Novel paracrine signaling mechanism in the ocular ciliary epithelium

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ABSTRACT The ciliary body contains an epithelial bilayer consisting of an outer pigmented cell layer (PE) and an inner nonpigmented cell layer (NPE) responsible for aqueous humor secretion. Secretion may be mediated in part by cytosolic Ca2+ concentration ([Ca2+]i), but whether or how the two layers could coordinate their Ca2+ signals to regulate secretion is unclear. To investigate interactions between PE and NPE, we examined [Ca2+]i, signaling in isolated intact ciliary epithelial bilayers using confocal microscopy. Phenylephrine selectively increased [Ca2+]i in PE and acetylcholine increased [Ca2+]i in NPE, but epinephrine increased [Ca2+]i, in both layers. This increase spread from PE to NPE, and [Ca2+]i signaling across the bilayer remained coordinated during [Ca2+]i oscillations. All epinephrine-induced-[Ca2+]i, signaling was blocked by the α1-adrenergic antagonist prazosin, whereas signaling in the NPE but not PE was blocked by the β-adrenergic antagonist propranolol, the gap junction blockers octanol and 18α-glycyrrhetinic acid, or the A kinase inhibitor Rp diastereomer of adenosine 3′,5′-cyclic monophosphothioate. The β-adrenergic agonist isoproterenol failed to increase Ca2+ by itself, but isoproterenol plus phenylephrine-induced-[Ca2+]i, signals across the bilayer similar to those induced by epinephrine. Finally, isoproterenol increased cell-to-cell spread of lucifer yellow via gap junctions, whereas cell-to-cell spread of [Ca2+]i, signals could be induced by photorelease of caged inositol 1,4,5-trisphosphate. Thus, calcium signals are coordinated in the epithelial bilayer so that adrenergic stimulation can increase [Ca2+]i in NPE, but only if NPE are primed by activation of endogenous adenyl cyclase, whereon they receive stimulation from adjacent PE via gap junctions. This novel interplay between endocrine and paracrine pathways may coordinate [Ca2+]i, signaling across the ciliary epithelial bilayer.

The epithelial bilayer of the ciliary body produces aqueous humor, a fluid essential for maintenance of intraocular pressure and nourishment of the avascular transparent tissues that comprise the anterior segment of the eye. Understanding the process of aqueous humor formation has been complicated by the unique anatomy of the epithelium. The two constituent layers, the inner aqueous-facing nonpigmented epithelium (NPE) and the outer serosal-facing pigmented epithelium (PE), originate from the neural crest during embryogenesis, then become apposed at their apices when the invagination of the optic vesicle occurs (1). The layers thereafter remain apposed to each other along their apical membranes, across which gap junctions are densely expressed to establish a heterocellular junctional path and a virtual syncytium (2–4).

Signal transduction to regulate aqueous humor secretion probably involves multiple pathways (1, 5), possibly including spontaneous (6) and induced elevations in cytosolic calcium concentration ([Ca2+]i) (7–10). Furthermore, different [Ca2+]i, signaling patterns occur in the ciliary body in response to agents that also affect aqueous humor formation, like adrenergic and muscarinic agonists (11–16). Although the spatial organization of [Ca2+]i, waves in ciliary epithelium is not known, [Ca2+]i, waves regulate secretion in other epithelial cells (17–21), and cell-to-cell spread of these waves via gap junctions may play a role in this regulation (20, 22). To understand whether there is similar coordination of [Ca2+]i, signaling in the epithelial bilayer of the ciliary body, we observed patterns of induced [Ca2+]i, signaling in intact segments of the bilayer (23) using time-lapse confocal microscopy (24).

MATERIALS AND METHODS

Animals and Materials. Male albino New Zealand rabbits weighing 2–3 kg obtained from Millbrook Farms (Amherst, MA) were used for all experiments. Acetylcholine, phenylephrine, epinephrine, isoproterenol, prazosin, propranolol, yohimbine, octanol, 18α-glycyrrhetinic acid (aGA), lucifer yellow (LY), and Hanks’ balanced salt solution were obtained from Sigma. Fluo-3 in acetoxymethoxylated form and pluron F-127 were obtained from Molecular Probes. Caged inositol 1,4,5-trisphosphate (IP3) and the Rp diastereomer of adenosine 3′,5′-cyclic monophosphothioate (RcAMP[S]), a protein kinase A inhibitor, were obtained from Calbiochem, and suramin was obtained from Biomol (Plymouth Meeting, PA). All other chemicals were of the highest quality commercially available.

