Adreno-Cholinergic Modulation of Junctional Communications Between the Pigmented and Nonpigmented Layers of the Ciliary Body Epithelium

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Purpose. Cell-to-cell communications between the epithelial layers of the ciliary body may be critical for aqueous humor production. The aim of this study was to identify pharmacologic agents that affect this path.

Methods. Whole New Zealand rabbit ciliary bodies were mounted in Ussing-type chambers with Ca²⁺-free and Ca²⁺-rich Tyrode's in the nonpigmented (NPE; aqueous) and pigmented (PE; serosa) epithelial side hemichambers, respectively. The NPE or the PE were then permeabilized, either selectively to monovalent ions with amphotericin B or nonselectively to small solutes with digitonin. Resultant active transport activities were tracked as short circuit currents (Iscs).

Results. Permeabilization of the NPE with either 10 μM amphotericin B or 10 μM digitonin led to an aqueous-to-serosa-positive Isc. This Isc was inhibited by serosal-side ouabain and heptanol, indicating movement of Na⁺ from the permeabilized NPE to the PE by the interlayer junctional path, followed by PE-to-serosa active Na⁺ transport. Permeabilization of the PE with amphotericin B elicited an Isc in the opposite direction. This Isc was abolished by aqueous-side ouabain and by heptanol, consistent with sequential PE to NPE Na⁺ translocation, followed by active, NPE-to-aqueous transport. Acetylcholine, epinephrine, norepinephrine, and the α-adrenergic agonist phenylephrine, but not brimonidine, an α₂-adrenergic agonist, each caused an approximately 50% reduction of these currents. The inhibitions were fully dependent on serosal-side Ca²⁺ and were blocked by one calmodulin inhibitor, trifluoperazine, but not by another, calmidazolium.

Conclusions. The above observations provide evidence that cholinergic or α₂-adrenergic activation of the PE causes Ca²⁺-dependent inhibition of the NPE–PE junctional path. A trifluoperazine-sensitive entity, which may be distinct from calmodulin, is involved in the inhibition. Invest Ophthalmol Vis Sci. 1996;37:1037–1046.

Aqueous humor production is thought to involve a functional interplay between the two apically apposed, distinct cell layers that form the ciliary body epithelium, namely, the aqueous-facing nonpigmented (NPE) and the serosal-facing pigmented (PE) epithelia. The two apical membranes are attached tightly by desmosomes and contain gap junctions that communicate the layers.1–3 Additionally, the NPE layer contains leaky type tight junctions that constitute the paracellular component of the blood–aqueous barrier. There are no such junctions between the PE cells.

Both cell types contain ouabain-sensitive rheogenic Na⁺ pumps within their basolateral membranes4; thus, each layer tends to generate an Na⁺ flux component in a direction opposite the other. These opposite actions are revealed by unilateral ouabain addition, when the tissue is mounted in electrophysiological Ussing-type chambers and maintained under the short-circuited condition.5 Addition of ouabain to the aqueous-side hemichamber, faced by the basolateral membrane of the NPE, induces a large positive aque-
ous-to-serosa short-circuit current (Isc). Addition of the inhibitor to the serosal side produces an Isc in the opposite direction.

On the other hand, measurements of other transport functions suggest cooperative interactions. Co-transporters and antiporters, which facilitate Na+ uptake, Cl− uptake, or both, predominate in the PE and anion channels in the NPE of the human and bovine cells. Such an asymmetric distribution of transporters can elicit serosa-to-aqueous Cl− and HCO3− fluxes. In the rabbit, a species in which the secretion includes a substantial bicarbonate component, intracellular pH measurements have demonstrated bicarbonate uptake at the serosal-facing PE and bicarbonate extrusion at the aqueous-facing NPE. Similarly, the extraordinary capacity of the ciliary body to accumulate ascorbic acid and secrete it into the aqueous appears to be dependent on the cooperation between a Na+-dependent uptake in the PE and facilitated efflux at the aqueous-facing NPE.

The cooperativeness of the two cell layers implies that the junctional path between them could play a critical role in the secretory process and could provide a unique site for exogenous intervention aimed at controlling the rate of aqueous formation. Accordingly, we have developed a macro-electrophysiological approach for the study of these junctions. The method is based on the ability of certain agents, such as amphotericin B or digitonin, to permeabilize, selectively and unselectively, respectively, the outermost cell membrane of a cell without affecting intracellular organelle barriers or contralateral membranes.

