Lens Beta-Adrenergic Receptors

Functional Coupling to Adenylate Cyclase and Photoaffinity Labeling

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Beta-adrenergic drugs affect lens epithelial and fiber cells. The regulation and cellular integration of lens beta-adrenergic responses are largely unknown. These studies further characterize beta-adrenergic receptors in lens cells with respect to cyclic adenosine monophosphate (cAMP) production and identification of receptor polypeptides. Stimulation of beta-adrenergic receptors in organ-cultured chick lenses resulted in dose-dependent increases in intracellular cAMP levels. Isoproterenol-elicited cAMP accumulation was found in both epithelial/superficial fiber cells and cortical fiber cells. Hormonal stimulation also apparently initiated additional mechanisms involved in the regulation of cAMP levels (i.e., phosphodiesterase activation/receptor desensitization). Individual receptor polypeptides were identified in epithelial and fiber membranes with the photoaffinity probe 125I-iodocyanopindolol diaziramine. The probe specifically labeled distinct populations of receptor polypeptides in the two cell types. Lens beta-adrenergic receptors were also shown to bind (−) stereoisomers of adrenergic ligands preferentially. These results indicate that differentiating fiber cells are hormonally sensitive to beta-adrenergic stimulation and that epithelial and fiber cells may respond differentially to beta-adrenergic drugs, at least in part, because of their distinct receptor polypeptides.

Materials and Methods

Reagents

How do most of the nonmitotic epithelial cells respond to beta agonists? Are lens beta receptors functionally coupled to adenylate cyclase? Do alpha-2 receptors exist in the lens to inhibit adenylate cyclase? What is the nature of beta receptor down-regulation? How is beta-adrenergic responsiveness maintained during the ongoing process of lens cell terminal differentiation? These questions become increasingly pertinent due to the unknown effect on lens metabolism of adrenergic drug administration for the treatment of both ophthalmic and systemic conditions.

Our studies were designed specifically to examine the functional coupling of lens beta-adrenergic receptors to cAMP production and to identify receptor polypeptides. We found that in isoproterenol-stimulated organ-cultured lenses both epithelial and fiber cells respond to hormonal stimulation by elevating their levels of cAMP. Fiber cells, however, show a reduced capacity to synthesize cAMP as a function of cellular differentiation/aging. Furthermore, photoaffinity labeling identifies distinct receptor polypeptides in epithelial and fiber cells which may contribute to observed differences in beta-adrenergic responsiveness of these cells.

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Animals

These experiments were done in accordance with the ARVO Resolution on the Use of Animals in Research. Heads from freshly killed 2-month-old white Leghorn chickens were obtained as previously described. Fertile eggs were obtained from a local supplier and incubated in a forced air incubator at 37°C. The embryos were used on the 7th, 14th, or 21st day of development.

Measurement of cAMP Levels

Lenses were removed from variously aged embryos by making an incision near the region of the scleral cartilage, grasping the vitreous humor with fine forceps, and pulling the vitreous, lens, and associated ocular tissues through the incision. This always included the entire vitreous body and most of the zonular processes. Juvenile lenses (from 2-month-old animals) were retrieved by removing the posterior globe, trimming away most of the vitreous body, and carefully dissecting out the lens, ciliary body/processes, and iris. Tissues were immediately placed in Medium 199 (Gibco, Grand Island, NY) supplemented with 1 mM sodium ascorbate, 0.2 mM 3-isobutyl-1-methylxanthine (IBMX), and kept at room temperature for 30 min. The IBMX enhances cAMP accumulation by inhibiting phosphodiesterase, the enzyme responsible for cAMP degradation. Isoproterenol was chosen to stimulate cAMP production in the lens since it is an agonist of both subclasses of beta-adrenergic receptors. Isoproterenol treatment was initiated by adding an appropriate volume from a freshly prepared stock (to give the final indicated concentrations) and placing the tissues in a 5% CO2/95% air incubator at 37°C for the indicated times. Incubations were terminated by boiling for 3 min; after this the samples were cooled on ice. Surrounding ocular tissues could then be easily dissected away and the entire lens placed in fresh, cold culture medium. When juvenile lenses were decapsulated from the posterior pole, the most superficial fibers always remained associated with the capsule/epithelial cells. This epithelial/superficial fiber fraction constituted approximately 20% of the total lens protein. Samples were sonicated briefly to release intracellular cAMP, tracer amounts of 3H-cAMP were added to monitor recovery, and then 1 N perchloric acid was added to precipitate protein. Samples were placed in a refrigerator for 16–18 hr to allow for complete protein precipitation before further processing. Cell sonicates were clarified by centrifugation and the supernate processed for the extraction of cAMP according to Zimmerman et al. Acid precipitates were assayed for protein as previously described. The cAMP content was determined according to Gilman using cAMP-dependent protein kinase in a competitive displacement binding assay. All treatments were done in duplicate and all samples assayed in triplicate.

