrine-induced inhibitory effects on these cells, showing that inhibition is mediated through α1-AR (Fig. 3A). However, in CHOα1D, DDA, DAD, and DAA, which had the region encompassing the amino-terminal extracellular tail to TMD V of α1D-AR, such an inhibitory effect of phenylephrine was not observed (Fig. 3A). Taken together, these results suggest that the domains within α1A-AR and α1D-AR which are responsible for the different subtype-specific effects on cell proliferation may reside in the region extending from the amino-terminal extracellular tail to TMD V.

cAMP production

As described above, we have recently observed that all of the α 1-AR subtypes share the inositol 1,4,5-triphosphate/Ca^{2+} signaling pathway, but only α1A- and α1B-ARs are coupled to the cAMP signaling pathway, and thereby inhibiting serum-promoted cell proliferation (submitted for publication). We first confirmed that all the chimeric α1-ARs couple to Ca^{2+} signaling pathway; hence, the $[Ca^{2+}]_i$ elevation response induced by NA

(10 μM) in CHOα1A, CHOα1D, and CHOα1A/α1D-CRs (data not shown). Next, we measured α1-AR-mediated cAMP production. In CHOα1A, AAD, ADA, and ADD, NA (10 μM) caused a 4.4–7.4-fold increase in cAMP production over basal levels. On the other hand, α1-AR activation did not promote cAMP synthesis in CHOα1D, DDA, DAD, and DAA (Fig. 3B). Furthermore, pretreatment with the α-AR antagonist phentolamine (10 μM) abolished this α1-AR-mediated cAMP production in CHOα1A, AAD, ADA, and ADD. These results indicate that the domains responsible for the difference between α1A-AR and α1D-AR in the outcome of α1-AR-mediated cAMP production may reside in the region stretching from the amino-terminal extracellular tail to TMD V. This region, which is important for the cAMP production signaling pathway, is the same as that responsible for the inhibition of serum-promoted cell proliferation.

cAMP production in the amino acid-substituted mutant receptors

To identify the amino acid(s) responsible for α1-AR subtype-specific cAMP signaling pathways, we first compared the sequences of wild-type human α1A-AR and α1B-AR with α1D-AR. In the region from the amino-terminal extracellular tail to TMD V of α1D-AR, we found 31 amino acids different from those common to the human α1A-AR and α1B-AR (Fig. 1). Among ICLs, which are considered to be important for coupling to G-proteins, although there are no such amino acid differences in ICL I of α1D-AR, we identified two such amino acids in ICL II, Thr-136, and Val-138. These two amino acids of α1A-AR were individually, as well as in combination, changed to the corresponding amino acids of α1D-AR in the different mutants. The positions of the mutated amino acids are indicated in Fig. 1. The K_d values of [^{125}I]HEAT for the amino acid-substituted mutant receptors were not significantly different from those of the wild-type human α1A- and α1D-ARs (K_d = 100–220 pM, B_{max} = 0.9–2.1 pmol/mg protein). The mutation of Thr-136 to Ala (T136A) resulted in NA (10 μM) stimulation causing a 3.6-fold increase in cAMP production over the level without stimulation (Fig. 4). This increased level was almost 80% of the stimulation of α1A-AR-induced cAMP production. However, mutation of Val-138 to Met (V138M) showed only a 2-fold increase in cAMP production that was less than 50% the simulation of α1A-AR induced cAMP production (Fig. 4). Furthermore, the double mutant (T136A/V138M) showed a 2.5-fold increase in cAMP production over basal levels, almost the same as V138M (Fig. 4). Pretreatment with phentolamine (10 μM) almost completely blocked NA-stimulated responses in the wild-type α1A-, α1D-AR, and the amino acid-substituted mutants (Fig. 4). Taken together, these results may indicate that the region extending from the amino-terminal extracellular tail to TMD V, and in particular, residue Val138 in ICL II of the α1A-AR appears to be intimately connected to the Gs/cAMP pathway.

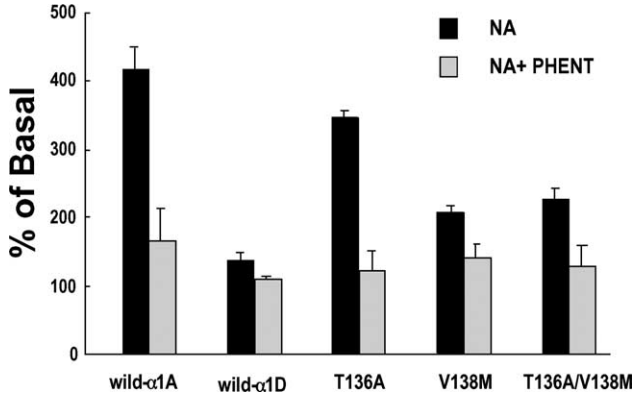

Fig. 4. Effect of α1-AR subtype activation on intracellular cAMP production in each HEK293 cell transiently expressing the wild-type α1A-AR, α1D-AR, T136A, V138M, or T136A/V138M. Phentolamine (10 μM) treatment was started 30 min prior to stimulation by noradrenaline. Values are means ± SEM of at least three different experiments each performed in duplicate. NA, noradrenaline; PHENT, phentolamine.

As described in the introductory material, α1-ARs mediate the effects of the sympathetic nervous system, especially those effects related to the regulation of cellular hypertrophy and proliferation. As the three different α1-AR subtypes are generally co-expressed, little is known regarding the physiological role of each receptor subtype at a cellular level and in the regulation of cell proliferation. Using a heterologous expression system, we have recently found that these highly homologous receptor subtypes have distinct signal transduction coupling properties and have differential effects on cell proliferation; thus, activation of α1A- or α1B-AR inhibits serum-promoted cell proliferation, whereas the activation of α1D-AR has a growth-promoting effect (submitted for publication). The present study further demonstrates that certain different amino acids between each α1-AR subtype may influence and differentiate functional responses mediated by α1-AR subtypes. This study provides valuable information on the α1-AR subtype and G-protein coupling.

Acknowledgments

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