Alpha_1-Adrenoceptors in Human Corneal Epithelium

Ronald J. Walkenbach,* Guo-Sui Ye,* Peter S. Reinach,t and Frances Boney*

Specific binding of the potent, selective alpha_1-adrenoceptor antagonist ^3H-prazosin was demonstrated in cultured human corneal epithelial cells. Specific binding of the radioligand was concentration-dependent between 0.5 and 6 nM, with apparent saturation of receptor sites seen at higher concentrations. The cells exhibited a maximum binding capacity for ^3H-prazosin of 225 fmol/mg of cellular protein and a dissociation constant of 2 nM. The binding of ^3H-prazosin was competitive with known alpha_1-adrenoceptor ligands and was reversible. Epithelium of intact human corneas also exhibited specific ^3H-prazosin binding, as did cultures of bovine and rabbit corneal epithelium.

The alpha-adrenergic agonist methoxamine significantly stimulated phosphatidylinositol 4,5-bisphosphate hydrolysis, measured as myoinositol trisphosphate accumulation in cultures of human corneal epithelium. This stimulation was inhibited by the presence of prazosin during the assays.

These findings indicate the existence of specific, reversible, high-affinity receptors for alpha_1-adrenoceptors that regulate inositol phosphate turnover in human, rabbit, and bovine corneal epithelial cells. Invest Ophthalmol Vis Sci 32:3067–3072, 1991

The cornea is innervated by adrenergic nerve fibers,1–3 but their role(s) in corneal physiology remain poorly understood. The existence of beta-adrenoceptors on corneal epithelial cells has been established4,5 and shown to be predominately of the beta_2 subtype.6 Corneal epithelial beta-adrenoceptors have been associated with stimulation of adenylate cyclase and cyclic AMP-dependent protein kinase,7–9 chloride secretion,10–14 as well as inhibition of mitotic rates15 and glycogen synthase activity.8

The existence of alpha-adrenoceptors on corneal epithelial cells is less well understood. Some preliminary reports using broken cell tissue preparations have suggested the absence of alpha-adrenoceptors,16,17 whereas other studies have used drugs with alpha-adrenoceptor agonist properties to demonstrate stimulation of ion transport12 in frog and inositol phosphate turnover18 in rabbit corneal epithelium.

The direct radioligand binding studies shown here indicate that intact corneal epithelial cells from rabbit, bovine, and human tissue exhibit high-affinity, specific alpha_1-adrenoceptors. These receptors regulate inositol phosphate turnover in human corneal epithelial cells, a finding analogous to that previously reported in rabbit corneal epithelium.

Methods and Materials

Rabbit and bovine eyes were obtained from local slaughterhouses within 2 hr after the animals were killed. The eyes were kept on ice for up to 4 hr more, until further processing occurred. Human eyes were obtained from the Missouri Lions Eye Tissue Bank. The corneas of human eyes were dissected within 12 hr postmortem and stored in Dexsol (Chiron Ophthalmics, Irvine, CA) or a similar medium composed of M-199 tissue culture medium supplemented with 1.35% chondroitin sulfate, 1% dextran (40,000 kDa), 17 mM Na bicarbonate, 20 mM HEPES buffer, brought to a final pH of 7.4 with 1 N NaOH. Corneas were stored at 4°C for up to 72 hr in one of these media before initiation of tissue fractionation, cell culture, or binding experiments.

Particulate fractions of native corneal epithelium from rabbit, bovine, or human corneas were prepared as previously described.19 Briefly, the corneas were rinsed with an ice-cold solution containing 10 mM K_2HPO_4 in 0.9% NaCl (PBS), and the corneal epithelium was removed from the isolated cornea (human) or eye (bovine and rabbit) with a scalpel blade. Tissue was homogenized in 1 ml per cornea of 25 mM glycylglycine buffer (pH = 7.6) with a Teflon/glass tissue grinder in the cold. The homogenate was centrifuged at 50,000 × g for 30 min at 4°C. The pellet was resuspended in fresh buffer, and the centrifugation and resuspension steps were repeated to produce each epithelial particulate fraction.

