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# Characterization of $\alpha_1$-adrenoceptors expressed in a novel vascular smooth muscle cell line cloned from p53 knockout mice, P53LMAC01 (AC01) cells

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**1** We pharmacologically studied the $\alpha_1$-adrenoceptor (AR) subtype(s) involved in receptor-mediated signalling in a novel vascular smooth muscle cell line cloned from p53 knockout mice, P53LMAC01 (AC01) cells.

**2** Radioligand binding studies with [$^{125}$I]-HEAT showed the existence of a homogeneous population of binding site with an affinity ($K_d$ value) of 0.4 nM and a maximum number of binding sites ($B_{max}$) of 100 fmol mg$^{-1}$ protein. Catecholamines competed for [$^{125}$I]-HEAT binding stereospecifically and with the characteristic $\alpha_1$-AR potency series.

**3** Displacement curves for BMY-7378 and KMD-3213 best fitted a one-site model with a $pK_i$ value ($-\log_{10}$ (equilibrium inhibition constant)) of 6.06 and 7.07, respectively.

**4** Reverse transcription-polymerase chain reaction (RT–PCR) assay detected $\alpha_{1B}$- and $\alpha_{1D}$-AR, but not $\alpha_{1A}$-AR transcript.

**5** Chlorethylclonidine (CEC) treatment nearly abolished (−)noradrenaline (NA) (10 $\mu$M)-induced inositol[1,4,5]trisphosphate ($IP_3$) production, and BMY-7378 inhibited the response with a $K_i$ value of 0.3 nM, which value was similar to that obtained in the cells expressing $\alpha_{1D}$-AR. In both AC01 cells and cells expressing $\alpha_{1D}$-AR, BMY-7378 protected $\alpha_1$-ARs from CEC alkylation while it had little protective effect on CEC alkylation and NA-induced $IP_3$ production in cells expressing $\alpha_{1B}$-AR.

**6** The results indicate that AC01 cells contain predominantly $\alpha_{1B}$-ARs and a small population of $\alpha_{1D}$-ARs; however, phosphoinositide (PI)/$Ca^{2+}$ signalling is mainly mediated through the minor population of $\alpha_{1D}$-ARs, rather than the $\alpha_{1B}$-ARs.

**Keywords:** $\alpha_1$-adrenoceptor; vascular smooth muscle cells; noradrenaline

**Abbreviations:** AC01, P53LMAC01; AR, adrenoceptor; $\alpha_{1B}$-AR CHO cells, CHO-K1 cells transfected with human $\alpha_{1b}$-AR cDNA; $\alpha_{1D}$-AR CHO cells, CHO-K1 cells transfected with human $\alpha_{1d}$-AR cDNA; $B_{max}$, maximum number of binding sites; bp base pair(s); $[Ca^{2+}]_i$, intracellular free $Ca^{2+}$ concentration; CEC, chlorethylclonidine; CHO, Chinese hamster ovary; DMEM, Dulbecco modified Eagle medium; EGTA, ethylene glycol bis ($\beta$-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FBS, foetal bovine serum; [$^{125}$I]-HEAT, 2-[2-(4-hydroxyl-3-[$^{125}$I]-iodophenyl)ethylaminomethyl]-α-tetralone; $IP_3$, inositol[1,4,5]trisphosphate; $K_i$, equilibrium inhibition constant; $pK_i$, $-\log_{10}$ (equilibrium inhibition constant); 5-MU, 5-methylurapidil; VSMC, vascular smooth muscle cells

## Introduction

The sympathetic nervous system plays an important role in regulating the tone of the peripheral blood circulation. Catecholamines bind and activate α-adrenoceptors (ARs), thereby inducing vascular smooth muscle contraction. It has now been clearly shown that postsynaptic α-ARs in the peripheral blood circulation are composed of $\alpha_1$- and $\alpha_2$-ARs, with both receptors mediating vasoconstriction (Minneman *et al*., 1992; Goodman & Gilman's, 1996). Considerable progress has been made toward elucidation of the molecular structures and signal transduction mechanisms of $\alpha_1$-ARs (Ruffolo *et al*., 1991). Molecular cloning studies have so far identified $\alpha_{1a}$, $\alpha_{1b}$, and $\alpha_{1d}$-AR cDNAs (Cotecchia *et al*., 1988; Schwinn *et al*., 1990; Lomasney *et al*., 1991; Perez *et al*., 1991; Hirasawa *et al*., 1993; 1995). The primary structure of the $\alpha_1$-ARs identified by molecular cloning corresponds to the predicted topographic model of the superfamily of G-protein-coupled receptors, and substantial evidence indicates the importance of agonist and G protein-regulated phospholipase C to generate phosphoinositide (PI)-derived second messengers for $Ca^{2+}$ signalling in response to $\alpha_1$-AR activation (Minneman *et al*., 1988). Moreover, three distinct $\alpha_1$-AR subtypes ($\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1D}$) have been distinguished pharmacologically (Hieble *et al*., 1995). The $\alpha_{1A}$ subtype has a high affinity for the agonist oxymetazoline and antagonists 5-methylurapidil (5-MU) and KMD-3213, and is relatively insensitive to inactivation by the alkylating agent chlorethylclonidine (CEC) (Han *et al*., 1987; 1990; Minneman, 1988; Boer *et al*., 1989; Hanft & Cross, 1989; Suzuki *et al*., 1990; Shibata *et al*., 1995). On the other hand, the $\alpha_{1B}$-subtype is highly sensitive to CEC inactivation (Minneman, 1988), and the $\alpha_{1D}$-subtype has a high affinity for the antagonist BMY-7378 (Piascik *et al*., 1995). Using CEC and BMY-7378 in a 'protection from alkylation' strategy to distinguish between $\alpha_{1B}$- and $\alpha_{1D}$-ARs has recently been developed (Xin *et al*., 1997). However, the physiological role of each $\alpha_1$-AR subtype is unclear, especially in the vascular smooth muscle cell (VSMC).

