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Short sequence-paper

Cloning, functional expression and tissue distribution of rabbit α_{1d}-adrenoceptor [1]

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Abstract

We have cloned a cDNA encoding rabbit α_{1d} adrenoceptor from the rabbit liver cDNA library. The deduced amino-acid sequence of this clone encodes a protein of 576 amino acids that shows strong sequence homology to previously cloned human, rat and mouse α_{1d} adrenoceptors. The pharmacological radioligand binding properties of this clone expressed in COS-7 cells were similar to those of rat α_{1d}-adrenoceptors. Competitive RT/PCR assays revealed wide tissue distribution of the α_{1d} adrenoceptor mRNA in rabbit, especially abundant in vas deferens, aorta, prostate and cerebral cortex.

Keywords: α_{1d}-Adrenoceptor; Gene cloning; Nucleotide sequence; Pharmacological characteristic; Tissue distribution; (Rabbit)

α_1-Adrenoceptors are widely distributed in many tissues, where they mediate a diversity of sympathetic adrenergic responses [1,2]. However, the α_1-adrenoceptors are not homogeneous; there is a great deal of uncertainty regarding the kind and nature of the α_1-adrenoceptors. Two distinct subtypes (α_{1A} and α_{1B}) were first demonstrated in the radioligand binding studies [1,3], but the presence of additional subtypes (α_{1D}, α_{1L} and α_{1N}) has been proposed in the following pharmacological studies [2,4–6]. Thereafter, three receptors corresponding to the α_{1A}, α_{1B} and α_{1D} subtypes have been cloned in molecular biological studies [2,7,8], and the recombinant subtypes are designated as α_{1a}, α_{1b} and α_{1d} with lowercase subscript letters, in contrast to the native receptors with uppercase subscript letters mentioned above [9]. The α_{1d}-adrenoceptor was originally cloned by Lomasney et al. [10] and Perez et al. [11]. This receptor shows wide tissue distribution in mouse [12], rat [10,11] and human [13] but the physiological roles are still not clear, although predominant involvement of α_{1D}-adrenoceptor in the contractile response to noradrenaline was more recently reported in the rat thoracic aorta [14,15]. In the present study, we first report the cloning, sequence, pharmacological characteristics and tissue distribution of rabbit α_{1d}-adrenoceptor.

We have constructed a rabbit liver cDNA library in λZAP II vector and screened it with a 333 bp

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[1] The nucleotide sequence reported in this paper has been submitted to the GenBank under the accession number U64032.

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DNA fragment of bovine α_{1a}-adrenoceptor gene labeled by nick-translation as a probe. The 333 bp fragment was PCR amplified from bovine cortex cDNA with a pair of primers; 5′-TTC TGC AAT GTC TGG GCG GCG-3′ and 5′-GTA GAC CCG GCA GTA CAT GAC-3′ coding for the third to fifth transmembrane domains [16]. After isolating a positive plaque, the inserted DNA fragment was subcloned into pBluescript according to the manufacturer's instruction (Stratagene) and the nucleotide sequence was determined using overlapping templates by the dideoxy chain termination method with the ABI 373A DNA sequencer. Then, the clone was ligated into a mammalian expression vector pSI (Pro-

