Identification of $\alpha_{1C}$-Adrenergic Receptor mRNA in Bovine Retinal Pigment Epithelium

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Purpose. To examine the localization of a novel $\alpha_1$-adrenergic receptor subtype of $\alpha_{1C}$ receptor in the eye, we compared the amount of $\alpha_{1C}$ receptor transcripts in bovine retinal pigment epithelium (RPE) and in neural retina by employing reverse transcription of mRNA and the polymerase chain reaction (RT-PCR) assay.

Methods. RT-PCR assay with specific primers for the $\alpha_{1C}$-adrenergic receptor and for $\beta$-actin showed linear relationships between input quantity of RNA and the amount of amplified PCR products for the $\alpha_{1C}$-adrenergic receptor and also for $\beta$-actin, when PCR was conducted for 24 and 15 cycles, respectively.

Results. The RT-PCR assay demonstrated that the spontaneous expression level of the $\alpha_{1C}$-adrenergic receptor mRNA was much higher in bovine RPE than in neural retina; the $\alpha_{1C}$-adrenergic receptor/$\beta$-actin ratio from RPE was 0.33 ± 0.15 (n = 4), whereas that from neural retina was virtually zero.

Conclusions. The RT-PCR assay is a sensitive semiquantitative method for a low abundance mRNA in a limited number of cells. Using the $\alpha_{1C}$ receptor as a model, we demonstrated the usefulness of this assay by showing the uneven distribution of the $\alpha_{1C}$ receptor transcripts in bovine RPE cells and neural retina. Invest Ophthalmol Vis Sci 1993:34:2769-2775.

The retinal pigment epithelium (RPE) is a monolayer of cells located on the Bruch's membrane interposed between the photoreceptor cells and the choroid. It is responsible for ion transport between the choroid and the outer retina, water transport away from the photoreceptor cells, and phagocytosis of shed photoreceptor outer segments. Catecholamines play an important role in regulating the physiologic functions of RPE through the interaction with specific adrenergic receptors.1 Transepithelial potential measurements in rabbit RPE indicate that the epinephrine- and phenylephrine-stimulated electrical responses are blocked by the nonselective $\alpha$-adrenergic receptor antagonist phentolamine and by the $\alpha_1$-adrenergic receptor-selective antagonist prazosin, but not by the $\alpha_2$-adrenergic receptor-selective antagonist yohimbine.2 Also, the epinephrine-stimulated fluid absorption of bovine RPE is found to be completely blocked by prazosin but not by propranolol.3 Therefore, these pharmacologic evidence suggest that $\alpha_1$-adrenergic receptors are considered to play functionally important roles in RPE.

Recent evidence increasingly suggests that $\alpha_1$-adrenergic receptors do not have the same properties in all tissues.4-7 Pharmacologic studies including radioligand binding and functional experiments have suggested that at least two subtypes of $\alpha_1$-adrenergic receptors of $\alpha_{1A}$ and $\alpha_{1B}$ receptors can be differentiated.4-7 Furthermore, using the molecular biologic approach, the cDNA that encodes a novel $\alpha_1$-adrener-
gic receptor subtype, the α_{1C} receptor, has been recently cloned from a bovine brain cDNA library; however, very little information is still available regarding the physiologic roles and the regulation of this new receptor subtype. These α_{1} receptor subtypes are all members of the GTP-binding protein-coupled receptor families, and have putative seven-spanning transmembrane domains in common. Because their amino acid residues are highly homologous with each other (~50% to 60% identity), it is difficult to distinguish between the α_{1} receptor subtypes: neither a subtype-specific pharmacologic tool nor antibodies are available yet. Thus, to detect mRNA specific for respective subtype using the subtype-selective base sequences would provide the most specific approach for identifying the expression of the distinct receptor subtype.

In addition to the receptor heterogeneity, expression of adrenergic receptors is regulated dynamically in a variety of physiologic and pathophysiologic conditions, which may generate functional variety. In the desensitization of adrenergic receptor-mediated responses, for example, functional uncoupling from covalent phosphorylation of receptor or downregulation has been well characterized. In addition to these regulatory mechanisms at the cell membrane level, recent evidence increasingly suggests that the regulation of receptor genes at the transcription level, for example, functional uncoupling from covalent phosphorylation of receptor or downregulation has been well characterized. Therefore, the development of a sensitive assay to monitor the amount of mRNA expression in a small number of cells would be required for further clinical and basic studies.

The present study was designed to monitor the spontaneous expression of a very small amount of mRNA, such as adrenergic receptor transcripts. Employing a reverse transcription-polymerase chain reaction (RT-PCR) assay, we demonstrate here the usefulness of this approach by showing a high amount of the α_{1c}-adrenergic receptor mRNA expression in RPE and markedly less expression in neural retina.

