

# Reports

## Beta-adrenergic receptors in ciliary processes of the rabbit. B. BRITT BROMBERG, DOUGLAS S. GREGORY, AND MARVIN L. SEARS.

*Identification and characterization of beta-adrenergic receptors were attempted in particulate membrane fractions derived from isolated ciliary processes\* (CP) of rabbit eyes. High-affinity binding sites for <sup>125</sup>I-hydroxybenzylpindolol (<sup>125</sup>I-HYP), a beta-adrenergic antagonist, were identified in particulate membrane fractions of homogenized CP that were recovered from discontinuous sucrose density gradients. Adenylate cyclase activity was recovered in the same fraction as the <sup>125</sup>I-HYP binding sites. The dissociation constant of <sup>125</sup>I-HYP for the high affinity site is 0.25 nM, with a minimum capacity of about 35 fmol/mg of protein. Adrenergic agonists and antagonists, including timolol, l-alprenolol, d,l-propranolol, l-isoproterenol, l-epinephrine, and phentolamine, were examined for their ability to displace <sup>125</sup>I-HYP from its binding site. The results were consistent with the identification of the high-affinity <sup>125</sup>I-HYP binding sites as beta-adrenergic receptors. This is the first report which identifies by ligand binding techniques beta-adrenergic receptors in CP exclusive of iridial or other uveal tissue and supports the possibility of direct action of beta-adrenergic agents on the formation of aqueous humor.*

Beta-adrenergic agents are assumed to alter the production of aqueous humor (AH) by occupying beta-adrenergic receptors in the ciliary processes (CP) and possibly elsewhere. Lahav et al.<sup>1</sup> found that a systemically delivered fluorescent analogue of propranolol was localized in the CP of the rat. Neufeld and Page<sup>2</sup> identified beta-adrenergic receptors in preparations of rabbit iris-ciliary body. This report describes the binding of <sup>125</sup>I-hydroxybenzylpindolol (<sup>125</sup>I-HYP), a beta-adrenergic antagonist,<sup>3</sup> to particulate fractions of rabbit CP that are free of iris smooth muscles and other uveal tissue. Competitive binding of other adrenergic agents and the presence of adenylate cyclase in the binding fractions support the identification of the <sup>125</sup>I-HYP binding site as the physiologically active beta-adrenergic receptor.

**Materials and methods.** *d,l*-Propranolol HCl, *l*-norepinephrine HCl, *l*-isoproterenol HCl, and

*l*-alprenolol-*d*-tartrate were purchased from Sigma Chemical Co. (St. Louis, Mo.). Hydroxybenzylpindolol, phentolamine mesylate, and timolol maleate were generously donated by Sandoz Pharmaceuticals (East Hanover, N. J.), CIBA Pharmaceutical Co. (Summit, N. J.), and Merck Sharp & Dohme (West Point, Pa.), respectively. <sup>125</sup>I-HYP, alpha-<sup>32</sup>P-ATP, and <sup>3</sup>H-cAMP were purchased from New England Nuclear (Boston, Mass.). Ultrapure sucrose, AG50W-X4 cation exchange resin, and neutral alumina were purchased from Schwarz/Mann (Orangeburg, N. Y.), Bio-Rad Laboratories (Rockville Center, N. Y.), and Alupharm Chemicals (New Orleans, La.), respectively.

**Tissue preparation.** Male albino New Zealand rabbits, 1 to 1.5 kg, were killed by intravenous injection of sodium pentobarbital or by intracardiac air embolism. The enucleated eyes were chilled in normal saline before dissection. All subsequent procedures were carried out at 0° to 5° C, unless otherwise stated. The anterior segment was isolated free of lens and zonule fibers. The preparation was everted and secured to a Plexiglas holder within a small Petri dish. The Petri dish was filled with ice-cold 0.9% NaCl so that the CP "floated" upward rather than lying adherent to one another. Under a dissecting microscope, individual CP from both eyes were cut free and pooled in a beaker of 0.9% saline.