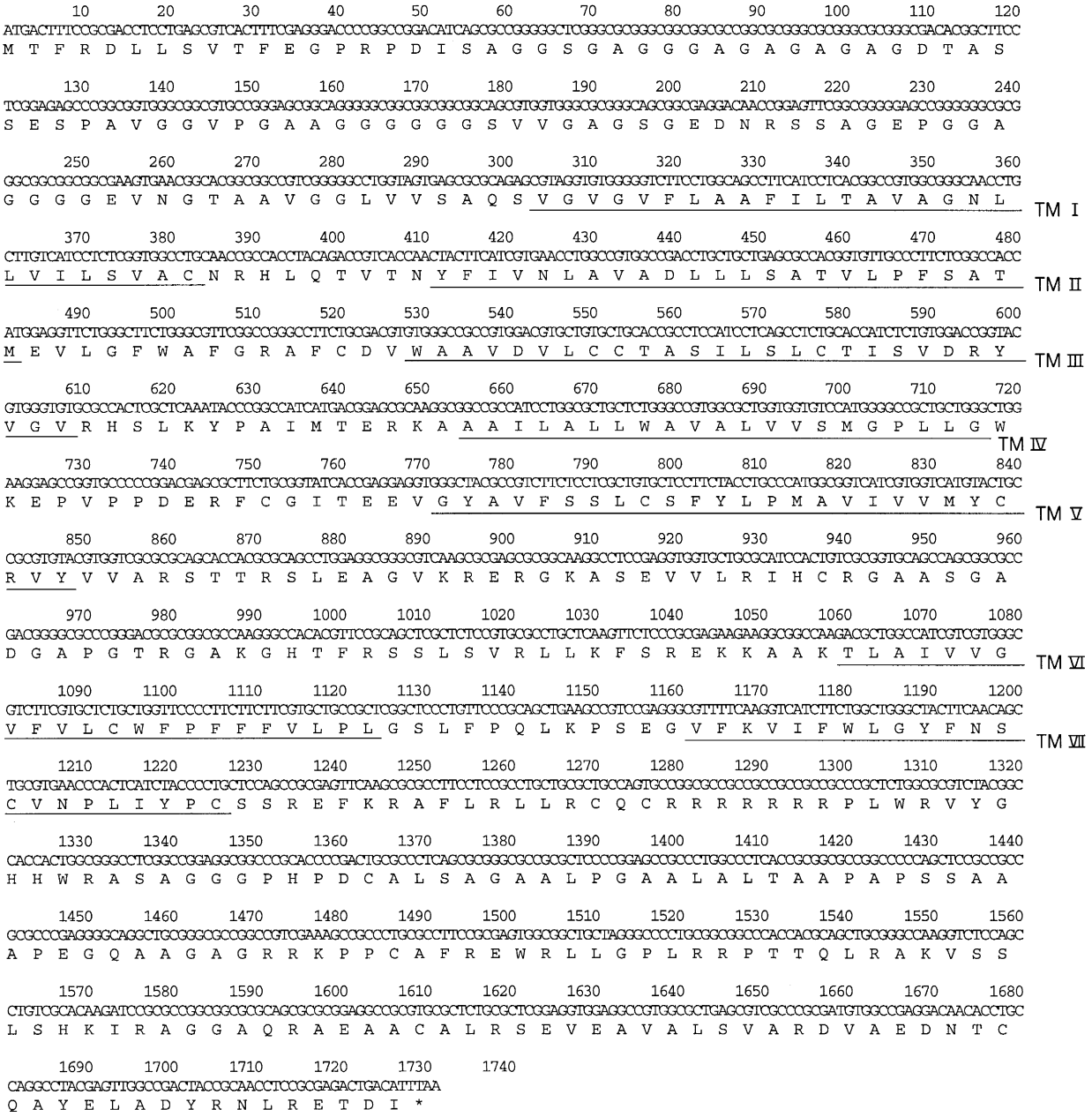

Fig. 1. Nucleotide and deduced amino-acid sequence of rabbit α_{1d} adrenoceptor. Deduced membrane-spanning segments TM I–TM VII are underlined.

mega) and transfected into COS-7 cells (RIKEN Cell Bank) by the DEAE-dextran method. As controls, human α_{1a}, hamster α_{1b} and rat α_{1d} adrenoceptor clones were also transfected [17]. Cells were harvested 72 h after transfection, homogenized and centrifuged at $80\,000 \times g$ for 30 min at 4°C. The resulting pellet was resuspended in the assay buffer (50 mM Tris-HCl, 1 mM EDTA, pH 7.4) and used for the binding assays, as described previously [18]. [^{3}H]YM617 (tamsulocin, NEN) was used as a radioligand for α_1-adrenoceptor binding, and nonspecific binding was defined under the presence of 0.3 μM prazosin in the reaction.