**MATERIALS AND METHODS**

**Preparation of RPE and Neural Retina**

Bovine eyes were obtained from a local slaughterhouse and preparation of RPE and neural retina was performed within 3 hours after enucleation. The anterior portion of the eye was removed by a circular cut just posterior to the limbus, and the vitreous body was discarded. Then the neural retina was cut at its attachment to theoptic nerve head. The neural retina was immediately frozen in liquid nitrogen and stored at -80°C. The exposed RPE was rinsed with Ca^{2+}-and Mg^{2+}-free phosphate-buffered saline and gently brushed from the underlying choroidal layer using a glass spatula. Cells were removed by aspiration and centrifuged. The pellet was immediately frozen in liquid nitrogen and stored at -80°C until assay. The number of RPE cells collected in this fashion was counted by a hemocytometer chamber under a microscope. The preparation contained more than 95% RPE cells; approximately 4 x 10⁶ cells were obtained from one eye.

**Isolation of Total Cellular RNA**

Total cellular RNA from RPE and neural retina were extracted by complete lysis with 5.5 M guanidine isocyanate and purified from 2.0 ml of lyase per tube by centrifugation through 0.8 ml of 5.7 M cesium chloride cushion in a TL100.3 fixed-angle rotor (Beckman Instruments, Inc., Palo Alto, CA) at 350,000 g for 4 hours. The RNA pellet was collected in 200 μl of TNES solution (0.1 M Tris-HCl, pH 7.5, 12.5 mM EDTA, 0.15 M NaCl, 1% SDS), extracted with phenol/chloroform (1:1 vol/vol), ethanol-precipitated, then vacuum-dried, resuspended in 50 μl of RNase-free water, and quantified by absorbance measurements at 260 nm. Isolated total cellular RNA of RPE from one eye was approximately 15–18 μg, which provided enough RNA for RT-PCR assay. In preliminary experiments, the integrity of the purified RNA collected with this method was confirmed by visualization of the 28S and 18S ribosomal RNA bands after the electrophoresis of RNA through a 1% agarose-formaldehyde ethidium bromide gel.

**Treatment of Total Cellular RNA With DNasei**

To eliminate contaminating genomic DNA, prepared total cellular RNA samples were further treated with RNase-free DNasel (Stratagene, La Jolla, CA). RNA samples (10 μg each) were incubated at 37°C for 30 minutes with 20 U of DNaseI in 30 μl of DNase buffer [40 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 2 mM CaCl₂, 2 U of placental RNase inhibitor (TaKaRa, Kyoto, Japan)]. The reaction was stopped by an extraction with phenol/chloroform (1:1 vol/vol), and RNA samples were ethanol-precipitated, then vacuum-dried, and resuspended in 14 μl of RNase-free water.

**Reverse Transcription of RNA**

RNAs from RPE and neural retina were reverse transcribed as follows: Each sample contained 10 μg of total cellular RNA; 50 μM Tris-HCl pH 8.3; 75 mM KCl; 0.5 mM MgCl₂; 10 mM dithiothreitol; 0.5 mM of each dNTP (dATP, dTTP, dGTP, dCTP); 20 U of RNase inhibitor; 100 pmol random hexamer (TaKaRa, Kyoto, Japan); and 200 U moloney murine leukemia virus reverse transcriptase (Gibco BRL, Life Technologies, Inc., Gaithersburg, MD) in a final volume of 25 μl. After incubation at 37°C for 60 minutes, the sam-
Preparation of Oligonucleotide Primers and Sequence of PCR Products

The primers were synthesized on a model 391A (Applied Biosystems, Inc., Foster City, CA) DNA synthesizer (using β-cyano-methylphosphoramidate derivatives), quantified by absorbance measurements at 260 nm, and stored at -20°C. Oligonucleotide primers were constructed from the published cDNA sequences of bovine α₁C-adrenergic receptor and rat β-actin cDNA. β-actin served as a cell cycle-independent standard for the efficacy of RNA isolation and cDNA synthesis.

The α₁C-adrenergic receptor primers were:
1. 5'-AAACACACCCTGGGCTACACGCTGCAC-GCA-3' (coding sense) corresponding to bases 1153-1182 of the cloned full-length sequence
2. 5'-GTGGGTACCTAAGATCACCCTCCCATCCTTC-3' (anticoding sense) which anneals to bases 1514-1544

The β-actin primers were:
1. 5'-ATCATGTTTGAGACCTTCAACCCCA-GCC-3' (coding sense) corresponding to bases 2158-2187 of the cloned full-length sequence
2. 5'-AAGAGAGCCTCGGGGCATCGGAACCGC-TCA-3' (anticoding sense) corresponding to bases 2550-2579

The predicted sizes of the amplified α₁C-adrenergic receptor and β-actin DNA products were 392 and 422 base pairs, respectively.