The CP were homogenized with 10 strokes of a motor-driven Teflon pestle in a glass Potter-Elvehjem homogenizer containing 1 ml of 50 mM Tris HCl (pH 7.5) and 1 mM *l*-ascorbic acid. After cooling in ice, the procedure was repeated twice, and the preparation was pelleted in the homogenizer at 3000 ×g in a Sorvall (Model RC-5) centrifuge. The supernatant, which contained virtually no ligand binding sites (unpublished observation), was discarded. The pellet was rehomogenized and filtered through a single layer of nylon mesh.

Filtrate aliquots of 0.2 ml were preincubated for 5 min at 37° C. Binding was initiated by the addition of 0.05 ml of <sup>125</sup>I-HYP and competing ligand when appropriate. After 15 min, the reaction mixture was placed on ice. Aliquots of 0.2 ml were then layered on 4.8 ml discontinuous sucrose gradients prepared as follows: 1 ml of 50%: 1 ml of 20%: 2.8 ml of 5%. The gradients contained 1 mM *l*-ascorbic acid and 50 mM Tris HCl, pH 7.5, throughout and were centrifuged for 1 hr at

\*Technically, the tissues used in this study are iridial processes which are cytologically similar to true ciliary processes of other mammals. For convenience, the term *ciliary processes* will be used.

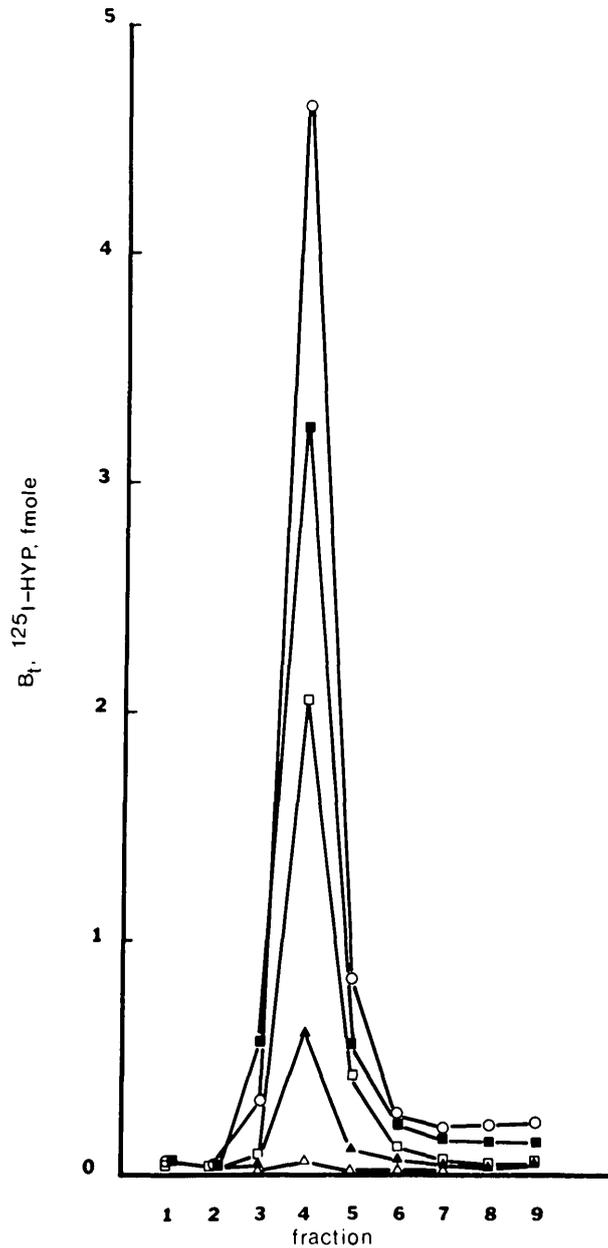


Fig. 1. Concentration-dependent binding of  $^{125}\text{I}$ -HYP to particulate membrane fractions of homogenized CP.  $^{125}\text{I}$ -HYP concentration: ○,  $7.5 \times 10^{-10}\text{M}$ ; ■,  $4.4 \times 10^{-10}\text{M}$ ; □,  $2.2 \times 10^{-10}\text{M}$ ; ▲,  $4.8 \times 10^{-11}\text{M}$ ; △,  $4.4 \times 10^{-12}\text{M}$ .  $B_t$ , Total binding.