From the *Missouri Lions Eye Research Foundation, Columbia, Missouri; the †Departments of Ophthalmology and Pharmacology, University of Missouri, Columbia, Missouri; and the ‡Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, Georgia.

Supported by National Institutes of Health grants EY 02597 and EY 04795.

Submitted for publication: March 20, 1991; accepted June 17, 1991.

Reprint requests: Ronald J. Walkenbach, PhD, The Missouri Lions Eye Research Foundation, 404 Portland Street, Columbia, MO 65201.
Rabbit, bovine, or human corneal epithelial cells were cultured in six-well multiplates as described previously. Briefly, epithelial tissue pieces (with some residual stroma) were placed in 5 ml per well as Eagle's minimum essential medium (MEM) with D-valine to inhibit keratocyte contamination of the epithelial cultures and 10% newborn calf serum. Media were changed after 1 week of culture and twice weekly thereafter. Each well typically contained 150–200 μg of cellular protein when used for experiments after 3–4 weeks of culture.

Particulate fractions of cultured epithelium were prepared as described except that a rubber spatula was used to remove cells from the wells.

The potent alpha,-adrenoceptor antagonist [3H]-prazosin (87 Ci/mmol; NEN Research Products, Boston, MA) was used to assess receptor binding activity in these tissue preparations. The protocol used for binding to epithelial particulate fractions was analogous to that described for [3H]-quinuclidinyl benzilate binding to muscarinic cholinceptors in this tissue. Briefly, total binding of the radioligand was assessed by running parallel assays with 100 nM norepinephrine added to the reaction mixtures during the incubation. Specific binding of [3H]-prazosin is defined as the difference between the measured total [3H]-prazosin bound and nonspecific bound in each set of parallel assays.

Binding studies with intact human corneas were performed analogously except that incubations were performed in culture media. After incubation, the corneas were rinsed briefly with ice-cold PBS, and the epithelium was removed quickly with a surgical scalpel blade and placed in 2 ml of 2 N NaOH. After digestion, the samples were neutralized before measuring their protein and radioactivity.

Binding protocols using intact cultured cells were identical except that 1 ml of 1 N NaOH was added to each well after the final PBS rinse.

Table 1. Comparison of [3H]-prazosin binding in different tissue preparations of corneal epithelium from the rabbit, bovine, and human

<table>
<thead>
<tr>
<th>Tissue preparation</th>
<th>[3H]-prazosin bound (fmol/mg)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Nonspecific</td>
</tr>
<tr>
<td>Rabbit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh, particulate fraction</td>
<td>435 ± 34</td>
<td>456 ± 33</td>
</tr>
<tr>
<td>Cultured, particulate fraction</td>
<td>189 ± 23</td>
<td>193 ± 15</td>
</tr>
<tr>
<td>Cultured, intact cells</td>
<td>295 ± 15</td>
<td>259 ± 19</td>
</tr>
<tr>
<td>Bovine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh, particulate fraction</td>
<td>243 ± 31</td>
<td>238 ± 25</td>
</tr>
<tr>
<td>Cultured, particulate fraction</td>
<td>210 ± 17</td>
<td>201 ± 27</td>
</tr>
<tr>
<td>Cultured, intact cells</td>
<td>299 ± 25</td>
<td>154 ± 20</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh, particulate fraction</td>
<td>117 ± 13</td>
<td>126 ± 19</td>
</tr>
<tr>
<td>Cultured, particulate fraction</td>
<td>165 ± 21</td>
<td>155 ± 18</td>
</tr>
<tr>
<td>Cultured, intact cells</td>
<td>275 ± 36</td>
<td>182 ± 33</td>
</tr>
<tr>
<td>Fresh, intact cornea</td>
<td>105 ± 7</td>
<td>64 ± 13</td>
</tr>
</tbody>
</table>