The versatility of the method allows the characterization of the effect of pharmacologic inputs on each of the cell layers on this unique interlayer junctional path. The current article focuses on agonist effects originating in the PE layer.

METHODS

Electrophysiology

Adult New Zealand rabbits were killed by CO2 narcosis and asphyxiation. Ciliary bodies were dissected and mounted in electrophysiological Ussing-type chambers in Tyrode’s solution containing, in mM: 103 NaCl, 4.5 KCl, 30 NaHCO3, 1.8 CaCl2, 1 NaH2PO4, 5.5 glucose at 37°C. The solution constantly was bubbled with 5% CO2 in air. The specialized methodologies used to dissect the intact, circular, iris–ciliary body and mount it between the two hemichambers while avoiding or minimizing edge and tissue damage have been described in detail.5,17 Mounted tissues were maintained under short-circuit conditions. For that purpose, each hemichamber contained two agar bridges. The tip of the voltage-sensing bridge was placed within 2 to 3 mm of the tissue surface and was connected at its other end to the electronic circuitry through calomel electrodes. The tip of the current injecting bridge was located at the rear of the hemichamber and was connected to the electrical circuit by a platinum-saline junction. The voltage clamp circuit injected an amount of current equal to that needed to maintain a null transepithelial voltage. The amount of current was displayed on real-time on a chart recorder.5 Transepithelial resistance (Rt) was determined throughout the experiments from the current deflections generated by the automated introduction of short duration (1-second), 1-mV voltage deflections, at 5-minute intervals.

In some of the experiments, according to specific needs, bicarbonate was replaced by Hepes, and CO2 was replaced by air. This solution is referred to henceforth as Hepes–Ringer’s. For the low-buffer capacity Ringer’s used to test the effects of pH, Hepes was reduced to 10 mM, and the osmolality was compensated by inclusion of 40 mM sucrose. For the Cl−-free Ringer’s, all NaCl and KCl were replaced by 51.5 mM Na2SO4, 2.25 mM K2SO4, and 55 mM sucrose. Depending on the specific experiment, CaCl2 (1.8 mM) was added to either the aqueous- or the serosal-side hemichamber, or to both. After constant electrical parameters became established, biologic agents were added as specified for each experiment.

Gallbladders were removed through a ventral incision immediately after the animals were killed. To remove the bile without risk of serosal exposure,18 the unopened sac was pierced at one end near the neck with a 25G-1-inch needle attached to a 20-ml syringe. A small orifice was made on the other side with a 20G-1-inch needle, and the bile was flushed away by gentle passage of Ringer’s from the syringe until a clear affluent was seen. The gallbladder was cut open and split into two symmetrical sections. Each section was mounted in Tyrode’s in the electrophysiological Ussing-type chamber used previously for studies of the rabbit conjunctiva.19