Preparation of Isolated Ciliary Epithelium. Isolated ciliary epithelium were prepared as described previously (23, 25) with slight modification. Briefly, rabbits were anesthetized with an i.m. injection of ketamine hydrochloride and xylazine and then euthanized by i.v. injection of pentobarbital sodium and phenytoin sodium. The eyes were enucleated promptly and then the anterior segments were isolated after careful removal of the lens. From the isolated anterior segment of the eye, ciliary processes were separated from the iris and cut into 10–20 strips, each 2–3 mm in length. All procedures conformed with National Institutes of Health recommendations as developed by the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: aGA, 18α-glycyrrhetinic acid; [Ca2+]i, cytosolic Ca2+ concentration; IP3, inositol 1,4,5-trisphosphate; LY, lucifer yellow; NPE, nonpigmented epithelium; PE, pigmented epithelium; Rp- cAMP[S], Rp diastereomer of adenosine-3′,5′-cyclic monophosphothioate; Fluoro-3, [1-2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-9- xanthenylidenephenoxy]-2-[2-amino-5-methylphenoxy]ethane-N,N,N’,N”-tetraacetate acid).

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Confocal Microscopic Measurements of \([\text{Ca}^{2+}]_{i}\). Isolated ciliary epithelia were loaded with fluo-3/acetoxy-methoxylated (50 \(\mu\)M) and pluronic F-127 for 1 hr at room temperature in Hanks’ balanced salt solution containing 10% fetal calf serum. Specimens were then placed between two glass coverslips in a gravity-driven perfusion chamber on the stage of a Zeiss Axiosvert microscope and perfused at a rate of 1–2 ml/min. The tissue was observed through a \(\times 63\) objective using a Bio-Rad MRC-600 laser scanning confocal imaging system. An argon laser was used to excite the dye at 488 nm and emission signals above 515 nm were collected. Optical sections 1–2 \(\mu\)m in thickness were obtained. Neither autofluorescence nor other background signals were detectable at the machine settings used, and there was no change in size, shape, or location of cells during the experiments. In most experiments, two-dimensional images consisting of 768 \(\times\) 512 pixels (0.26 \(\mu\)m/pixel) were recorded at a rate of 1 frame/s on an optical disc recorder and analyzed subsequently using the mean pixel values of prese-}

\[\text{RESULTS}\]

Agonist-Induced \([\text{Ca}^{2+}]_{i}\) Rises. Stimulation of isolated ciliary bilayer epithelium with agonists caused a rise in \([\text{Ca}^{2+}]_{i}\) as described previously (14, 15). Epinephrine increased \([\text{Ca}^{2+}]_{i}\) in both epithelial layers (Fig. 1A–G); the \([\text{Ca}^{2+}]_{i}\) increase in the PE preceded the NPE \([\text{Ca}^{2+}]_{i}\) signal in 12 of 18 experiments, with no measurable time lag in the remaining 6 experiments. In contrast, the \(\alpha\_1\)-adrenergic agonist phenylephrine increased \([\text{Ca}^{2+}]_{i}\) only in the PE layer (\(n = 15\) experiments; Fig. 1H), whereas acetylcholine increased \([\text{Ca}^{2+}]_{i}\), only in the NPE layer in 25 of 35 experiments, with increases in \([\text{Ca}^{2+}]_{i}\) observed in both layers in the remaining 10 experiments (Fig. 1I). Furthermore, acetylcholine but not epinephrine increased \([\text{Ca}^{2+}]_{i}\) in isolated NPE monolayers (\(n = 5\) each, data not shown).

These findings show that PE and NPE each independently has the capacity to increase \([\text{Ca}^{2+}]_{i}\), in response to an appropriate stimulus.