One of the major difficulties that have hindered progress in studying the regulation of vascular $\alpha_1$-ARs is that the phenotypic characteristics of VSMC change when cultured. It

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has been reported that cultured VSMC often de-differentiate, which is accompanied by changes in the expression of smooth muscle-specific protein markers (Owens, 1995). Recently, we established the P53LMAC01 (AC01) cell line from the aorta of p53 knockout mice (Ohmi *et al*., 1997). AC01 cells have an extended bipolar shape and expressed several protein markers of differentiated smooth muscle (e.g., h-caldesmon, calponin and $\alpha$-smooth muscle actin). Further, we observed that in AC01 cells noradrenaline (NA) and phenylephrine induce an increase in the intracellular calcium concentration in an oscillatory manner, and exert a slow contractile response. Hence, the purpose of the current study was to determine which $\alpha_1$-AR subtype is expressed and mediates the signal transduction in this unique cell line of mouse AC01 cells.

## Methods

### *Materials*

The following drugs were used: [$^{125}$I]-HEAT (specific activity, 2200 Ci mmol$^{-1}$; NEN, Boston, MA, U.S.A.); KMD-3213 [(−)-(R)-1-(3-hydroxypropyl)-5-[2-[2-[2-(2,2,2-trifluoroethoxy) phenoxy]-ethylamino]propyl]indoline-7-carboxamide]dihydrobromide (Kissei Pharmaceutical Co., Matsumoto, Japan); BMY-7378 8-[2-[4-(2-Methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride (Research Biochemicals Inc., Natick, MA, U.S.A.); prazosin hydrochloride (Pfizer, Groton, CT, U.S.A.); phentolamine mesylate (Ciba-Geigy, Summit, NJ, U.S.A.); CEC, 5-MU 5-metyl-6 [[3-[4-(2-methoxyphenyl)-1-piperazinyl]propy] amino] -1,3 -dimethyluracil (Research Biochemicals Inc., Natick, MA, U.S.A.); (−)-NA bitartrate, (−)-adrenaline bitartrate, (+)-adrenaline bitartrate, and oxymetazoline (Sigma Chemical Co., St. Louis, MO, U.S.A.); Dulbecco modified Eagle medium (DMEM), F-12, and G418 (Gibco Life Technologies, Gaithersburg, MD, U.S.A.). All other chemicals were of reagent grade.

### *Cell culture*

AC01 was prepared from the aorta of p53 knock out mice as described previously (Ohmi *et al*., 1997). The AC01 cells were grown to confluence in monolayers on 100 mm culture dishes in DMEM with 10% foetal bovine serum (FBS), 100 $\mu$g ml$^{-1}$ streptomycin and 100 units ml$^{-1}$ penicillin-G in a 5% $CO_2$-95% air atmosphere at 37°C. The cells were subcultured every 3 days after trypsinization.

### *Transfection of CHO-K1 cells with human $\alpha_{1b}$-AR or $\alpha_{1d}$-AR cDNA*

Wild type chinese hamster ovary (CHO)-K1 cells were transfected with human $\alpha_{1b}$-AR or $\alpha_{1d}$-AR cDNAs and stable transformants were obtained as described previously (Shibata *et al*., 1995). The pharmacological profile of the $\alpha_{1B}$-AR and $\alpha_{1D}$-AR in the CHO-K1 cells was as follows: saturable binding of [$^{125}$I]-HEAT; $B_{max}$ values were $5.5 \pm 0.1$ and $1.1 \pm 0.1$ pmol mg$^{-1}$ of protein, with $K_d$ values of $60 \pm 1$, and $300 \pm 26$ pM for the $\alpha_{1B}$-ARs and $\alpha_{1D}$-ARs, respectively. The transformants were maintained in F-12 medium with 10% FBS and 200 $\mu$g ml$^{-1}$ G418.

### *[$^{125}$I]-HEAT binding assay*

*Membrane preparation* Membrane preparation of the cells was performed as described previously (Hirasawa *et al*., 1993; Horie *et al*., 1994). Briefly, the collected cells were placed in ice-cold buffer A (mM: sucrose 250, Tris-HCl 5, $MgCl_2$ 1, (pH 7.4)), and disrupted in a sonicator model SONIFER 250 (Branson, Danbury, CT, U.S.A.) at setting 5 for 8 s. They were then centrifuged at $3000 \times g$ at 4°C for 10 min to remove the nuclei. The supernatant fraction was centrifuged at $35{,}000 \times g$ for 20 min at 4°C. The resulting pellet was resuspended in binding buffer B [mM: Tris-HCl 50, $MgCl_2$ 10, ethylene glycol bis ($\beta$-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA 10), (pH 7.4) and was frozen at −80°C until assay.