Nucleotide sequences of the amplified α₁C-adrenergic receptor and β-actin DNA products were determined as follows: Fragments of PCR products were gel purified and inserted into pBluescript II KS(+) (Stratagene, La Jolla, CA), and subcloned clones were analyzed on an automated fluorescence-based GENESISTM 2000 sequencing system (Applied Biosystems, Inc., Foster City, CA) using a fluorescent dideoxy sequencing techniques.

Amplification of α₁C-Adrenergic Receptor and β-Actin cDNAs

Each reverse transcription mixture was diluted 1:5 in RNase-free water and 5 μl (for α₁C-adrenergic receptor) or 2 μl (for β-actin) was then transferred to fresh tubes for amplification. Each sample contained the upstream and downstream primers (0.2 μM of each primer) spanning the given sequence for amplification; 200 μM of each dNTP (dTTP, dATP, dCTP, dGTP, dTTP); 50 mM KCl; 10 mM Tris-HCl; pH 8.3; 10 mM MgCl2; 0.01% (wt/vol) gelatin; 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a final volume of 25 μl. The reaction mixture was then overlaid with 3 drops (25 μl) of mineral oil and amplified for 12–39 cycles in a model PJ 2000 DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT). The amplification profiles consisted of denaturation at 94°C for 1 minute, primer annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute for α₁C-adrenergic receptor, whereas for β-actin, 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes, respectively. Negative control reactions without template were routinely included in PCR amplifications with both primer sets.

Quantification of the Amplified Products

After completion of the RT-PCR, each amplified DNA was electrophoresed through a 2% (wt/vol) agarose gel containing 0.5 μg/ml of ethidium bromide in 40 mM Tris-acetate buffer, pH 8.0, containing 1 mM EDTA.

For Southern blots, PCR products were blotted onto HybondN+ nylon membranes (Amersham, United Kingdom), prehybridized in 50 ml of buffer composed of 5 × saline-sodium citrate buffer (SSC), 5 × Denhardt’s solution, 0.5% (wt/vol) SDS, and heat-denatured salmon sperm DNA (100 μg/ml). Hybridization was conducted in 50 ml of buffer as described in the presence of 5 × 10⁶ dpm of the 32P-labeled probe prepared by random primer labeling (TaKaRa, Kyoto, Japan) for overnight at a temperature 65°C. Final wash condition of hybridization was 0.1 × SSC for 20 minutes at 65°C. The autorograms of Southern blots were analyzed using BAS 2000 (Fuji, Tokyo, Japan).

RESULTS

Verification of PCR Products as Predicted Segments of α₁C-Adrenergic Receptor and β-Actin mRNAs

To verify that the amplified products derived from the bovine α₁C-adrenergic receptor and β-actin primers were authentic, we determined the sizes of the amplified products and prepared Southern blot analyses of the amplified DNAs, which we then probed with labeled sequenced PCR products and confirmed by sequencing the RT-PCR products. The amplified products of both α₁C-adrenergic receptor and β-actin were identical to the predicted sizes for the mRNA template of 391 and 421 base pairs, respectively. Southern blot analyses of the amplified products hybridized with labeled α₁C-adrenergic receptor and β-actin cDNA probes produced signals that corresponded in size to the ethidium bromide-stained gel (data not shown). The sequence of PCR product of α₁C-adrenergic receptor from RPE cDNA was identical to nucleotide
positions 1153–1544 of bovine α1C-adrenergic receptor cDNA (data not shown).

To confirm that the amplified products were originated from mRNA rather than from contaminating genomic DNA, we compared the amounts of RT-PCR products with or without DNasel or RNaseA treatment, or both. As shown in Figure 1, using bovine genomic DNA as a template for PCR reaction, the predicted sizes of 392 base pairs product for the α1C receptor was obtained (lane 9), which was abolished with DNasel treatment (lane 8). The identical size of products as the amplified product of the genomic DNA were obtained from RT-PCR in RPE (lanes 2 and 3) and neural retina (lanes 6 and 7), indicating that the α1C gene has no intron in this fragment. The RT-PCR products obtained in RPE were abolished with RNaseA pretreatment of RNA before being reverse transcribed either with or without DNasel treatment (lanes 4 and 5), showing that the amplified products were originated from mRNA rather than from contaminating genomic DNA. In neural retina, on the other hand, the same PCR amplification used for RPE (for 30 cycles) produced a much weaker signal compared to RPE (lane 7), and the signal was substantially reduced with DNasel treatment (lane 6), indicating a substantial genomic DNA contamination in this case.