Photoaffinity Labeling

Unless otherwise noted, all procedures were done at 4°C. Lenses from 2-month-old chickens were placed in ice-cold buffer containing 5 mM Tris HCl, pH 8.1, 1 mM ethyleneglycol-bis-(β-aminoethyl-ether)N,N,N',N'-Tetraoletic Acid (EGTA), 10 μg/ml of phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin (Buffer A) before further dissection. The calcium chelator EGTA plus the protease inhibitors PMSF, leupeptin, and pepstatin were included in all buffers to inhibit any potential proteolytic degradation of the receptor polypeptides. Lenses were left on ice for 1 hr to facilitate subsequent retrieval of epithelial cell sheets (composed of both central epithelial plus annular pad cells) after lens decapsulation. Epithelial cell sheets and fiber cell masses were separately homogenized in 20 ml of Buffer A and centrifuged at 500 × g for 5 min. The resulting supernates were diluted to 40 ml with Buffer A and centrifuged at 40,000 × g for 20 min. The crude plasma membranes were rehomogenized and recentrifuged three additional times before being resuspended in N2-saturated buffer containing 50 mM Tris HCl, pH 8.1, 15 mM MgCl2, 1 mM EGTA, 1 mM sodium ascorbate, 10 μg/ml of PMSF, 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin (Buffer B) at 0.5–1 mg/ml.

Photoaffinity labeling was done essentially as previously described. Samples containing membranes (100–200 μg) and the beta-adrenergic antagonist 125I-iodocyanopindolol diazarine (ICYP-da) (specific activity: 2200 Ci/mmol; Amersham, Arlington Heights, IL) were incubated for 60 min in the dark, diluted with Buffer B, and photolysed by irradiation at 366 nm for 60 min using a 4-W ultraviolet light lamp (Ultra-Violet Products, San Gabriel, CA) positioned 3 cm above the open sample tubes. After photolysis (which covalently links the iodinated probe to the receptors it is occupying by generating a reactive carbene group), samples were centrifuged, washed with Buffer B, and dissolved in sodium dodecyl sulfate sample buffer before electrophoresis. Immediately before electrophoresis, samples were adjusted to a final concentration of 50 mM dithiothreitol. After staining, the gels were dried between two sheets of cellophane and placed on Kodak XRP-1 X-ray film (Rochester, NY) at −70°C with Ilford Fast Tungstate Intensifying Screens (Ilford Ltd., Mobberley, Che-
isoproterenol showed a slight, total increase of 8 ± 5%. A 100-fold molar excess of propranolol, a beta-adrenergic antagonist, was completely effective in inhibiting any isoproterenol-stimulated increases in cAMP (data not shown). These controls indicate that cAMP levels are balanced by basal rates of adenylyl cyclase activity and phosphodiesterase-mediated degradation, at least during short-term organ culture. As shown in Table 1, increases in isoproterenol-stimulated intracellular cAMP levels were found throughout the juvenile lens with epithelium/superficial fibers having a somewhat greater total-fold increase than cortical/nuclear fibers. When expressed as a percentage of the entire lens, the epithelium/superficial fibers (which comprise 20% of total lens protein) account for 31% of the resting and 39% of the isoproterenol-stimulated cAMP levels. However, it should be noted that concentrations of isoproterenol of 10 μM or greater were required to stimulate measurable cAMP accumulation in the juvenile chicken lens. Although it appears from Figure 1 and Table 1 that the capacity to generate cAMP diminishes during development, the total amount of cAMP/lens rises nearly as dramatically as protein content/lens (Table 2). The time course of isoproterenol-stimulated cAMP accumulation is shown in Figure 2. Large increases in cAMP levels were noted as little as 5 min after the initiation of treatment with maximum levels occurring by 30 min. Longer periods of stimulation resulted in a steady decline in cAMP content. Raising the IBMX concentration to 2 mM effectively abolished the decreases in lens cAMP brought about by long-term isoproterenol stimulation (Fig. 2B).