192,000  $\times$  g in a Beckman L-2 ultracentrifuge (SW 50.1 rotors). Fractions (15 drops) were collected after puncturing the bottom of the polyallomer centrifuge tube. The amount of  $^{125}\text{I}$  in each fraction was determined in a Beckman Gamma 4000 automatic gamma counter. Protein was measured by the method of Lowry et al.<sup>4</sup> Dissociation constants ( $K_d$ 's) for several adrenergic agonists and antagonists were determined by the concentra-

tion-dependent displacement of  $^{125}\text{I}$ -HYP. In competitive assays, the concentration of competing ligand which removed 50% of the specifically bound  $^{125}\text{I}$ -HYP ( $2 \times 10^{-10}\text{M}$ ) was used to calculate the  $K_d$  as described by Maguire et al.<sup>3</sup>

Adenylate cyclase activity was determined by a modification of the technique of Salomon et al.<sup>5</sup> A 20  $\mu\text{l}$  aliquot of each fraction was preincubated for 10 min at 30° C with 5  $\mu\text{l}$  of  $4 \times 10^{-3}\text{M}$  (final)

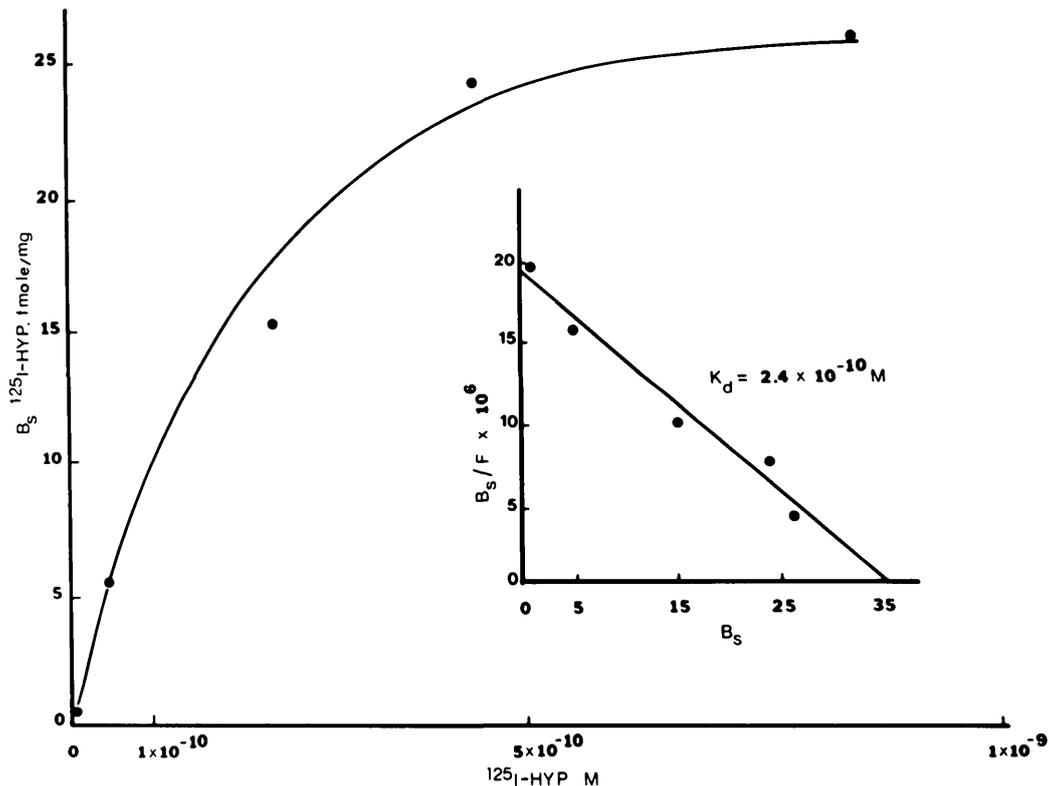


Fig. 2. Net binding of  $^{125}\text{I}$ -HYP to particulate membrane fractions from homogenized CP. Data are derived from Fig. 1.  $B_s$ , specific binding. Inset, Scatchard plot.  $F$ , unbound concentration of  $^{125}\text{I}$ -HYP.