Radiometric Fluorophotometry of Fura-2-Loaded Ciliary Body Epithelium

Ciliary body segments from Dutch belted rabbits were dissected as described by Wolosin et al,15, loaded with the [Ca2+] sensitive dye fura-220 in its acetoxymethoxy form (Molecular Probes, Eugene, OR), and set on a perfusion chamber. Changes in relative intracellular [Ca2+] and fura-2 content were then determined using microscope-aided dual excitation wavelength photometry. All the procedures used are described in detail in Schütte et al,21 except that Rm/380, the ratio of the intensity of the emissions (A > 515 nm) derived from two alternating excitations at 340 nm (I340) and 380...
FIGURE 1. The effects of aqueous-side amphotericin B, digitonin, Ca\(^{2+}\), ouabain, and heptanol on the I\(_c\) across whole iris–ciliary body preparations mounted in a bicameral chamber. (A) Studies with amphotericin B. Representative experiments are shown. Unmodified tissues (with Ca\(^{2+}\) present in both solutions) generated a negative serosal-to-aqueous I\(_c\) of 3 to 5 nA. This I\(_c\) was not affected by replacement of aqueous-side Ca\(^{2+}\) by 100 \(\mu\)M EGTA (solid trace; Ca\(^{2+}\)-free; \(n = 15\)). Addition of ouabain (10 \(\mu\)M; dashed trace) to the aqueous elicited an I\(_c\) increase (\(n = 6\)) consistent with the inhibition of the rheogenic NPE Na\(^{+}\)-pump. Amphotericin B induced a substantially larger I\(_c\) increase (\(n = 42\)). This amphotericin B-increased I\(_c\) was not sensitive to aqueous-side ouabain (\(n = 6\)). It was abolished by 3 mM heptanol (solid trace; \(n = 29\)). On the other hand, the ouabain-pre-enhanced I\(_c\) underwent further increase with the addition of amphotericin B (dashed line; \(n = 6\)). Sodium dodecyl sulfate (SDS; 0.2%) was added to both hemichambers to destroy the epithelium and to confirm the null point for the I\(_c\). (B) Studies with digitonin. Representative experiments are shown. Five minutes before the addition of digitonin (10 \(\mu\)M), the aqueous-side Tyrodes was replaced by an homologous solution containing no CaCl\(_2\) and complemented with 100 \(\mu\)M EGTA (Ca\(^{2+}\)-free). Addition of the detergent induced a large increase in the I\(_c\) (\(n = 72\)). This I\(_c\) was abolished by the addition of 2 mM Ca\(^{2+}\) (solid trace; \(n = 27\)) or 100 \(\mu\)M \(\alpha\)-18-glycyrrhetinic acid (inset; \(n = 4\)) to the aqueous or, more gradually, by the addition of 100 \(\mu\)M ouabain to the serosa (dashed trace; \(n = 7\); the bulky underlying serosa separating the basolateral membrane surface from the serosal solution slows the rate of ouabain action). (thin trace) Open bar = 5 minutes (all figures).

nm (I\(_{980}\)), a relative measure of [Ca\(^{2+}\)], was used for data analysis without further conversion into [Ca\(^{2+}\)] by the in situ calibration method.\(^{20}\) Brominidine (UK 14304; UK), yohimbine, prazosin, and calmidazolium were purchased from RBI (Natick, MA). All other chemicals were obtained from Sigma (St. Louis, MO). All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RESULTS

The rabbit ciliary body of the adult (2 to 3 kg) albino rabbit mounted in a modified Ussing chamber displays a positive aqueous-to-serosa I\(_c\), which is the result of the sum of various net ionic currents, including a serosa-to-aqueous HCO\(_3\)\(^{-}\) transport\(^{1,17}\) and a R\(_s\) of approximately 140 \(\Omega\). Addition of the antibiotic amphotericin B (10 \(\mu\)M) to the aqueous side resulted in a substantial I\(_c\) increase\(^{2}\) (Fig. 1A) and a 15 to 20 \(\Omega\) decrease in the R\(_s\). The I\(_c\) enhancement occurred equally whether the aqueous side contains Ca\(^{2+}\) or is Ca\(^{2+}\)-free (compare Fig. 1A, dashed and solid traces). This electrical change can be explained on the basis of the well-known properties of amphotericin B and the structural and functional characteristics of the ciliary body. Amphotericin B is a monovalent cation ionophore that permeabilizes outermost-exposed membranes of cells without affecting contralateral membranes.\(^{5,22,23}\) Thus, it can be expected to eliminate rapidly the NPE basolateral membrane barrier and thereby equalize the intracellular cation concentration in this cell with that of the aqueous-side medium. Given the existence of tight junctions between the NPE cells, it stands to reason that any NPE cell Na\(^{+}\) able to reach the PE cell cytosol will be translocated by the PE Na\(^{+}\)-K\(^{-}\) -ATPase. Under zero-voltage clamp (short-circuit) conditions, this process will lead to the generation of a positive aqueous-to-serosa I\(_c\). The observation that the addition of ouabain to the serosa (to inhibit the PE Na\(^{+}\)-K\(^{-}\) - ATPase) abolished the induced I\(_c\) (Fig. 1A; dashed trace) provides substantial support to the proposed mechanism. (The slow rate of inhibition results from the use of a limited ouabain concentration coupled to a thick sero-
sal barrier. Using a high inhibitor concentration\(^5\) accelerates the inhibition). Further confirmation of the role of Na\(^+\) was derived from the observation that the amphotericin B and digitonin-induced \(I_\text{sc}\) were completely abolished by bilateral replacement of Na\(^+\) by N-methyl-d-glucamine (not shown). Additionally, it is pertinent to note that aqueous-side ouabain, which normally increases the control \(I_\text{sc}\) caused by inhibition of the rheogenic, aqueously directed, NPE Na\(^+\) pump\(^5\) (Fig. 1A, dashed trace), had no effect on the post-amphotericin B \(I_\text{sc}\) (Fig. 1A, solid trace). This lack of effect is consistent with the notion that after the permeabilizing treatment, the basolateral membrane of the NPE was essentially short circuited, i.e., changes in current across it could not elicit a transmural electromotive force.