### *[$^{125}$I]-HEAT binding*

[$^{125}$I]-HEAT binding assay was performed as described previously (Horie & Tsujimoto, 1995). Briefly, membrane aliquots ($\sim$10 $\mu$g of protein for AC01 cells and 0.1 – 0.5 $\mu$g of protein for CHO cells expressing $\alpha_{1B}$-ARs and $\alpha_{1D}$-ARs, respectively) were incubated in a final volume of 250 $\mu$l of binding buffer B containing [$^{125}$I]-HEAT for 60 min at 25°C. The incubation was terminated by adding the ice-cold binding buffer B and immediate filtration through Whatmann GF/C glass-fibre filters with a Brandel Cell Harvester (Model-30, Gaithersburg, MD, U.S.A.). Each filter was collected and the radioactivity was measured. Binding assays were always performed in duplicate, and specific [$^{125}$I]-HEAT binding was experimentally determined from the difference between counts in the absence and presence of 10 $\mu$M phentolamine. $B_{max}$ and $K_d$ values were obtained by fitting rectangular hyperbolic functions to the experimental data, using computer-assisted iterative nonlinear regression analysis. The protein concentration was measured using the BCA protein assay kit (PIERCE, Rockford, IL, U.S.A.). Data were analysed by computer with the iterative nonlinear regression program LIGAND (Munson & Rodbard, 1980).

### *CEC treatment*

Intact cell treatment: $5 - 10 \times 10^6$ cells suspended in 1 ml of the buffered salt solution (mM: NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.25, $NaHPO_4$ 1, HEPES 5, glucose 11 and 0.1% BSA, pH 7.4) were incubated with or without CEC (100 $\mu$M) at 37°C for 30 min. After incubation, cells were washed three times with the buffered salt solution and used for inositol phosphate assay.

### *$IP_3$ production*

AC01 cells were grown to confluence on collagen-coated 35 mm dishes. The cells were washed two times with buffered salt solution (mM: NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 5, glucose 5 (pH 7.4)). $IP_3$ was measured quantitatively in NA-stimulated cells pretreated or non-treated with related compounds by $IP_3$ competition assay. $IP_3$ production peaked within 5 s after NA addition. The reaction was terminated at 5 s after 10 $\mu$M NA stimulation by addition of $HClO_4$ (final concentration 5%). The mixture was kept on ice for 20 min. The cells were then harvested and centrifuged at $2000 \times g$ for 15 min. The supernatant was neutralized with ice-cold 1.5 M KOH/60 mM HEPES for 20 min. The sample was then centrifuged at $2000 \times g$ for 10 min to remove the $KClO_4$ precipitate. The supernatant samples (100 $\mu$l) were assayed for $IP_3$ with an $IP_3$ assay kit (DuPont New England Nuclear, Boston, MD, U.S.A.). The standard curve was linear from 0.12 to 12.0 pmol of $IP_3$.

### *RT–PCR*

Total cellular RNA was extracted from the AC01 cells using the Total RNA extraction kit (QIAGEN Inc., Santa Clara, CA, U.S.A.). The RNA samples were precipitated in ethanol, then vacuum-dried, and resuspended in RNase-free water. Reverse transcription was carried out as follows: each RNA sample contained (in mM) Tris-HCl (pH8.3) 50, KCl 75, $MgCl_2$ 0.5, dithiothreitol 10, dNTP (dATP, dTTP, dGTP, dCTP) 0.5 each, 10 $\mu$g of total cellular RNA, 20 units of RNase inhibitor, 100 pmol random hexamer (TaKaRa, Kyoto, Japan) and 200 units Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD, U.S.A.) in a final volume of 20 $\mu$l. After incubation at 37°C for 60 min, the samples were heated at 94°C for 5 min to terminate the reaction. The reactions were carried out in the presence or absence of reverse transcriptase, as indicated in the Results. Oligonucleotide primers were constructed from the cDNA sequences of mouse $\alpha_{1a}$-AR, $\alpha_{1b}$-AR and $\alpha_{1d}$-AR cDNAs as described below. As positive controls, pBluescript plasmid inserted with mouse $\alpha_{1a}$-AR, $\alpha_{1b}$-AR or $\alpha_{1d}$-AR cDNA was used (gifts from Dr M.A. Chinchetru) (Alonso-Llamazares *et al*., 1995). In all assays, $\beta$-actin served as a control for assessing the efficacy of RNA isolation and cDNA synthesis. Plasmid inserted with mouse $\beta$-actin cDNA was used as a positive control.

Primers used for $\alpha_{1a}$-AR were derived from the sequences of the fifth and sixth transmembrane domains of bovine $\alpha_{1a}$-AR (Schwinn *et al*., 1990). The sequences of the $\alpha_{1a}$-AR primers were (coding sense), corresponding to bases 349–375, and the 5′-(anticoding sense), which anneals to bases 840–870 of the cloned bovine $\alpha_{1a}$-AR full-length sequence. The sequences of the $\alpha_{1b}$-AR primers were 710–733 bp (coding sense), and 806-829 (anticoding sense) of the cloned mouse $\alpha_{1b}$-AR cDNA sequence (Alonso-Llamazares *et al*., 1995). $\alpha_{1d}$-AR primers were 109–130 (coding sense), and 1295–1315 (anticoding sense) of the cloned mouse $\alpha_{1d}$-AR cDNA sequence (Alonso-Llamazares *et al*., 1995).