Pharmacology of the Adrenergic Response. Additional studies were performed to determine why the PE and NPE respond jointly to epinephrine. In five consecutive experiments, the \(\beta\_adrenergic agonist isoproterenol alone had no significant effect on \([\text{Ca}^{2+}]_{i}\) in the epithelial bilayer (\(F_{\text{max}}/F_0 = 104.8 \pm 2.9\%\) in the PE and 111.5 \(\pm\) 1.7\% in the NPE; \(n = 5\) each), but
isoproterenol plus phenylephrine induced serial \([\text{Ca}^{2+}]_i\) signals in the PE \((F_{\text{max}}/F_0 = 199.1 \pm 15.6\% , P < 0.01\) relative to isoproterenol alone by paired \(t\) test and then NPE \((F_{\text{max}}/F_0 = 182.6 \pm 5.3\% , P < 0.0005\) relative to isoproterenol alone), similar to the pattern induced by epinephrine (Fig. 2A and B). Similarly, the \(\beta\)-adrenergic antagonist propranolol (Fig. 2C and D) blocked the epinephrine-induced \([\text{Ca}^{2+}]_i\) increase in the NPE \((F_{\text{max}}/F_0 = 122.1 \pm 3.0\% \) in the presence of propranolol and 236.3 \pm 14.9\% with epinephrine alone; \(n = 5, P < 0.005\) but not the PE \((221.5 \pm 18.9\% \) and 245.3 \pm 15.6\%, respectively). The \(\alpha_1\)-adrenergic antagonist prazosin (Fig. 2E and F) blocked the epinephrine-induced \([\text{Ca}^{2+}]_i\) increase in both layers \((F_{\text{max}}/F_0 = 217.7 \pm 17.9\% \) vs. 113.4 \pm 3.6\% in the PE without and with prazosin, respectively, and \(F_{\text{max}}/F_0 = 211.7 \pm 21.5\% \) vs. 108.8 \pm 3.0\% in the NPE without and with prazosin, respectively; \(n = 5, P < 0.01\), whereas the \(\alpha_2\)-adrenergic antagonist yohimbine (Fig. 2G and H) did not affect the epinephrine-induced \([\text{Ca}^{2+}]_i\) increase in either layer \((F_{\text{max}}/F_0 = 255.8 \pm 24.4\% \) vs. 234.2 \pm 32.1\% in the PE without and with yohimbine, respectively, and \(F_{\text{max}}/F_0 = 193.7 \pm 23.4\% \) vs. 182.4 \pm 14.0\% in the NPE without and with yohimbine, respectively; \(n = 5, P > 0.1\)). These findings show that the sequential signaling induced in the PE, then NPE, by epinephrine requires both \(\alpha_1\) and \(\beta\)-adrenergic stimulation.

**Organization of the \([\text{Ca}^{2+}]_i\), Waves Within Each Layer.** The temporal pattern of \([\text{Ca}^{2+}]_i\), signaling in the bilayer was examined in greater detail with confocal line scanning microscopy. In each layer, epinephrine triggered an abrupt increase in \([\text{Ca}^{2+}]_i\), in the apical region, and this \([\text{Ca}^{2+}]_i\), rise then spread rapidly to the basal region (Fig. 3). The speed of epinephrine-induced \([\text{Ca}^{2+}]_i\) waves was 26.6 \pm 2.2 \mu m/s \((n = 15)\) in the PE and 25.9 \pm 1.9 \mu m/s \((n = 10)\) in the NPE. The time lag between the onset of \([\text{Ca}^{2+}]_i\), rises in the PE and NPE cells was easier to quantify by line scanning, given the increased temporal resolution. The initial rise in epinephrine-induced \([\text{Ca}^{2+}]_i\), signals began 1.93 \pm 0.49 s \((n = 10)\) sooner in the PE \((P < 0.0001\) by paired \(t\) test).

**Signaling During \([\text{Ca}^{2+}]_i\), Oscillations.** To further evaluate intercellular \([\text{Ca}^{2+}]_i\), signaling in this tissue, adjacent pairs of PE and NPE cells within the epithelial bilayer were examined during \([\text{Ca}^{2+}]_i\), oscillations. Oscillations were detected in 65% \((13\) of 20) of ciliary specimens stimulated with epinephrine. In each case, oscillations were synchronized across the bilayer (Fig. 4). The duration of each individual \([\text{Ca}^{2+}]_i\), spike and the oscillation period were 2.50 \pm 0.27 s and 4.49 \pm 0.43 s, respectively \((n = 13)\). These results further demonstrate that epinephrine-induced \([\text{Ca}^{2+}]_i\), signals are coordinated in the bilayer.