### Photoaffinity Labeling

The radio-iodinated, beta-adrenergic photoaffinity probe ICYP-da labeled four polypeptides of molecular weights 44,000, 34,000, 28,000, and 23,000 in 2-month-old chick lens epithelial cell membranes and a single polypeptide of 39,000 in lens fiber cell membranes (Fig. 3). Specificity of labeling was established by observing reduced incorporation of ICYP-da in the presence of 10 μM propranolol. Nonspecific binding of the probe was unaffected by the presence of propranolol. Specifically labeled bands were cut

### Results

#### cAMP Measurements

The accumulation of cAMP by variously aged embryonic chick lenses in response to isoproterenol treatment is shown in Figure 1. During a 30-min incubation, all lenses showed dose-dependent increases in intracellular cAMP content. Controls where both isoproterenol and IBMX were omitted from the incubation had a reduction in cAMP levels of 12 ± 6% of initial values. Additional controls omitting only

| TABLE 1. Isoproterenol-stimulated increases in cAMP content in juvenile chicken lenses.*† |
|---------------------------------|-----------------|----------------|----------------|
|                                 | mg Protein (%) | pmoles cAMP/mg protein | x-Fold stimulation |
| Epithelium/superficial fibers   | 4.2 (20)        | 35              | 2.0           |
| Cortical/nuclear fibers         | 13.9 (80)       | 23              | 1.4           |
| Whole lens                      | 18.1 (100)      | 26              | 1.6           |

* Lenses were stimulated for 30 min with 10 μM isoproterenol.
† Values are the averages from a single experiment that was typical of four others.
Table 2. cAMP content of variously aged chicken lenses

<table>
<thead>
<tr>
<th>Age</th>
<th>pmoles cAMP/mg protein*</th>
<th>mg Protein/lens*</th>
<th>pmoles cAMP/lens</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-Day embryo</td>
<td>199 ± 32</td>
<td>0.053 ± 0.02</td>
<td>10.5</td>
</tr>
<tr>
<td>14-Day embryo</td>
<td>73 ± 15</td>
<td>0.385 ± 0.06</td>
<td>28.1</td>
</tr>
<tr>
<td>21-Day embryo</td>
<td>46 ± 12</td>
<td>1.170 ± 0.16</td>
<td>53.8</td>
</tr>
<tr>
<td>Juvenile (2 mo)</td>
<td>26 ± 9</td>
<td>17.740 ± 1.28</td>
<td>461.2</td>
</tr>
</tbody>
</table>

* Values are the average (±SD) from at least three separate experiments.

from the dried gels after autoradiography and quantitated by liquid scintillation counting (Fig. 4). Corresponding counts from identical areas of the propranolol-treated samples were subtracted from the nonpropranolol-treated samples for receptor quantitation. The ratio of specific ICYP-da incorporation into epithelial polypeptides was approximately 2:3:1:4 (from higher to lower molecular weight polypeptides, respectively). The ratio of specific-receptor polypeptide labeling in epithelial versus fiber cell membranes was approximately 3:1, in good agreement with the number of dihydroalprenolol binding sites found in these preparations. The stereospecificity of lens beta-adrenergic receptors was established by observing reduced ICYP-da labeling only in the presence of the (−) stereoisomer of a competing ligand (Fig. 5).

Discussion

Cyclic nucleotide metabolism in the lens is regulated in accordance with well-characterized mechanisms. A Ca++/calmodulin-regulated adenylate cyclase has been characterized in both epithelial and fiber cells as has phosphodiesterase activity. Both stimulatory and inhibitory guanosine triphosphate-binding proteins are present in fiber cells, but similar studies have not yet been conducted with epithelial cells. More recently, beta-adrenergic receptors have been identified in lens cells using specific radiolabeled ligands. Although beta-adrenergic drugs do not stimulate adenylate cyclase in bovine

Fig. 2. Time course of and IBMX concentration effect on isoproterenol-stimulated cAMP accumulation in 14-day embryonic chick lenses. Lenses were stimulated at 37°C with 1 μM isoproterenol for the indicated periods of time in the presence of 0.2 mM IBMX (A) or 2.0 mM IBMX (B). Values shown are the means (±SD) from three such experiments. Note that increasing IBMX concentrations (in B) effectively eliminates the long-term reduction in cAMP levels which occurs in the presence of lower IBMX concentrations (in A).