The sequence of the $\beta$-actin primers was 5′-GCATCCT-CACCCTGAAGTACCCCA-3′ (coding sense), which corresponds to bases 268–292 of the cloned full-length sequence, and 5′-ACTCGTCATACTCCTGCTTGCTGAT-3′ (anticoding sense), corresponding to bases 1149–1173 (Tokunaga *et al*., 1986). The predicted sizes of the amplified $\alpha_{1a}$-AR, $\alpha_{1b}$-AR, $\alpha_{1d}$-AR, and $\beta$-actin PCR products was 522, 120, 1207, and 906 base pairs (bp), respectively.

Each reverse transcription mixture was diluted 1 : 5 in RNase-free water and 2 $\mu$l aliquots were then transferred to fresh tubes for amplification. Each sample contained the upstream and downstream primers (0.2 $\mu$M each primer) spanning the given sequence for amplification, (in mM) KCl 50, Tris-HCl (pH8.3) 10, $MgCl_2$ 10, 200 $\mu$M of each dNTP (dATP, dCTP, dGTP, dTTP), 0.01% (V/V) gelatin, and 2.5 units of ExTaq DNA polymerase (TaKaRa, Kyoto, Japan) in a final volume of 25 $\mu$l. The reaction mixture was amplified for 30 cycles in a Perkin Elmer Cetus thermal cycler (Norwalk, CT, U.S.A.). The amplification profiles consisted of denaturation for 1 min at 94°C; primer annealing for 30 s at 55°C, and extension for 1 min at 72°C. Negative controls without the reverse transcription reaction were routinely included in PCR amplifications with both primer sets.

### *Statistical analysis*

The results obtained are expressed as the mean ± s.e.mean. Statistical analyses were performed by one-way analysis of variance (ANOVA), and the statistical significance among groups was determined by Dunnett's multiple comparisons test, using Stat View 4.0 (Abacus Concepts, Inc., Berkeley, CA, U.S.A.). A $P$ value of $<0.05$ was considered statistically significant.

## Results

### *Binding studies*

As shown in Figure 1, specific binding of [$^{125}$I]-HEAT to a high affinity site on membranes prepared from AC01 cells is saturable with a $K_d$ of 400 ± 63 pM and $B_{max}$ of 110 ± 20 fmol mg$^{-1}$ of protein ($n=3$). Competition for [$^{125}$I]-HEAT binding sites by adrenergic ligands was examined (Table 1). The rank order of potency was (−)adrenaline ⩾ (−)NA > > (−)isoprenaline. Also, the binding of adrenaline was stereoselective. The affinity of (−)adrenaline was approximately 34 times that of the (+) isomer. Prazosin [$-\log_{10}$(equilibrium inhibition constant) ($pK_i$) = 9.64] was more potent than phentolamine ($pK_i = 6.87$) and much more potent than yohimbine ($pK_i = 5.60$), suggesting that the $\alpha$-AR identified is predominantly of $\alpha_1$-subtype. Pretreatment with CEC (100 $\mu$M, 30 min) completely eliminated the [$^{125}$I]-HEAT binding sites (data not shown).

In order to determine the relative percentage of each $\alpha_1$-AR subtype in AC01 cell membranes, we performed competition experiments using the $\alpha_{1A}$-selective antagonist, KMD-3213 and the $\alpha_{1D}$-selective antagonist, BMY-7378. Nonlinear regression analysis with LIGAND showing that inhibition curves for both KMD-3213 and BMY-7378 best fitted a one-site model, and the $pK_i$ values were 7.07 ± 0.09 and 6.06 ± 0.09, respectively (Figure 2). The values obtained were in good agreement with those previously reported as lower affinity sites.

### *Expression of $\alpha_1$-AR mRNA in AC01 cells*

We next performed a RT–PCR study to determine the subtype of $\alpha_1$-AR expressed in AC01 cells. As shown in Figure 3, only the $\alpha_{1b}$- and $\alpha_{1d}$-AR, but not $\alpha_{1a}$-AR, were visible in the PCR product of AC01 cells (Figure 3, lane 1). For $\alpha_{1a}$-AR, in

**Table 1** Pharmacological profile of AC01 cells

| *Drug* | $pK_i$ |
|---|---|
| *Agonists* | |
| (−)Noradrenaline | 5.04 ± 0.08 |
| (−)Adrenaline | 5.37 ± 0.05 |
| (+)Adrenaline | 3.84 ± 0.10 |
| Oxymetazoline | 6.08 ± 0.05 |
| (−)Isoprenaline | 3.37 |
| *Antagonists* | |
| Prazosin | 9.64 ± 0.07 |
| Phentolamine | 6.87 ± 0.06 |
| 5-methylurapidil | 6.86 ± 0.05 |
| Yohimbine | 5.60 ± 0.06 |

AC01 cells membrane were incubated with [$^{125}$I]-HEAT, in the absence or presence of interesting concentrations of various agonists and antagonists. At least ten concentrations of each ligand were tested, and the points were chosen to be the linear portion of displacement curve. $pK_i$ values were generated using the iterative curve-fitting program LIGAND (Munson & Rodbard, 1980). Each value represents the mean ± s.e.mean of the data from three to five different data performed in duplicate.