Essentially identical \(I_\text{sc}\) enhancements and \(R_\text{i}\) decreases to those induced by amphotericin B were generated by the addition of 10 \(\mu\)M of the detergent digitonin to the aqueous-side hemichamber (Fig. 1B, dashed and solid trace), but only in the absence of Ca\(^{2+}\). This detergent shares with amphotericin B the ability to permeabilize an outermost-exposed membrane without affecting intracellular\(^24\) or contralateral membranes.\(^23,25\) However, unlike the permeabilization with amphotericin B, the permeabilization with digitonin is nonselective; the higher the concentration, the larger the disruption of the exposed membrane and, thus, the size of the permeable solutes. The extent of permeabilization at a given concentration is dependent on the specific membrane treated. Thus, to assess the detergent effect on the NPE, we performed measurements of intracellular [Ca\(^{2+}\)] in fura-2 loaded segments of pigmented ciliary body (Fig. 2). From the results of these studies, it could be concluded that the addition of 10 \(\mu\)M digitonin to the medium causes a rapid permeabilization of the NPE to Ca\(^{2+}\) and, concurrently, a much slower and gradual increase in the permeability of the entrapped fura-2 anion. The increase in the rate of influx of this approximately 800-kD polyanionic organic anion (the exact molecular weight and charge depends on the extent of intracellular de-esterification)\(^20\) indicates that essential metabolites in the similar and lower molecular weights, which are normally retained within the cell because of their electrical charge and/or polarity (e.g., adenosine triphosphate, cyclic adenosine monophosphate, and so on) will undergo a similar dissipative efflux. The increase in the rate of influx of Ca\(^{2+}\) suggests that for monovalent ions, a large facilitation will occur. Thus, with respect to such ions, digitonin can be expected to induce rapidly a similar equalization of intracellular (NPE) and extracellular composition to that generated by amphotericin B and, by extension, a similar aqueous-to-serosa, serosal-side, ouabain-inhibitable, flux of Na\(^+\).

**FIGURE 2.** Effect of digitonin on relative [Ca\(^{2+}\)] and on the rate of leak of entrapped fura-2 in the nonpigmented epithelial cells. (**A**) Hepes Ringer's solution. Previous studies\(^7\) demonstrate that under these conditions, the intracellular [Ca\(^{2+}\)] is less than 0.1 \(\mu\)M. (**B**) Ringer's containing 10 \(\mu\)M digitonin. (**C**) Ringer's with 10 mM Ca\(^{2+}\) plus digitonin and 2 mM thapsigargin, the inhibitor of the endoplasmic reticulum Ca\(^{2+}\)-ATPase. (**D**) Ca\(^{2+}\)-free Ringer's with 10 mM EGTA. The lack of effect of the increase in extracellular [Ca\(^{2+}\)] from 1.8 mM to 10 mM on \(R_{340/380}\) implies that intracellular [Ca\(^{2+}\)] has exceeded the saturation point for the dye (approximately 5 \(\mu\)M). **(bottom panel)** \(I_{340}\) and \(I_{380}\). At constant intracellular [Ca\(^{2+}\)], the decrease in each of these two intensities reflects the loss of fura-2 from the cell. The dashed line joins two equimolar [Ca\(^{2+}\)] time points (identical \(R_\text{i}\) values). Thus, it represents the average rate of dye loss over this period, in the presence of 10 \(\mu\)M digitonin. The dotted line illustrates typical dye losses observed in control conditions in similar experiments.