NaF. The reaction was initiated by addition of  $15 \mu\text{l}$  of  $2.67 \text{ mM}$  alpha- $^{32}\text{P}$ -ATP (20 to 40 Ci/mol) and  $2.67 \text{ mM}$   $^3\text{H}$ -cAMP (8 Ci/mol). The final concentrations of both ATP and cAMP were  $1 \text{ mM}$ . The start solution also contained components to yield the following final concentrations:  $5 \times 10^{-3} \text{ M}$   $\text{MgSO}_4$ ,  $2 \times 10^{-3} \text{ M}$  dithiothreitol,  $1 \times 10^{-2} \text{ M}$  creatine phosphate, 1 to 2 U of creatine kinase, and  $0.1 \text{ M}$  Tris HCl, pH 7.5. After 20 min the reaction was stopped by addition of  $0.1 \text{ ml}$  of  $40 \text{ mM}$  ATP, 2% sodium dodecyl sulfate, and  $50 \text{ mM}$  Tris HCl, pH 7.5. The stopped solutions were diluted and passed through AG50W-X4 and alumina columns as described by Salomon et al.<sup>5</sup> for the recovery of  $^{32}\text{P}$ -cAMP and  $^3\text{H}$ -cAMP. The final eluates and aliquots of the original substrate solution were mixed with ACS (Amersham/Searle, Arlington Heights, Ill.), and  $^3\text{H}$  and  $^{32}\text{P}$  activities were determined in a Chicago Mark II liquid scintillation counter. After correction for quenching and recovery, the rates were expressed as picamoles per minute.

**Results.** A single peak of  $^{125}\text{I}$ -HYP binding activity appeared at the 50%:20% sucrose interface

Table I. Dissociation constants for adrenergic agents

Antagonists	$K_d(\text{nM})^*$
<i>d,l</i> -Hydroxybenzylpindolol	$0.25 \pm 0.18$ (6)†
<i>l</i> -Alprenolol	0.18 (2)
Timolol	$0.63 \pm 0.33$ (3)
<i>d,l</i> -Propranolol	$1.93 \pm 0.51$ (3)
Phentolamine	>50,000
Agonists	$K_d(\mu\text{M})^*$
<i>l</i> -Isoproterenol	$0.17 \pm 0.17$ (3)
<i>l</i> -Norepinephrine	$5.1 \pm 4.4$ (3)

\*Mean  $\pm$  S.D.

†Number of experiments in parentheses.

(Fig. 1). When  $^{125}\text{I}$ -HYP was centrifuged without membranes, no peak was present (data not shown). Total binding was calculated as the sum of binding activity in fractions 3 to 6. Background binding, defined as binding of  $^{125}\text{I}$ -HYP in the presence of an excess ( $1.5$  to  $3.0 \times 10^{-8} \text{ M}$ ) of unlabeled HYP, was shown to be linearly related to the concentration of  $^{125}\text{I}$ -HYP from  $8.5 \times 10^{-11} \text{ M}$