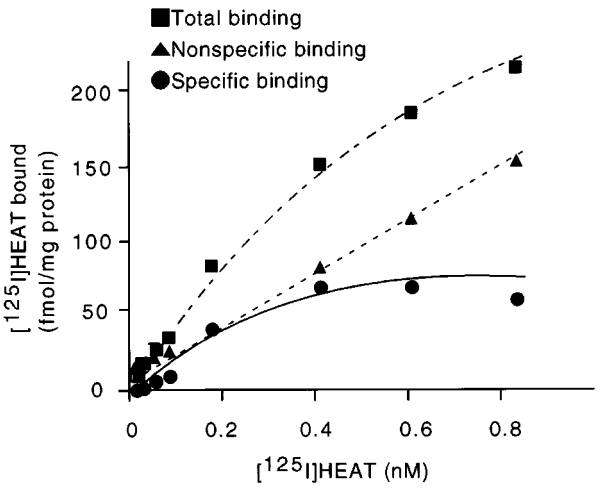


**Figure 1** Saturation of [$^{125}$I]-HEAT binding sites from AC01 cell membranes. The assay was performed as described in Methods. Specific binding is the difference between total binding and nonspecific binding determined in the presence of 10 $\mu$M phentolamine. Each value shown is the mean of a duplicate determination. The experiment shown is representative of three such experiments.

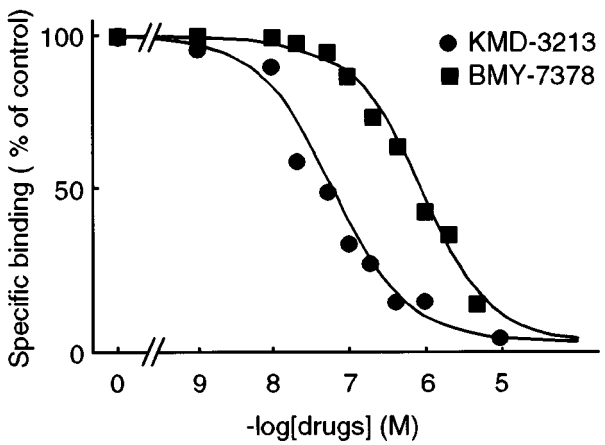


**Figure 2** Competition curves for KMD-3213 and BMY-7378 with [$^{125}$I]-HEAT in the AC01 cell membranes. AC01 cell membranes were incubated with [$^{125}$I]-HEAT ($\sim$0.4 nM) and varying concentrations of the subtype-selective agents, KMD-3213 and BMY-7378. Data thus obtained were subjected to computer modelling as described in Methods. Data points represent the means of duplicates from a representative experiments for each of the two subtype-selective compounds ($n=3-5$). In all cases, modelling of the data to one site produced a significantly better fit than modelling the data to two sites.

the positive control using mouse cDNA fragment as a template, PCR product was visible (Figure 3, lane 2). The sequences of the PCR products obtained in AC01 cells were confirmed to be identical to the nucleotide positions of 710–829 bp of the cloned mouse $\alpha_{1b}$-AR cDNA sequence (Alonso-Llamazares *et al*., 1995), and 109–1315 bp of the cloned mouse $\alpha_{1d}$-AR cDNA sequence (Alonso-Llamazares *et al*., 1995). No products were detected in the negative controls experiments performed without the reverse transcription reaction (Figure 3, lane 3), assuring that the amplified products all originated from mRNA rather than from contaminating genomic DNA. In all of the RT–PCR samples obtained from AC01 cells, $\beta$-actin PCR products were present (Figure 3).

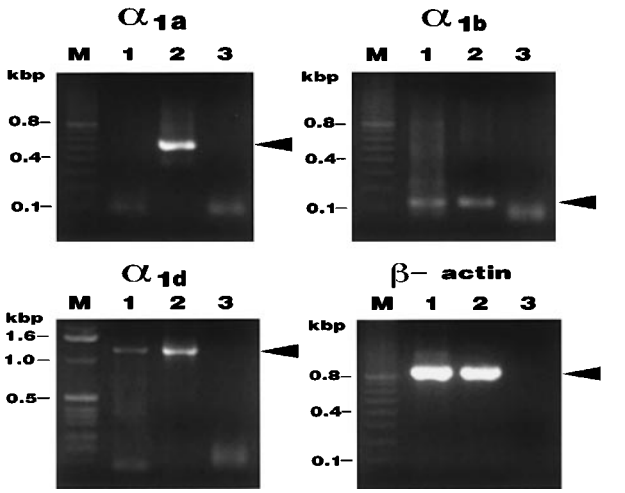


**Figure 3** The $\alpha_{1a}$-, $\alpha_{1b}$-, $\alpha_{1d}$-ARs and $\beta$-actin mRNA expression in AC01 cells. RT–PCR was performed with total RNA from AC01 cells using the primer sets as described in Methods. In each pannel PCR products using cDNAs from AC01 cells as templates are shown (lane 1, with reverse transcription; lane 3, without reverse transcription). Also, positive controls using mouse cDNAs of $\alpha_{1a}$-, $\alpha_{1b}$-, $\alpha_{1d}$-ARs or $\beta$-actin as templates are shown (lane 2). PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. Arrowhead shows the position of the bands by PCR amplification. M, DNA fragment size marker. kbp, kilo base pair.