Intact cells or particulate fractions were assayed using 2 nM [3H]-prazosin without or with 100 μM norepinephrine. Each tissue preparation's total and nonspecific [3H]-prazosin binding value represents the mean ± SEM of 12 to 20 assays, using tissue from at least three different harvest dates. Statistical significance was determined using the Student's t-test for unpaired samples. Protein levels ranged from 75 to 100 μg per assay.
nated by aspirating the incubation medium and adding 1 ml of ice-cold 10% HClO₄ to each well. The cells in the wells were frozen, thawed, and kept on ice for 30 min to complete the extraction of 3H-inositol phosphates from the cells, then decanted. The residue in each well was saved for subsequent protein assays; the extract was neutralized using approximately 1 ml of 75 mM HEPES in 2 N KOH. The neutralized extract was centrifuged at 2,000 rpm for 2 min at 4°C. One milliliter of each supernatant fraction was applied to columns with a 3 cm × 0.7 cm diameter bed of Dowex 1×8-200 resin. 3H-inositol, 3H-inositol-1-phosphate, 3H-inositol-1,4-bisphosphate, and 3H-inositol-1,4,5-trisphosphate were isolated from each column by retaining the effluents after sequential applications of 6 ml of 0.1 M formic acid; 10 ml of 0.2 M ammonium formate in 0.1 M formic acid; 10 ml of 0.6 M ammonium formate in 0.1 M formic acid; and 7.5 ml of 1.0 M ammonium formate in 0.1 M formic acid, respectively. A sample (2.5 ml) of each effluent was mixed with 10 ml of Scintiverse BOA cocktail, stored in the dark for ≥1 hr and counted using liquid scintillation techniques. In preliminary experiments, 3H-inositol and standard samples of each of the 3H-inositol phosphate derivatives (NEN Research Products) were used to determine the optimal separation conditions and the efficiency of isolating each product from the columns.

![Graph](image1.png)

**Fig. 1.** Time course of 3H-prazosin binding to human corneal epithelial cells in culture. Cells were incubated using 1 nM 3H-prazosin alone (—•—) to assess total binding or with 1 nM 3H-prazosin + 100 μM norepinephrine in parallel assays to measure nonspecific binding (—△—) of the radioligand. Specific binding (—□—) was calculated as the difference between the levels of total and nonspecific binding. Each symbol represents the mean of nine determinations. Brackets indicate the SEM.

![Graph](image2.png)

**Fig. 2.** Concentration-dependence and saturation of 3H-prazosin-specific binding to human cornea epithelial cells in culture. Cells were incubated with the concentration of 3H-prazosin indicated under otherwise standard binding conditions. Specific binding was calculated for each 3H-prazosin concentration as described in Figure 1. The data were treated according to the method of Rosenthal (inset) to determine a maximal binding capacity (Bmax) for 3H-prazosin of 225 fmol/mg and the concentration at which one-half of the receptors are occupied (Kd), which was 2 nM.

Protein was measured using the method of Lowry et al for all tissue preparations.

Unless otherwise indicated, the data are presented as the means ± SEM of three experiments, each using triplicate assays per experimental condition.

All reagents were purchased from Sigma Chemical Co., and all supplies were obtained from Fisher Scientific unless specifically indicated.

**Results**

The kinetics of total, nonspecific, and specific binding of 3H-prazosin to human corneal epithelial cells in culture are shown in Figure 1. A steady state of binding was reached within 20 min of incubation at 37°C, and binding levels remained stable for at least 30 min thereafter. Although nonspecific binding of 3H-prazosin under these conditions was substantial, a significant difference between total and nonspecific binding (specific binding) was consistently observed.

The level of specific 3H-prazosin binding to cultured human corneal epithelial cells was proportional to the radioligand concentration between 0.5 and 6 nM, as shown in Figure 2. Rosenthal analysis of these data (inset, Fig. 2) indicated a maximal binding capacity (Bmax) for 3H-prazosin of 225 fmol/mg protein and a dissociation constant (Kd) of 2 nM.