**Role of Gap Junctions and cAMP.** To understand the mechanism by which \([\text{Ca}^{2+}]_i\), signaling is coordinated across the bilayer, specimens were pretreated with octanol, \(\alpha\)GA, or \(R_g\)-cAMP[S]. In specimens pretreated with octanol (Fig. 5A and B), epinephrine-induced \([\text{Ca}^{2+}]_i\), signaling was unchanged in the PE \((F_{\text{max}}/F_0 = 207.9 \pm 6.1\% \) vs. 196.9 \pm 7.0\% without and with octanol, respectively; \(n = 8, P > 0.15\)) but was nearly abolished in the NPE \((F_{\text{max}}/F_0 = 183.8 \pm 11.5\% \) vs. 110.7 \pm 3.8\% without and with octanol, respectively; \(P < 0.001\)).
[Ca\(^{2+}\)]\text{w} \text{ spikes have higher amplitudes in the PE cell in this particular example, but this was not a general feature.}

Similarly, in specimens pretreated with aGA (Fig. 5 C and D), epinephrine-induced [Ca\(^{2+}\)]\text{w signals} was unchanged in the PE (F\(_{\text{max}}/F_0\) = 212.5 ± 14.2% vs. 201.4 ± 17.9% without and with aGA, respectively; n = 5, P > 0.3) but was nearly abolished in the NPE (F\(_{\text{max}}/F_0\) = 174.7 ± 13.8% vs. 105.2 ± 2.0% without and with aGA, respectively; P < 0.005). In specimens pretreated with R\(_{\text{P}}\)-cAMP[S] (Fig. 5 E and F), the epinephrine-induced [Ca\(^{2+}\)]\text{w increase} was abolished in the NPE (F\(_{\text{max}}/F_0\) = 187.8 ± 18.1% vs. 105.4 ± 2.3% without and with R\(_{\text{P}}\)-cAMP[S], respectively; P < 0.01), but was reduced only slightly (by 8%) in the PE (F\(_{\text{max}}/F_0\) = 217.5 ± 20.1% vs. 200.9 ± 17.6% without and with R\(_{\text{P}}\)-cAMP[S], respectively; n = 5, P = 0.02). [Ca\(^{2+}\)]\text{w signals} in epithelia also may spread from cell to cell via ATP secretion coupled to activation of extracellular P2 ATP receptors (24), but the P2 receptor antagonist suramin (100 μM; Fig. 5 G and H) did not inhibit epinephrine-induced [Ca\(^{2+}\)]\text{w signals} in either the PE (F\(_{\text{max}}/F_0\) = 237.2 ± 19.4% vs. 225.8 ± 19.2% without and with suramin, respectively; n = 5, P > 0.2) or NPE (F\(_{\text{max}}/F_0\) = 192.2 ± 12.4% vs. 197.8 ± 14.5% without and with suramin, respectively; P > 0.7). These findings demonstrate that epinephrine-induced [Ca\(^{2+}\)]\text{w signals} in the PE depend only on activation of \(\alpha_1\)-adrenergic receptors, but that spread of the signal to the NPE further depends on gap junction conductance and A kinase activation.