Further, we observed that mouse aorta expresses transcripts of $\alpha_{1b}$- and $\alpha_{1d}$-AR by using the same RT–PCR protocol (data not shown), indicating that the expression pattern of $\alpha_1$-AR subtypes in AC01 cells resembles that of native aortic VSMC.

### *$IP_3$ production in cultured AC01 cells*

The effect of NA on the $IP_3$ production in AC01 cells was examined. $IP_3$ production peaked within 5 s after NA addition (data not shown). NA (10 $\mu$M) significantly induced $IP_3$ production ($32.0\pm2.7$ pmoles dish$^{-1}$) relative to the basal value without NA stimulation ($11.7\pm1.6$ pmoles dish$^{-1}$). Pretreatment with phentolamine (10 $\mu$M) and CEC (100 $\mu$M) almost completely inhibited the NA-induced $IP_3$ production (Figure 4). Effects of subtype-selective antagonists KMD-3213 and BMY-7378 on the NA-induced $IP_3$ production were further examined. Lower concentration of KMD-3213 (1 nM) had no inhibitory effect, while BMY-7378 (10 nM) significantly ($P<0.01$) inhibited the NA-induced $IP_3$ production. Higher concentrations (BMY-7378, 10 $\mu$M; KMD-3213, 10 $\mu$M) reduced NA-induced $IP_3$ production to 32 and 6% of the control, respectively.

### *Selective alkylation of $\alpha_{1B}$-ARs and protection of $\alpha_{1D}$-ARs with CEC in the presence of BMY-7378*

Selective alkylation of $\alpha_{1B}$-ARs by CEC is critically dependent on the concentration of CEC, incubation time, and temperature; increasing any of the these factors results in progressive alkylation of $\alpha_{1A}$ and $\alpha_{1D}$-ARs (Michel *et al*., 1995). Therefore, results obtained with CEC alone to differentiate among $\alpha_1$-ARs may be inconclusive. Hence, we adopted a very recently developed, a 'protection from alkylation' approach using CEC and BMY-7378 to identify the receptor subtype of $\alpha_1$-AR which mediates $IP_3$ production (Xin *et al*., 1997). To determine the selectivity of BMY-7378 against $\alpha_1$-AR-mediated $IP_3$ production, we first determined its potency

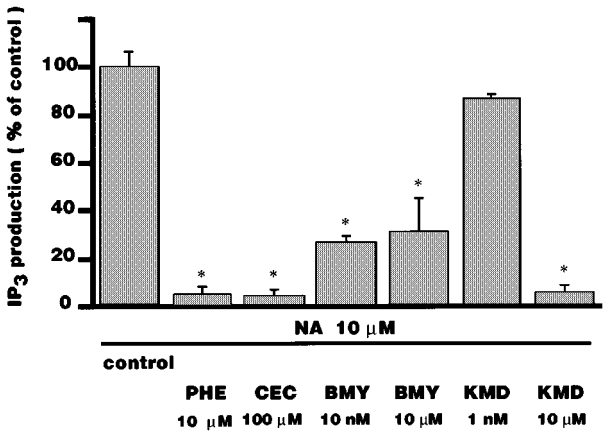


**Figure 4** Inhibition of NA-induced $IP_3$ production by antagonists in AC01 cells. The indicated antagonists were incubated 30 min prior to the 10 $\mu$M NA stimulation. In case of CEC, after incubation, cells were washed three times with the buffered salt solution and then stimulated by NA. The actual value of $IP_3$ production before and after stimulation NA were $11.7 \pm 1.6$ pmoles dish$^{-1}$ and $32.0 \pm 2.7$ pmoles dish$^{-1}$, respectively ($n=3$). Values are expressed as the relative value to the value of the increase in $IP_3$ production induced by NA. *$P<0.01$ versus control. NA, noradrenaline; PHE, phentolamine; CEC, chlorethylclonidine; BMY, BMY-7378; KMD, KMD-3213.

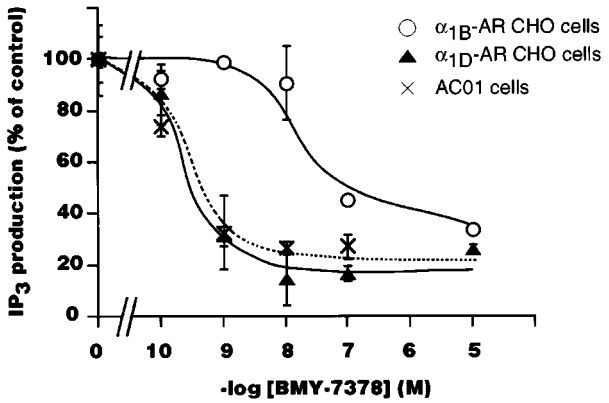


**Figure 5** Inhibitory effect of BMY-7378 on NA-induced $IP_3$ productions in $\alpha_{1B}$-AR CHO cells, $\alpha_{1D}$-AR CHO cells, and AC01 cells. Cells were pretreated with each concentration of BMY-7378 or vehicle at 37°C for 30 min, followed by adding 10 $\mu$M NA for 5 s. Then $IP_3$ production was measured as described in Methods. Responses were normalized as percentage of maximal response obtained with 10 $\mu$M NA ($n=3$ each).