Agents with known alpha-adrenoceptor potency were able to compete with 3H-prazosin binding during incubation with cultured human corneal epithelium (Fig. 3). Unlabeled prazosin was the most effec-
Fig. 3. Inhibition of 3H-prazosin binding to cultured human corneal epithelial cells by alpha adrenergic agents. Control cells were incubated with 1 nM 3H-prazosin alone. Other cells were incubated with 1 nM 3H-prazosin and the indicated concentration of either unlabeled prazosin (—•—), yohimbine (—*—), norepinephrine (—A—), methoxamine (—O—), or phenylephrine (—○—). Each symbol represents the mean binding level of 3H-prazosin at its respective experimental condition, expressed as a percent of the control level of binding.

The levels of 3H-prazosin binding were compared in several types of epithelial preparations from rabbit, bovine, and human corneas. Table 2 shows that significant levels of specific 3H-prazosin binding could not be observed in particulate fractions of fresh or cultured corneal epithelium from any species tested. However, specific binding was seen in all of the intact cell preparations tested. Cultured cells from rabbit epithelium exhibited relatively small, but statistically significant, levels of specific 3H-prazosin binding. Cultured bovine corneal epithelium showed the highest specific 3H-prazosin binding activity tested—145 fmol/mg protein. Cultured human corneal epithelium displayed less 3H-prazosin binding activity (93 fmol/mg) than bovine tissue, but the level of specific binding was still highly significant. Moreover, specific binding of 3H-prazosin also could be observed in fresh, intact human corneal epithelium, if whole corneas were incubated with the radioligand before isolation of the epithelium.

In most tissues studied, alpha-adrenoceptors are associated with regulating the formation of two intracellular second messengers: inositol-1,4,5-trisphosphate and 1,2-diacylglycerol. Accordingly, the effect of an alpha adrenoceptor agonist and antagonist were tested on the inositol phosphate turnover in cultured human corneal epithelial cells. Table 1 shows that incubation of the cells with 100 µM methoxamine significantly elevated levels of inositol-1,4,5-trisphosphate, as well as its bisphosphate and monophosphate metabolites in these cells. When cells were incubated with methoxamine in the presence of 0.1 µM prazosin, the level of inositol-1,4,5-trisphosphate was significantly reduced compared with that seen with methoxamine alone.
The experiments described here indicate that corneal epithelial cells exhibit physiologically relevant alpha-adrenoceptors that are associated with the regulation of inositol-1,4,5-trisphosphate formation. The receptors displayed a high affinity for a well-recognized alpha-adrenoceptor antagonist, $^3$H-prazosin (Fig. 2), and exhibited saturation of the receptors at radioligand concentrations greater than 6 nM. Known alpha-adrenoceptor antagonists and agonists competed for binding sites with $^3$H-prazosin during incubations with epithelial cells (Fig. 3). The relative affinities and the order of potencies seen among the competing alpha-adrenoceptor ligands in Figure 3 are consistent with those seen in many other tissues with demonstrated alpha-adrenoceptor systems.25

In Figure 3, the selective alpha$_1$-adrenoceptor antagonist yohimbine was a much less potent competitor for $^3$H-prazosin binding sites than was the selective alpha$_1$-adrenoceptor antagonist prazosin. Moreover, our laboratory has been unable to demonstrate specific $^3$H-yohimbine binding (not shown) using the same tissue preparations and experimental conditions employed for these experiments. Thus, it appears that corneal epithelial alpha-adrenoceptors are primarily, if not exclusively, of the alpha$_1$, subtype.