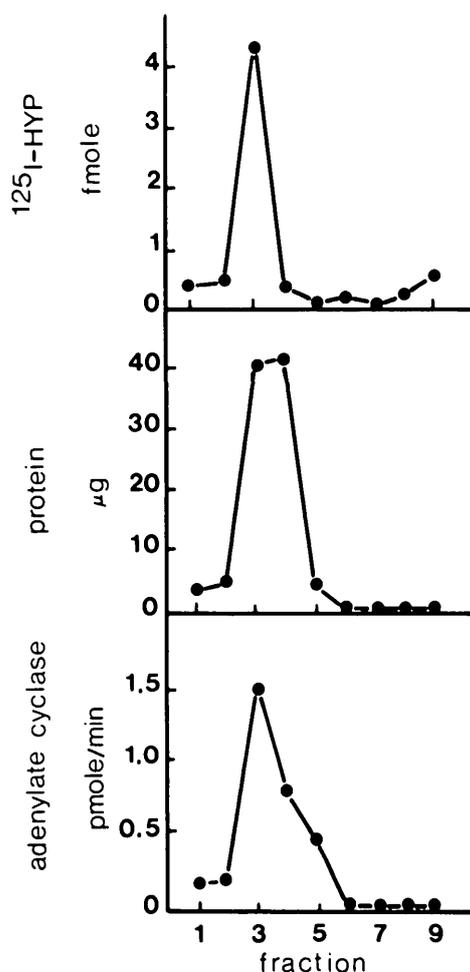


Fig. 3. Distribution of  $^{125}\text{I}$ -HYP binding sites, protein, and adenylate cyclase in particulate membrane fractions from homogenized CP.  $^{125}\text{I}$ -HYP concentration is  $2.4 \times 10^{-10}\text{M}$ .

to  $3.4 \times 10^{-9}\text{M}$ . Thus background binding was calculated routinely for each point from that observed when  $8 \times 10^{-10}\text{M}$   $^{125}\text{I}$ -HYP was incubated in the presence of  $1.5 \times 10^{-8}\text{M}$  unlabeled HYP. The concentration-dependent binding curve for the data in Fig. 1 is shown in Fig. 2. Saturation of specific  $^{125}\text{I}$ -HYP binding sites occurred at about  $10^{-9}\text{M}$   $^{125}\text{I}$ -HYP. The Scatchard plot (Fig. 2, inset) indicated a single set of binding sites for  $^{125}\text{I}$ -HYP with a capacity of 35 fmol/mg of protein and a  $K_d$  of  $2.4 \times 10^{-10}\text{M}$ . The mean  $K_d \pm \text{S.D.}$  of six experiments was  $2.5 \pm 1.8 \times 10^{-10}\text{M}$  (Table I).

*K<sub>d</sub>'s of some adrenergic agents.* Timolol, *l*-alprenolol, and *d,l*-propranolol all had low  $K_d$ 's, which are consistent with their physiological actions as beta-adrenergic receptor antagonists (Table I). On the other hand, phentolamine, an

alpha-adrenergic antagonist, had virtually no ability to displace bound  $^{125}\text{I}$ -HYP. The adrenergic agonists, *l*-isoproterenol and *l*-norepinephrine, also displaced  $^{125}\text{I}$ -HYP. The  $K_d$  for *l*-isoproterenol,  $0.17 \mu\text{M}$ , was 30 times lower than that for *l*-norepinephrine.

*Association of  $^{125}\text{I}$ -HYP receptors, adenylate cyclase activity, and protein.* The  $^{125}\text{I}$ -HYP binding fraction also had a fluoride-responsive adenylate cyclase (Fig. 3). This activity was not present in other fractions of the gradient which did not specifically bind  $^{125}\text{I}$ -HYP. Responsiveness of the cyclase to catecholamines was not tested because of the small amount of cyclase present. Associated with  $^{125}\text{I}$ -HYP binding sites and adenylate cyclase was a peak of protein concentration.

*Discussion.* The CP of rabbits are especially convenient for biochemical investigation because they are easily harvested free of extraneous tissue such as ciliary muscle and iris.  $^{125}\text{I}$ -HYP is a particularly good ligand for studying beta-adrenergic receptors because the high specific activity permits the assay to be conducted with low concentrations of membrane and ligand.