against NA-induced $IP_3$ production in AC01 cells, CHO-K1 cells transfected with human $\alpha_{1b}$-AR cDNA ($\alpha_{1B}$-AR CHO cells), and CHO-K1 cells transfected with human $\alpha_{1d}$-AR cDNA ($\alpha_{1D}$-AR CHO cells). As shown in Figure 5, BMY-7378 inhibited NA-induced $IP_3$ production in a concentration-dependent manner; the $K_i$ values in AC01 cells, $\alpha_{1B}$-AR CHO cells, and $\alpha_{1D}$-AR CHO cells was 0.3, 50, and 0.3 nM, respectively (Figure 5).

We next tested the efficacy of BMY-7378 in protecting of $\alpha_{1D}$-ARs from CEC alkylation in an effort to increase the selectivity of CEC for $\alpha_{1D}$-AR inhibition. AC01 cells, $\alpha_{1B}$-AR CHO cells, and $\alpha_{1D}$-AR CHO cells were first incubated at 37°C with 0.1 $\mu$M BMY-7378 for 30 min, followed by CEC treatment. After extensive washing, the cells were allowed to equilibrate at 37°C for 30 min and then stimulated with NA (10 $\mu$M). As illustrated in Figure 6, in $\alpha_{1B}$-AR CHO cells, NA-induced $IP_3$ production was almost completely inhibited by

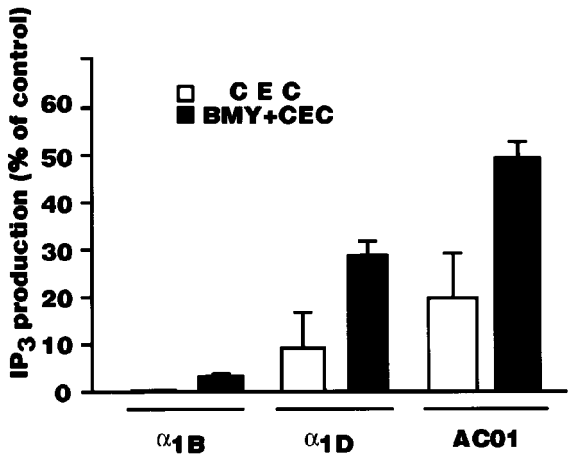


**Figure 6** BMY-7378 protects against CEC inhibition of NA-induced $IP_3$ productions in $\alpha_{1B}$-AR CHO cells, $\alpha_{1D}$-AR CHO cells and AC01 cells. Cells were preincubated with BMY-7378 (0.1 $\mu$M) and/or CEC (100 $\mu$M) before the addition of 10 $\mu$M NA. In CEC treatment (left), cells were incubated with CEC (100 $\mu$M) at 37°C for 30 min, then cells were washed three times with the buffered salt solution and used for inositol phosphate assay. In CEC-treated cells, $IP_3$ productions after NA stimulation were $0.2 \pm 0.1$, $1.0 \pm 0.8$, and $2.5 \pm 1.3$ pmoles dish$^{-1}$ for $\alpha_{1B}$-AR CHO cells, $\alpha_{1D}$-AR CHO cells, and AC01 cells, respectively ($n=3$). In BMY+CEC groups (right), cells were first incubated with 0.1 $\mu$M BMY-7378 at 37°C for 30 min, followed by CEC treatment for further 30 min. Cells were then washed three times and received fresh media containing 10 $\mu$M NA. In BMY+CEC groups, $IP_3$ productions after NA stimulation were $1.8 \pm 0.4$, $3.0 \pm 0.3$, and $6.3 \pm 0.5$ pmoles dish$^{-1}$ for $\alpha_{1B}$-AR CHO cells, $\alpha_{1D}$-AR CHO cells, and AC01 cells, respectively ($n=3$). BMY, BMY-7378; $\alpha_{1B}$, $\alpha_{1B}$-AR CHO cells; $\alpha_{1D}$, $\alpha_{1D}$-AR CHO cells; AC01, AC01 cells.

CEC treatment alone ($0.5 \pm 0.2$% of the control). When the protocol for BMY-7378 protection from CEC alkylation was used, NA-induced $IP_3$ production was $3.5 \pm 0.7$% of the control. In $\alpha_{1D}$-AR CHO cells, NA-induced $IP_3$ production after treatment with CEC alone was $9.2 \pm 7.4$% of the control, while NA-induced $IP_3$ production using the protocol for BMY-7378 protection from CEC alkylation was $28.8 \pm 2.7$% of the control. In AC01 cells, NA-induced $IP_3$ production was $19.7 \pm 9.9$% of the control by treatment with CEC alone, while NA-induced $IP_3$ production using the protocol for BMY-7378 protection from CEC alkylation was $49.2 \pm 3.6$% of the control.