Fig. 3. Photoaffinity labeling of beta-adrenergic receptor polypeptides in 2-month-old chick lens cells. SDS-PAGE (A) and corresponding autoradiogram (B) of plasma membranes from epithelial (E) and fiber (F) cells labeled in the presence of 500 nM ICYP-da. Specifically labeled polypeptides (arrowheads in B) showed reduced ICYP-da incorporation in the presence of 10 μM propranolol (+). Samples were separated on the same gel and are representative of six separate experiments. The position of molecular weight standards (in kilodaltons) is indicated at the left.
Fig. 4. Receptor quantitation. Specifically labeled bands were cut from dried gels after autoradiography and quantitated by liquid scintillation counting. Receptor polypeptides are identified by their cell of origin (E—epithelial, F—fiber) and by their apparent molecular weight (in kilodaltons). Values are the means (±SD) of three such determinations.

Fig. 5. Stereospecificity of lens beta-adrenergic receptors. Autoradiogram of epithelial cell membranes after photoaffinity labeling and SDS-PAGE. ICYP-da concentration during labeling was 500 nM. Membranes were labeled in the presence of ICYP-da only (A), ICYP-da plus the (−) stereoisomer of isoproterenol at 10 μM (B), or ICYP-da plus the (+) stereoisomer of isoproterenol at 10 μM (C). Only the (−) stereoisomer (in B) inhibited ICYP-da labeling of receptor polypeptides (arrowheads). Results are representative of three such experiments. The position of molecular weight standards (in kilodaltons) is indicated on the left. Similar results were obtained with fiber cell membranes (not shown).

Although cAMP levels measured in these studies are somewhat higher than those in previous reports, species differences, the use of phosphodiesterase inhibitors, and more rapid processing of tissues could easily account for the higher values. Interestingly, isoproterenol stimulated cAMP increases in cortical fibers (Table 1), indicating that terminally differentiating fiber cells are hormonally responsive. Others noted that cortical fibers retain the ability to generate and degrade cAMP but do not respond to appropriate beta agonists. However, the conditions during fiber-membrane isolation in those studies may not support extensive maintenance of receptor/guanosine triphosphate-binding protein/adenylate cyclase complexes. In our study, intact lenses were

Fiber membranes, isoproterenol (in the presence of a nonhydrolyzable guanosine triphosphate analogue) stimulates adenylate cyclase in bovine epithelial membranes. A common focus for lens cAMP effects is the activation of cAMP-dependent protein kinase which phosphorylates various substrate proteins. Our study indicates that beta-adrenergic drugs are capable of stimulating cAMP production in several regions of the lens by interacting with distinct receptor molecules on epithelial and fiber cells.

cAMP Production

Lens cAMP production was stimulated in a dose-dependent manner by isoproterenol in chickens of all ages examined (Fig. 1, Table 1). Although it appears that earlier developmental stages are capable of much more extensive cAMP generation, this is probably not true. Although cAMP content per lens increases 40-fold during development, protein content per lens (against which cAMP levels are measured) increases over 300-fold, thereby giving the impression that the ability to synthesize cAMP decreases during development (Fig. 1, Table 2). However, at all developmental stages examined, an intact lens was capable of a roughly twofold increase in cAMP content in response to isoproterenol stimulation (Fig. 1, Table 1). This indicates that a sustained ability to generate cAMP characterizes those stages of most rapid lens growth. Perhaps significantly, juvenile lenses become refractory to stimulation and require higher agonist concentrations to elicit comparable increases in cAMP production. How these findings translate into a possible beta-adrenergic control of lens growth by receptor-mediated changes in local concentrations of cAMP must be determined.
stimulated before the isolation of cAMP, thereby avoiding this possible complication. Still, an alteration in the ability of older fiber cells to regulate cAMP levels is supported by this and other studies.6,13

A final point about isoproterenol-stimulated cAMP production in the lens is that several down-regulatory processes are also concomitantly activated. Long-term stimulation (>1 hr) consistently resulted in a decrease in cAMP levels after an initial rise (Fig. 2). This indicates that in lens cells, as in other cell types,20,21 hormonal stimulation resulting in increased cAMP production also stimulates phosphodiesterase activity. Since both epithelial and cortical fiber cells have significant phosphodiesterase activity,11-13 increased concentrations of IBMX were used during isoproterenol stimulation to counter receptor-mediated phosphodiesterase activation (Fig. 2). This treatment effectively abolishes the long-term decreases in cAMP accumulation and indicates that homologous desensitization of lens beta-adrenergic receptors is occurring. Whether receptor phosphorylation or internalization contributes to receptor desensitization remains to be determined.1 It may also be that the cellular export of cAMP (first described by Davoren and Sutherland23), in response to prolonged stimulation, contributes to the stabilizing of cAMP levels at higher IBMX concentrations. Cellular export of cAMP could not be measured with the current experimental protocol since extralenticular tissues (retained so as to avoid excess mechanical damage) were always present during culture.