Tissue distribution of rabbit α_{1d}-mRNA was examined with competitive RT-PCR assays. Briefly, after extraction of total cellular RNA [19], the RNA was treated with RNase-free DNase I (Pharmacia) for 30 min at 37°C and was extracted again. The competitor RNA was transcribed with T7 RNA polymerase (Gibco BRL) from a template DNA which was constructed by inserting a 60 bp *Sma*I/*Rsa*I fragment of pBluescript DNA into *Msc*I site of rabbit α_{1d} clone (at 1066 base, see Fig. 1). The sample RNA (250 ng) was mixed with the competitor RNA (30 pg) and were reverse transcribed with Tth polymerase (Perkin Elmer) using an antisense primer (5′-GGG TAG ATG AGT GGG TTC AC-3′, 1223–1204 base) at 70°C for 15 min. The resulting cDNA was amplified in Programmable Thermal Controller PTC-100 (MJ Research) with Pwo DNA polymerase

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rabbit     1:MTFRDLLSVTFEGPRPDISAGGSGAGGGAGAGAGAGDTASSESPAVGGVPGAAGGGGGGS 60
human      1:.........S.......S.....S....-.-.-S..GA.P..G........G.-.....- 55
rat        1:.....I.........SSS.T.........-------.VGP.GG.......-.--T...A 50
mouse      1:.....I.........ASS.T.........-------.VGP.G........-.--T..SA 50

rabbit    61:VVGAGSGEDNRSSAGEPGGAGGGGEVNGTAAVGGLVVSAQSVGVGVFLAAFILTAVAGNL 120
human     56:..................S..A..D..............G............M..... 115
rat       51:...T......Q..T....-.AAS.....S...........G.................. 109
mouse     51:...T......Q..TA.A.-.AAS.....S...........G.................. 109

rabbit   121:LVILSVACNRHLQTVTNYFIVNLAVADLLLSATVLPFSATMEVLGFWAFGRAFCDVWAAV 180
human    116:.......................................................... 175
rat      110:.................................A..................T........ 169
mouse    110:.................................A..............P...T........ 169

rabbit   181:DVLCCTASILSLCTISVDRYVGVRHSLKYPAIMTERKAAAILALLWAVALVVSMGPLLGW 240
human    176:.............................................V......V...... 235
rat      170:.....................................................V...... 229
mouse    170:.....................................................V...... 229

rabbit   241:KEPVPPDERFCGITEEVGYAVFSSLCSFYLPMAVIVVMYCRVYVVARSTTRSLEAGVKRE 300
human    236:................A.......V.................................. 295
rat      230:.....................I...V.............................I... 289
mouse    230:.....................I...V.............................I... 289

rabbit   301:RGKASEVVLRIHCRGAASGADGAPGTRGAKGHTFRSSLSVRLLKFSREKKAAKTLAIVVG 360
human    296:................T.....H.M.S.............................. 355
rat      290:P..............TS.K.Y...QSS....L.......................... 349
mouse    290:P..............TS.K.N...QSS....L.......................... 349

rabbit   361:VFVLCWFPFFFVLPLGSLFPQLKPSEGVFKVIFWLGYFNSCVNPLIYPCSSREFKRAFLR 420
human    356:.......................................................... 415
rat      350:.......................................................... 409
mouse    350:.......................................................... 409

rabbit   421:LLRCQCRRRRRRRPLWRVYGHHWRASAGGGPHPDCALSAGAALPGAALALTA-A-PAPSS 478
human    416:.......................T-S.LRQ...P.S.D.P...P.....LPD.D.EP 474
rat      410:......-......-..A........-ST.DARS...P.PRI.P...P...-T-.H.GAG. 464
mouse    410:......-......-..PSLRPPLASLDRRPALRL.PQP.HRTPR.SPSPHCT-PR.GL-R 465