## Discussion

As described in the Introduction, considerable progress in understanding vascular smooth muscle biology has been made using cultured VSMCs; however, one drawback has been that the cells lose differentiated properties in an unpredictable manner after repeated subcultures (Owens, 1995). Therefore, many studies have been performed on primary or very early subculture cells (Wikberg *et al*., 1983; Colucci *et al*., 1984; 1985; Okazaki *et al*., 1994; Chen *et al*., 1995; Hu *et al*., 1996; Xin *et al*., 1997). The experimental limitations of this approach are that only a small number of cells can be obtained from a single artery of a small experimental animal, and each primary culture contains a heterogenous population of cells that may have different characteristics including growth properties, receptor type and number, and expression of smooth muscle-specific genes. In an effort to establish an VSMC culture system that would be more homogenous and would provide a large number of cells for continued study, we had tried to

isolate and culture VSMC from mouse aorta. Limited dilution of enzymatically isolated mouse aortic cells was utilized to clone VSMC; however, only VSMC from p53 knockout mice, but not wild mice, can proliferate through multiple subcultures, and cells prepared from p53 knockout mice can be passaged for a long period (over 6 months, at least over 60 passages) (Ohmi *et al*., 1997). Also, even after multiple passages, cells revealed extended bipolar shape and expressed h-caldesmon and calponin as well as α-smooth muscle actin as protein markers of differentiated smooth muscle. Further, NA induced a concentration-dependent intracellular calcium response and a slowly developing contractile response in AC01 cells (Ohmi *et al*., 1997). In parallel experiments, a single cell imaging analysis revealed that NA did not cause any calcium response in either an established rat aortic VSMC line of A10 cells or primary cultured rat aortic VSMC (data not shown). The AC01 cell line, hence, is a cloned population that maintain VSMC characteristics, and appears to be a unique cell line for vascular smooth muscle biology experiments.

The present study pharmacologically characterized the subtype(s) of $\alpha_1$-AR which mediate the signalling pathway in a novel AC01 cell line. RT–PCR analysis detected the $\alpha_{1B}$- and $\alpha_{1D}$-AR transcripts, but not $\alpha_{1A}$-AR transcripts. Radioligand binding studies with subtype-selective ligands suggested that AC01 cells expresses predominantly $\alpha_{1B}$-AR. Similar to AC01 cells, the expression of $\alpha_{1B}$- and $\alpha_{1D}$-ARs was further confirmed in the native tissue of mouse aorta by RT–PCR as well as immunoblotting analysis (data not shown). However, the $\alpha_1$-AR-mediated $IP_3$ production in AC01 cells appears to be mainly mediated by the $\alpha_{1D}$-AR. Together, the results showed that AC01 cells express predominantly $\alpha_{1B}$-ARs with a small population of $\alpha_{1D}$-AR; however, the minor population of $\alpha_{1D}$-AR, rather than $\alpha_{1B}$-AR are important for $IP_3$ production.

Our conclusion that $\alpha_{1D}$-ARs, rather than $\alpha_{1B}$-ARs are functionally important is based on the two lines of pharmacological evidence. First, the inhibitory effect of BMY-7378 on NA-stimulated $IP_3$ production and the extent of 'protection by BMY-7378 from CEC alkylation' in AC01 cells were more similar to those observed in $\alpha_{1D}$-AR CHO cells than in $\alpha_{1B}$-AR CHO cells. Both assays depend on the selectivity of BMY-7378. An important consideration is potential species-related difference in the binding properties of BMY-7378 to the $\alpha_1$-ARs. We used AC01 cells originally derived from mouse aortic VSMCs, and cloned human receptors as controls in the present study ($\alpha_{1B}$-AR CHO cells and $\alpha_{1D}$-AR CHO cells). In fact, species-related difference between human and rodent $\alpha_1$-ARs was previously noted for (+)-niguldipine (Laz *et al*., 1994; Weinberg *et al*., 1994) and KMD-3213 (Shibata *et al*., 1995) when human and rat $\alpha_{1A}$-ARs expressed in COS-7 cells were compared.

Many types of tissues and cells have been shown to co-express $\alpha_1$-AR subtypes which share the signal transduction and play a similar functional role (Chen *et al*., 1995; Eckhart *et al*., 1996). Very recently, however, it has been noted that each $\alpha_1$-AR subtype might mediate the transduction of a distinct signal and have a distinct cellular function such as in VSMC growth (Xin *et al*., 1997). Also, $\alpha_1$-AR subtypes may be differentially regulated in many respects, such as in their cellular localization and agonist-promoted desensitization (Hirasawa *et al*., 1997; Awaji *et al*., 1998). Interestingly, Xin *et al*. (1997) demonstrated that growth of rat aorta VSMC (which express $\alpha_{1B}$- and $\alpha_{1D}$-ARs) is mediated by $\alpha_{1D}$-ARs, which is similar to our current observation. Our present observations further support the idea that each $\alpha_1$-AR subtype can mediate a distinct cellular function even though they are co-expressed.

Taken together, the development of a cultured VSMC line, AC01 cells, has enabled us to pharmacologically characterize the $\alpha_1$-AR-mediated signal transduction *in vitro*, but the experimental conditions are nearly the same as those *in vivo*. Moreover, we can manipulate the cellular environment and maintain precisely controlled experimental conditions for prolonged periods of time, allowing assessment of the influence of the cellular milieu on adrenergic receptor expression and function even after long periods of time. This unique VSMC model may provide a potentially useful tool for elucidating the functional specificity of different $\alpha_1$-AR subtypes, to better understand the effects of adrenergic drugs.

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