Kinetics and Yield of Amplification of α1C-Adrenergic Receptor and β-Actin mRNAs

First, we have varied the number of cycles of amplification to determine the points at which amplification is within the exponential phase. In Figure 2, the amplification of the α1C-adrenergic receptor mRNA derived from bovine RPE. The extent of the α1C-adrenergic receptor amplification was measured by the autoradiograms with BAS 2000. The open circle and the closed circle indicate 500 ng and 50 ng of total cellular RNA equivalent, respectively.

FIGURE 2. Amplification of the α1c-adrenergic receptor mRNA derived from bovine RPE. The extent of the α1c-adrenergic receptor amplification was measured by the autoradiograms with BAS 2000. The open circle and the closed circle indicate 500 ng and 50 ng of total cellular RNA equivalent, respectively.

Second, we also determined the relationship between input RNA and the amount of amplified PCR product. Increasing amounts of input RNA were reverse transcribed, amplified for 24 cycles, and the amount of PCR product was quantified by BAS 2000 (Fuji, Tokyo, Japan). As shown in Figure 3, a linear relationship between input RNA and PCR product was observed.

To determine that differences in the α1C-adrenergic receptor mRNA measurements between samples were not the result of random variations in the input quantity of RNA or in the efficiency of the reverse transcription, we amplified a β-actin mRNA. In Figure 4A, the amplification of β-actin mRNA remained in the exponential phase at 18 and 21 cycles, whereas the plateau of amplification was reached at 24 cycles. Increasing amounts of input RNA were reverse transcribed, amplified for 15 cycles, and quantified by BAS 2000. As shown in Figure 4B, a linear relationship between input RNA and PCR product was observed.

Comparison of the Expression of α1C-Adrenergic Receptor mRNA in RPE and Neural Retina

Using the RT-PCR assay described above, we compared the level of the α1C-adrenergic receptor mRNA
a1c-Adrenergic Receptor mRNA in Bovine RPE

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FIGURE 3. Quantitative analysis of the a1c-adrenergic receptor mRNA levels generated from bovine RPE for 24 cycles of amplification. (top) The autoradiograms of Southern blots of PCR products amplified from the indicated amount of total cellular RNA. (bottom) The radioactivity of the corresponding amplified products plotted against the input quantity of RNA.

DISCUSSION

In this study, we adopted a sensitive semiquantitative assay for a very small amount of mRNA in a limited number of cells. For one assay of a1c-adrenergic receptor amplification, approximately 500 ng of total cellular RNA is required. This amount could be extracted from less than one twentieth of the RPE sheet (approximately 2 x 10^5 cells) obtained from one bovine eye. Our study showed the effectiveness of the assay by determining the uneven distribution of the a1c-adrenergic receptor mRNA expression in RPE and neural retina; thus, the spontaneous expression level of the a1c-adrenergic receptor mRNA was much higher in RPE than in neural retina. By contrast, the conventional analysis of mRNA, Northern blot analysis, could not detect the a1c-adrenergic receptor mRNA in bovine RPE even though 5 µg of poly(A)+-selected mRNA collected from more than 50 eyes was used (data not shown). Because the RT-PCR method can monitor the amount of mRNA in a limited number of cells, it should provide a powerful approach to investigate the alterations of the targeted cells at the transcription level in various disease states.

With the RT-PCR assay, we found a substantially lower expression of a1c receptor mRNA in neural retina than RPE. Previous radioligand binding studies, however, have suggested the presence of a1-adrenergic receptors in both bovine RPE and neural retina with an almost equal amount or an even higher level of expression in neural retina.\textsuperscript{24,25} Therefore, a1 receptors in neural retina may contain a1A receptors, a1B receptors, or both and very few, if any, a1C receptors; whereas in RPE, other a1 receptor subtypes could exist besides a1C receptors. Further studies clearly are

FIGURE 4. (A) Amplification of β-actin mRNA derived from bovine RPE. The open circle and the closed circle indicate 100 ng and 10 ng of total cellular RNA equivalent, respectively. The amplification of β-actin mRNA remained in the exponential phase at 18 and 21 cycles, whereas the plateau of amplification was reached at 24 cycles. (B) Quantitative analysis of β-actin mRNA level generated from bovine RPE for 15 cycles of amplification. The radioactivity of amplified products is plotted against input quantity of total cellular RNA.

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