rabbit   479:AAAPEGQAAGAGRRKPPCAFREWRLLGPLRRPTTQLRAKVSSLSHKIRAGGAQRAEAACA 538
human    475:PGT..M..PV.S.....S..........F.............................. 534
rat      465:.DT..T.DSVSSS...AS.L.........Q.................S-..R...T... 523
mouse    466:RH.GGAGFGLRPS-.ASLRL.........Q.............F.S...R...T... 524

rabbit   539:LRSEVEAVALSVARDVAEDNTCQAYELADYRNLRETDI                      576
human    535:Q.......S.G.PHE...GA..........S.......                      572
rat      524:........S.N.PQ.G..AVI.....PG..S.......                      561
mouse    525:........S.N.PQ.G..AVI.....PG.LS.......                      562
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Fig. 2. Comparison of amino-acid sequences of the rabbit (U64032), human (L31772), rat (L31771) and mouse [14] α_{1d} adrenoceptors. Identical amino acids are substituted with dots, and gaps in the sequences are indicated with horizontal bars.

Table 1
Pharmacological characteristics of rabbit α_{1d}- and other α_1-adrenoceptors expressed in COS-7 cells

Drug	pK_i			
	rabbit α_{1d}	rat α_{1d}	human α_{1a}	hamster α_{1b}
Prazosin	9.15 ± 0.03	9.94 ± 0.04	9.71 ± 0.07	10.32 ± 0.05
WB4101	8.75 ± 0.03	9.61 ± 0.01	9.74 ± 0.02	8.74 ± 0.03
5-Methylurapidil	7.33 ± 0.13	7.88 ± 0.05	8.78 ± 0.03	7.34 ± 0.03
JTH-601	8.39 ± 0.05	8.97 ± 0.06	9.85 ± 0.04	9.17 ± 0.10
BMY7378	8.60 ± 0.05	9.05 ± 0.03	7.01 ± 0.05	7.29 ± 0.02
HV723	8.62 ± 0.06	8.94 ± 0.02	8.97 ± 0.01	8.80 ± 0.08
Rauwolscine	< 6	< 6	< 6	< 6
Propranolol	< 5	< 5	< 5	< 5
Noradrenaline	7.19 ± 0.06	7.12 ± 0.04	5.77 ± 0.04	5.90 ± 0.02
Oxymetazoline	5.96 ± 0.07	6.16 ± 0.04	8.03 ± 0.01	6.52 ± 0.04
correlation coefficient	–	0.98	0.60	0.82

Concentrations of [^{3}H]YM617 used were 200 pM, 100 pM, 70 pM and 500 pM for rabbit α_{1d}, rat α_{1d}, human α_{1a} and hamster α_{1b} adrenoceptors, respectively.
Data shown are mean ± standard error of 3–5 experiments.
pK_i: negative log of the equilibrium dissociation constant for each drug.
correlation coefficient: correlation between the affinities for tested drugs (except rauwolscine and propranolol) of rabbit α_{1d} subtype and the other subtypes.

(Boehringer) employing a primer set of a sense primer (5′-CTC CGT GCG CCT GCT CAA GT-3′, 1104–1033 base) and the antisense primer in the following condition; initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 30 sec and a final extension at 72°C for 4 min. PCR product was separated in 6% polyacrylamide gel electrophoresis and was stained with ethidium bromide. The intensity of the product band was quantified under UV light using Densitograph System (ATTO).

A single positive clone having a DNA insert of 2.6 kbp was isolated from the analysis of 3×10^5 recombinants. The nucleotide sequence of the cDNA clone (Fig. 1) contained an open reading frame of 1731 bp encoding a 576-amino-acid peptide. The amino-acid sequence had 84, 79 and 89% identity with rat [10,11], mouse [12] and human [13] α_{1d}-adrenoceptors, respectively (Fig. 2). The homologues of α_{1d}-adrenoceptor including this rabbit clone share high homology not only in the membrane spanning region but also in amino and carboxyl termini including two asparagine residues (70 and 87 residues) that could serve as *N*-glycosylation target. However, the homology of the amino-acid sequences between this rabbit clone and homologues of α_{1a} and α_{1b} subtypes was recognized only in the membrane spanning region and was less than 60%. We concluded that this clone encodes a rabbit α_{1d}-adrenoceptor. The result of sequence comparison suggests that α_{1d}-specific char-