Photoaffinity Labeling

As identified by photoaffinity probes, beta-adrenergic receptors have a variety of molecular weights between 36,000-75,000 depending on their cell or tissue of origin.24-26 This heterogeneity in apparent molecular weight is due to multiple gene products,27-30 proteolysis during sample preparation,31-34 and varying degrees of receptor glycosylation.35-37 Deduced primary sequences from beta receptor genes yield a range of possible molecular weights between 46,000-54,000;27-29 nonproteolyzed, deglycosylated receptors have molecular weights of 40,000-46,000.26,33,37-39

Based on these findings, the 44,000 polypeptide found in chick lens epithelial cells may represent a nonglycosylated form of an intact beta-adrenergic receptor. The remaining specifically labeled receptor polypeptides in epithelial and fiber cells may represent proteolytic degradation products. However, modifying all buffers to include 5 µg/ml each of leupeptin and pepstatin plus including additional protease inhibitors 1,10 phenanthroline at 10 mM, diisopropyl fluorophosphate at 1 µM, and aprotinin at 4 KI units/ml had no effect on the pattern of specifically labeled polypeptides (data not shown). Further support for the nonglycosylated nature of lens beta receptors was obtained with lectin-affinity chromatography. Columns of concanavalin A or wheat-germ agglutinin, which bind the carbohydrate moieties most commonly found on beta-adrenergic receptors for subsequent elution with competing sugars,35-37 did not retain any specifically photolabeled polypeptides (not shown). These preliminary experiments do not preclude the possibility that chick lens beta-adrenergic receptors are modified by carbohydrates which do not bind concanavalin A or wheat-germ agglutinin. Although proteolysis and deglycosylation of beta receptors during membrane preparation before photoaffinity labeling are often difficult to inhibit,31-33 studies with intact cells indicate that receptor fragments are present in situ at the cell surface and are accessible to photoaffinity probes.32,33 Endogenously occurring receptor fragments and proteolytic degradation products derived during sample preparation are still capable of high-affinity interactions with ligands30,41 and can undergo functional coupling to guanosine triphosphate-binding proteins.41 It is therefore possible that all or some of the specifically labeled polypeptides in our study represent naturally occurring, functional beta receptors. Although lens beta-adrenergic receptors exhibit typical stereospecificity,32,33 they appear somewhat unique in that glycosylated forms are not present in any great abundance. However, the role of glycosylation in receptor function is controversial.26,41,42

Photoaffinity labeling indicates that beta-adrenergic receptors in epithelial and fiber cells function in dissimilar environments. The fiber-cell receptor polypeptide is unique to the differentiating phenotype in terms of its molecular weight and lower affinity for beta-adrenergic drugs.8 This finding is curious because fiber cells are all directly derived from epithelial cells and presumably possessed the epithelial complement of receptor polypeptides. This indicates that during terminal differentiation of the fiber cells, distinct receptor processing occurs to yield a unique profile of receptors or else a different gene product begins to be expressed. These results provide a physical basis for the reduced ability of fiber cells to synthesize cAMP.

Conclusion

In summary, beta-adrenergic stimulation of the lens in organ culture was shown to elevate cAMP levels in a dose-dependent manner. Increases in intracellular cAMP content were found in epithelial,
superficial fiber, and cortical fiber cells. The lenswide regulation of cAMP levels appears to be modulated by several interdependent mechanisms. Individual receptor polypeptides were identified by photoaffinity labeling and were found to differ between epithelial and fiber cells. Distinct receptor polypeptides may serve as a possible mechanism for the differential responses to beta-adrenergic stimulation observed in the two cell types comprising the ocular lens.

Key words: lens beta-adrenergic receptors, adenylate cyclase, photoaffinity labeling, iodocyanopindolol diazirane

References