**DISCUSSION**

The spatial pattern of [Ca\(^{2+}\)]\text{w} signals, both within individual cells (19, 30, 31) and from cell to cell (22, 32, 33), plays a role in the regulation of cell and tissue function. We examined the spatial organization of [Ca\(^{2+}\)]\text{w} signals in the secretory ciliary epithelial bilayer of the eye. The NPE and PE layers each exhibited the capacity to generate [Ca\(^{2+}\)]\text{w} signals through...
stimulation of muscarinic receptors on the NPE and α₁-adrenergic receptors on the PE. Stimulation with the endogenous agonist epinephrine increased [Ca²⁺] in both cell layers, though. This increase reflects sequential [Ca²⁺] signaling, first in the PE, then in the adjacent NPE. Epinephrine-induced signaling in the PE resulted from α₁-adrenergic stimulation of those cells, whereas signaling in the NPE required not only α₁ stimulation of the PE but β-adrenergic stimulation, presumably of the NPE. The spread of [Ca²⁺] signals to the NPE further depended on gap junctional communication between the two layers, plus A kinase activation through β-adrenergic stimulation. Thus, epinephrine-induced [Ca²⁺] signaling in the NPE appears to require a “priming” signal imparted through direct neuroendocrine stimulation of the NPE, followed by paracrine stimulation of these cells by the PE, via gap junctions. The priming signal is activation of A kinase, and this effect may act by increasing the sensitivity of NPE IP₃ receptors (34), or, more likely, by increasing the conductance of gap junctions (35, 36) that couple the NPE to the PE (37). Direct evidence in support of the latter mechanism was provided by the observation that isoproterenol increases dye transfer in the bilayer (Fig. 6D). Previous work in T84 colonic epithelia has also shown that cAMP can increase the conductance of gap junctions (38). In the ciliary bilayer this increased conductance likely results from phosphorylation of connexin 43 (37), the principal connexin isofrom of the gap junctions linking PE to NPE (39).

Cell-to-cell spread of [Ca²⁺] signals has been examined in several epithelia, including hepatocytes (20, 27), pancreatic acinar cells (22), and respiratory epithelia (40, 41). Both [Ca²⁺] and IP₃ can cross gap junctions in these cell types; therefore, increases in [Ca²⁺] or IP₃ in a single cell lead to an increase in [Ca²⁺] in neighboring cells (22, 40–42). Here, we showed that IP₃-mediated [Ca²⁺] signals can spread from cell to cell within the ciliary epithelial bilayer as well. In other tissues, simultaneous stimulation of cells linked via gap junctions results in highly synchronized [Ca²⁺] signals (20, 27). This coordinated response depends not only on gap junctions but on agonist-induced increases in basolateral levels of IP₃ in each of the cells (22, 27). Communication via gap junctions potentiates cAMP signaling as well (43). The current work extends these observations in two ways. First, this work shows that signaling via [Ca²⁺] and cAMP can work synergistically to induce the spread of [Ca²⁺] waves. Second, this study shows that [Ca²⁺] waves can spread, via gap junctions, from one type of epithelium to another.

We used two different agents, octanol and αGA, to block gap junction conductance. Long-chain alcohols block gap junctions as demonstrated by both cell-to-cell transfer of dyes (44) and direct electrophysiologic measurements (26, 45). Although nonspecific inhibitory effects of octanol have been described (46), we have not observed such effects in epithelia transiently exposed to octanol (20, 47), as done here. αGA is an alternative agent to block gap junctions, perhaps more specific than octanol (27), and αGA also blocked epinephrine-induced [Ca²⁺] signaling in the NPE but not the PE.

What is the functional significance of coordinated PE-to-NPE [Ca²⁺] signaling in the eye? In single epithelial cells, polarized movement of [Ca²⁺] waves is thought to direct fluid and electrolyte secretion by sequential activation of apical, then basolateral chloride channels (17, 19). Intercellular [Ca²⁺] signaling may also organize mechanical actions to facilitate secretion, such as ciliary beating in the lung (40) or canalicular peristalsis in the liver (32). In the exocrine pancreas, intercellular [Ca²⁺] signals may serve to coordinate and thus decrease the threshold for secretion (22). Secretion by the ciliary body may be more complex because it results from the action of two separate but communicating epithelia. The PE is responsible for extracting selected substances from the blood, whereas the NPE must then secrete these substances into the posterior chamber to form the aqueous humor (48). Ca²⁺-sensitive transport mechanisms have been identified in both the PE (49) and NPE (50). For example, Ca²⁺-activated potassium channels (51–53) may provide the electrical driving force known to be required for vectorial chloride secretion across the bilayer (51, 52). The sequential PE-to-NPE [Ca²⁺] signaling demonstrated here also depends on β-adrenergic activation of A kinase, consistent with previous observations in both rabbit and humans that β-adrenergic stimulation modulates aqueous humor formation (1, 54). Thus, this paracrine regulation of [Ca²⁺], signaling may provide a mechanism whereby the PE can direct the NPE to complete the transport of aqueous humor constituents initiated by the PE.

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