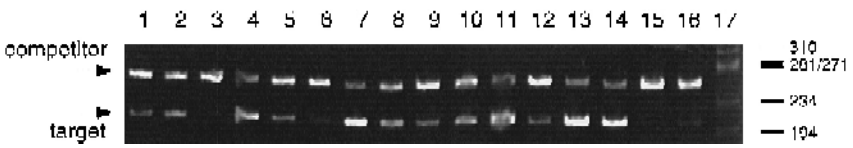

Fig. 3. Competitive RT-PCR analysis of α_{1d}-adrenoceptor mRNA from rabbit, various tissues. Total RNA from rabbit tissues were mixed with competitor RNA and were reverse transcribed, PCR amplified and separated in electrophoresis as described in the text. The product from tissue RNA (210 bp) and that from competitor RNA (270 bp) are indicated by arrows on the left. Analyzed rabbit tissues are heart, kidney, liver, lung, spleen, skeletal muscle, aorta, cerebellum, hippocampus, brain stem, cerebral cortex, thalamus, vas deferens, prostate, submaxillary gland and parotid gland, from lanes 1 to 16, respectively. The ϕX174 DNA/*Hae*III marker was run in lane 17.

Table 2
Distribution of α_{1d}-adrenoceptor mRNA in rabbit tissues

Tissue	Relative level
Heart	0.3 ± 0.2
Kidney	0.6 ± 0.0
Liver	< 0.1
Lung	2.5 ± 0.1
Spleen	0.5 ± 0.0
Skeletal muscle	< 0.1
Thoracic aorta	5.2 ± 0.9
Cerebellum	1
Hippocampus	0.4 ± 0.1
Brain stem	0.8 ± 0.3
Cerebral cortex	3.1 ± 0.7
Thalamus	0.4 ± 0.1
Vas deferens	5.6 ± 2.1
Prostate	4.2 ± 1.2
Submaxillary gland	< 0.1
Parotid gland	< 0.1

Relative levels of expression of α_{1d}-adrenoceptor mRNA in various tissues were listed using the level of that in cerebellum as 1.
Values in the table represent mean ± standard error of three independent experiments.

acter depends not only on the membrane spanning region but on the extracellular as well as the intracellular tails.

Transfection of COS7 cells with the cDNA clones of rabbit α_{1d} and other α_1-adrenoceptors resulted in specific binding of the [^{3}H]YM617. Analysis of saturation binding data for [^{3}H]YM617 resulted in a single dissociation constant, $pK_D = 9.73 \pm 0.05$, 10.17 ± 0.01, 10.63 ± 0.04 and 9.45 ± 0.06 for rabbit α_{1d}, rat α_{1d}, human α_{1a} and hamster α_{1b} adrenoceptors, respectively. The pharmacological binding profile of rabbit α_{1d}-adrenoceptor was characteristic of that expected for an α_{1d} subtype (Table 1). Especially, BMY7378, an α_{1d}-selective antagonist [20], and noradrenaline showed higher affinity for the α_{1d}-subtypes than for α_{1a} and α_{1b} subtypes. This pharmacological analysis also supported our conclusion above based on the peptide sequence.

In the competitive RT/PCR assay used, we confirmed linear relationship between the ratios of amplified target and competitor signals changing the competitor doses from 3 pg to 100 pg (data not shown). This range of linearity is compatible with other reports [21–23]. The representative results of competitive RT/PCR for rabbit tissues are shown in Fig. 3. The α_{1d}-adrenoceptor mRNA was expressed most abundantly in the rabbit vas deferens, prostate, aorta and cerebral cortex (Table 2). This distribution pattern was consistent with those in rat [10,11,22], human [13,23] and mouse [12]. Since the physiological roles of α_{1d}-adrenoceptor are little known except the functional involvement in noradrenaline-induced contraction of rat aorta [14,15], the result presented here would be an important clue to uncover its biological functions.