The  $K_d$  for  $^{125}\text{I}$ -HYP,  $0.25 \text{ nM}$ , is in the same range as those reported for rat glioma<sup>3</sup> and turkey erythrocytes.<sup>6</sup> The value is possibly in slight error because the competing ligand for  $^{125}\text{I}$ -HYP was noniodinated rather than iodinated HYP, which may have a different  $K_d$ . However, Maguire et al.<sup>3</sup> found that the  $K_d$ 's for the iodinated and noniodinated forms were similar in rat glioma membranes. All the physiologically active beta-adrenergic antagonists have  $K_d$ 's in the nanomolar range. As observed in other systems,<sup>7</sup> beta-adrenergic antagonists bind to beta-adrenergic receptors in rabbit CP better than do beta-adrenergic agonists. Neufeld and Page,<sup>2</sup> using membranes prepared from rabbit iris/ciliary body, found  $K_d$ 's for adrenergic agonists and antagonists in general agreement with those reported in Table I. Except for timolol, this correlation is remarkable, given the difference in starting tissue, tissue preparation, and assay technique. Our value for timolol ( $K_d = 0.63 \text{ nM}$ ) is about 10 times lower than Neufeld and Page reported. Our number of observations is small ( $n = 3$ ), but the difference may be a true reflection of starting tissue composition. The significance of an especially high affinity of timolol for the beta-adrenergic receptors in rabbit CP is not immediately evident. The efficacy of timolol in lowering the pressure in normotensive rabbit eyes was first reported as marginal,<sup>8</sup> but Radius et al.<sup>9</sup> found a significant effect.

We did not detect low-affinity binding sites in

our experiments with  $^{125}\text{I}$ -HYP (Scatchard plot in Fig. 2 indicates a single class of high-affinity binding sites) because only enough unlabeled HYP ( $1.5 \times 10^{-8}\text{M}$ ) was added to displace  $^{125}\text{I}$ -HYP bound to a site with a  $K_d$  considerably less than  $10^{-7}\text{M}$ .  $^{125}\text{I}$ -HYP not displaced by  $1.5 \times 10^{-8}\text{M}$  HYP could represent binding to low-affinity binding sites.

Waitzman and Woods<sup>10</sup> first reported the presence of a catecholamine-stimulated adenylate cyclase in rabbit CP. We have shown that  $^{125}\text{I}$ -HYP binding activity and adenylate cyclase activity appear in the same fractions of our discontinuous sucrose gradients. Because other experiments in our laboratory have shown that fluoride-stimulated adenylate cyclase activity is routinely three to four times the catecholamine-stimulated activity, stimulation of the adenylate cyclase recovered in the sucrose density gradient was not tested with adrenergic amines. Nevertheless, the recovery of  $^{125}\text{I}$ -HYP binding sites and adenylate cyclase from the same fractions is consistent with the identification of the high-affinity  $^{125}\text{I}$ -HYP binding sites in the rabbit CP as a physiologically active beta-adrenergic receptor.

The data presented in this paper demonstrate the presence of beta-adrenergic receptors in the tissue responsible for AH formation. The result is consistent with the idea that catecholamines can regulate the rate of AH formation by direct interaction with receptors in the CP. Resolution of the specific cellular location of these receptors, e.g., afferent or efferent vessels, pigmented cell layer or nonpigmented cell layer, may depend upon adapting the ligand binding technology to light or electron microscopic autoradiography.

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**Key words:** beta-adrenergic receptor, ciliary processes, catecholamines, ligand binding, adenylate cyclase,  $^{125}\text{I}$ -hydroxybenzylpindolol, aqueous humor, beta-adrenergic antagonist

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## Human tear responses to alkali. RICHARD M. HILL AND LEO G. CARNEY.

*With the use of a closed-chamber microelectrode system, the buffering capacities of over 100 tear samples were measured in response to alkali challenge of several strengths. Although distinctive variations were found among the seven subjects studied, a substantially greater buffer capacity was consistently noted in all their tear responses to pH conditions of near 10 and above. These results are consistent with the early observations of Friedenwald,<sup>11</sup> who demonstrated the buffering capacity of corneal tissue when challenged in this higher pH range, and may here be due to the onset of protein denaturation in the tears.*

Although the clinical literature reflects a sustained concern for the effects of alkaline substances on the eye tissues, little attention has been given to the interaction of tears with such agents.<sup>1-4</sup>