The data in Table 1 clearly show that particulate fractions of fresh or cultured epithelium, unlike intact cells, failed to exhibit specific $^3$H-prazosin binding. Similarly, there was no specific $^3$H-prazosin binding in other subcellular fractions of this tissue or after treatment of intact cells with trypsin (data not shown). It appears that cellular disruption, at least as employed in these studies, destroys or masks the corneal epithelial cell’s ability to specifically bind $^3$H-prazosin. This is an unusual finding because many tissues show greater alpha$_1$-adrenoceptor binding activity in broken cell preparations versus intact cells.26

### Table 2. Inositol phosphates in human corneal epithelial cultures

<table>
<thead>
<tr>
<th>Condition</th>
<th>IP</th>
<th>IP$_2$</th>
<th>IP$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>470.1 ± 70.9</td>
<td>221.2 ± 38.7</td>
<td>27.4 ± 3.2</td>
</tr>
<tr>
<td>Methoxamine, 100 µM</td>
<td>558.1 ± 52.8</td>
<td>265.0 ± 50.6</td>
<td>39.8 ± 7.4</td>
</tr>
<tr>
<td>Methoxamine, 100 µM + prazosin, 0.1 µM</td>
<td>506.0 ± 67.4</td>
<td>170.3 ± 56.8</td>
<td>32.8 ± 4.1</td>
</tr>
</tbody>
</table>

Human corneal epithelial cultures were incubated without drugs (control) or with the drugs indicated above followed by isolation of $^3$H-inositol-1-phosphate (IP), $^3$H-inositol-1,4-bisphosphate (IP$_2$), and $^3$H-inositol-1,4,5-trisphosphate (IP$_3$). The data indicate the mean ± SEM of nine assays. The Student’s t-test was used to ascertain significant differences in IP, IP$_2$, levels between control and methoxamine-treated cultures (P < 0.01) and between methoxamine-treated and methoxamine + prazosin-treated cultures (P < 0.05).

However, the corneal epithelium may be a unique tissue in this sense because our laboratory has obtained a similar finding with muscarinic cholinoreceptor binding activity in intact versus broken cells.19 The possibility that the corneal epithelium releases protein inactivating agents upon cellular disruption seems plausible and may be a physiologically important defense mechanism to protect the rest of the cornea from foreign infiltration.

Conneal epithelial alpha$_1$-adrenoceptors appear to be associated with regulation of inositol-1,4,5-trisphosphate formation because the alpha adrenoceptor agonist methoxamine increased turnover of inositol-1,4,5-trisphosphate and its metabolites in human corneal epithelial cells (Table 2) in the same concentration range that it was able to effectively compete with $^3$H-prazosin (Fig. 3). Moreover, prazosin inhibited the methoxamine-induced effects on inositol phosphate turnover at a concentration expected from its receptor-binding potency shown in Figures 2 and 3. The magnitudes of inositol phosphate turnover by methoxamine (Table 2) were similar to those previously reported in rabbit corneal epithelial tissue in response to norepinephrine.18

The finding of alpha$_1$-adrenoceptors in bovine, rabbit, and human corneal epithelium and their regulation of inositol phosphate turnover in human tissue is in accord with their previously defined role in this tissue as modulators of active ion transport17 and inositol phosphate turnover using drugs with alpha-adrenergic potency. Alpha$_1$-adrenoceptors appear to play a role in corneal epithelial cell homeostasis by regulating levels of intracellular second messengers such as Ca$^{2+}$, inositol 1,4,5-trisphosphate, and 1,2-diacylglycerol, as previously demonstrated in many other tissues.26

The physiologic responses to alpha$_1$-adrenoceptor activation and the second messengers described here have not been determined in the corneal epithelium. They may contribute to the regulation of corneal de-turgescence, although this effect would not appear to be quantitatively important because our laboratory has not detected an effect of alpha$_1$-adrenergic agents on rabbit corneal thickness when the corneas were cultured in vitro (data not shown). Nevertheless, these agents may have other physiologically important transport effects within the epithelium. It also is possible that alpha$_1$-adrenoceptors contribute to the regulation of epithelial mitosis or the migration of epithelium toward the anterior surface. The latter possibility is being investigated in our laboratory.

**Key words:** cornea, epithelium, alpha$_1$-adrenoceptors, alpha$_2$-adrenergic, receptors, human, inositol phosphate
Acknowledgments

The authors thank Cheryl Dake and Nels Holmberg for their expert assistance in the execution of these experiments.

References