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References

[1] Minneman, K.P. (1988) Pharmacol. Rev. 40, 87–119.
[2] Graham, R.M., Perez, D.M., Hwa, J. and Piascik, M.T. (1996) Circ. Res. 78, 737–749.
[3] Morrow, A.L. and Creese, I. (1986) Mol. Pharmacol. 29, 321–330.
[4] Ford, A.P.D.W., Williams, T.J., Blue, D.R. and Clarke, D.E. (1994) Trends Pharmacol. Sci. 15, 167–170.
[5] Muramatsu, I., Ohmura, T., Hashimoto, S. and Oshita, M. (1995) Pharmacol. Commun. 6, 23–28.
[6] Muramatsu, I., Ohmura, T., Kigoshi, S., Hashimoto, S. and Oshita, M. (1990) Br. J. Pharmacol. 99, 197–201.
[7] Lomasney, J.W., Cotecchia, S., Lefkowitz, R.J. and Caron, M.G. (1991) Biochim. Biophys. Acta 1095, 127–139.
[8] Bylund, D.B. (1992) FASEB J. 6, 832–839.
[9] Hieble, J.P., Bylund, D.B., Clarke, D.E., Eikenburg, D.C., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P. and Ruffolo, R.R. (1995) Pharmacol. Rev. 47, 267–270.
[10] Lomasney, J.W., Cotecchia, S., Lorenz, W., Leung, W.-Y., Schwinn, D.A., Yang-Feng, T.L., Brownstein, M., Lefkowitz, R.J. and Caron, M.G. (1991) J. Biol. Chem. 266, 6365–6369.
[11] Perez, D.M., Paisick, M.T. and Graham, R.M. (1991) Mol. Pharmacol. 40, 876–883.
[12] Alonso-Llamazares, A., Zamanillo, D., Casanova, E., Ovalle, S., Calvo, P. and Chinchetru, M.A. (1995) J. Neurochem. 65(6), 2387–2392.
[13] Weinberg, D.H., Trivedi, P., Tan, C.P., Mitra, S., Perkins-Barrow, A., Borkowski, D., Strader, C.D. and Bayne, M. (1994) Biochem. Biophys. Res. Commun. 201(3), 1296–1304.
[14] Kenny, B.A., Chalmers, D.H., Philpott, P.C. and Naylor, A.M. (1995) Br. J. Pharmacol. 115, 981–986.

[15] Buckner, S.A., Oheim, K.W., Morse, P.A., Knepper, S.M. and Hancock, A.A. (1996) Eur. J. Pharmacol. 297, 241–248.
[16] Schwinn, D.A., Lomasney, J.W., Lorenz, W., Szklut, P.J., Fremeau, R.T., Jr., Yang-Feng, T.L., Caron, M.G., Lefkowitz, R.J. and Cotecchia, S. (1990) J. Biol. Chem. 265(14), 8183–8189.
[17] Muramatsu, I., Takita, M., Suzuki, F., Miyamoto, S., Sakamoto, S. and Ohmura, T. (1996) Eur. J. Pharmacol. 300, 155–157.
[18] Oshita, M., Kigoshi, S. and Muramatsu, I. (1993) Br. J. Pharmacol. 108, 1071–1076.
[19] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156–159.
[20] Goetz, A.S., King, H.K., Ward, S.D.C., True, T.A., Rimele, T.J. and Saussy, D.L., Jr. (1995) Eur. J. Pharmacol. 272, R5–R6.
[21] Gilliland, G., Perrin, S., Blanchard, K. and Bunn, H.F. (1990) Proc. Natl. Acad. Sci. USA 87, 2725–2729.
[22] Scofield, M.A., Liu, F., Abel, P.W. and Jeffries, W.B. (1995) Pharmacol, J.. Exp. Ther. 275(2), 1035–1042.
[23] Faure, C., Gouhier, C., Langer, S.Z. and Graham, D. (1995) Biochem. Biophys. Res. Commun. 213(3), 935–943.