After full permeabilization of the NPE basolateral membrane with either of the two agents used, the most likely route of aqueous-side Na\(^+\) movement into the PE is provided by the NPE-PE gap junctions.\(^1-5\) However, other cellular or paracellular routes could conceivably exist, in particular after the introduction of the permeabilizing agents. Several observations suggested, nonetheless, that the junctional path accounts for all or nearly all of this flux.

First, the digitonin-induced \(I_\text{sc}\) was rapidly and irreversibly abolished with the addition of Ca\(^{2+}\) to the aqueous side (Fig. 1B; solid trace), consistent with the measured Ca\(^{2+}\) permeability induced by digitonin and the known inhibitory effect of high [Ca\(^{2+}\)] on gap
Regulation of Ciliary Body Gap Junctions

FIGURE 3. Representative traces of the effect of amphotericin B or digitonin and of bilaterally added heptanol on the I_sc in the rabbit gallbladder. All experimental details are described in the Methods section. (solid trace) Gallbladder was mounted in Tyrodes. The additions of 50 µM amphotericin B (Amph. B), 3.5 mM heptanol to the serosal and mucosal compartments, 1 mM ouabain to the serosa, and 0.1% sodium dodecyl sulfate bilaterally, are indicated. The mean amphotericin B-induced I_sc at peak was 26.8 µA/cm² (n = 3). The mean transepithelial resistance was 0.16 Ω·cm². These values yield a calculated transmural difference (I•R) of 4.4 mV, in good agreement with the original values reported by Rose and Nahrwold.22 (dashed trace) Gallbladder was mounted with Ca²⁺-free Tyrodes in the luminal side. The addition of 10 fM digitonin and 50 µM amphotericin B to the lumen are indicated.

In contrast, as can be seen from Figure 1A with amphotericin B, which does not transport divalent cations, the extracellular Ca²⁺ status was irrelevant.

Second, the I_sc induced with either amphotericin B (Fig. 1A; solid trace) or digitonin (Fig. 1B, inset) were abolished by the addition of two gap junction inhibitors, heptanol²⁷,²⁸ and 18α-glycyrrhetinic acid.²⁹ For heptanol, the concentration required (3 mM) to attain complete inhibition of the I_sc was similar to that required in other cellular systems to block fully the junctional communications.¹⁹,²⁵,²⁷ Furthermore, in contrast to its effect in the ciliary body, in the simple epithelium of the rabbit gallbladder, where luminal-side Na⁺ does not have to transverse a junctional barrier to reach the serosal-side Na⁺ pump, heptanol at 3.5 mM had essentially no effect on the I_sc induced by luminal-side amphotericin B,²² even when the alkanol was added bilaterally to ensure its equilibration across the tissue (Fig. 3; solid trace). As seen in the case of the ciliary body, the induced I_sc was abolished rapidly by the addition of 1 mM ouabain to the serosal side, demonstrating the involvement of the Na⁺ pump in the process and by extension, the lack of sensitivity of the rabbit Na⁺-K⁺-ATPase to heptanol.

Third, aqueous-side acidification caused rapid and reversible changes in the digitonin-induced I_sc of the ciliary body epithelium (Fig. 4); the sensitivity to H⁺ (47% ± 5% [± SD] of the maximal inhibition was attained at pH 6.7; n = 4; the maximal inhibition was reached at pH levels below 5.8) conformed to the known sensitivity of junctional permeability to acidification.³⁰ Changes in pH also could conceivably inhibit the pump; however, H⁺ will have to enter the PE cell from its apical side at rates high enough to completely overcome the pH regulatory mechanism of this cell.⁶,⁷,¹⁴

Thus, digitonin- and amphotericin B-induced I_sc appear to reflect a two-step sequential process, heptanol-sensitive NPE to PE Na⁺ movement through gap junctions (and, presumably, K⁺ movement in the opposite direction) and ouabain-sensitive active Na⁺ transport from PE to serosal side. Figure 5 provides a graphic depiction of this concept.

Two additional observations pertinent to this model are as follows. First, the near complete elimination of the ciliary body I_sc by heptanol occurred without any measurable change in Rₑ. This is in stark contrast to the events in the corneal epithelium, in which a decrease in the amphotericin B-enhanced I_sc is accompanied by a corresponding large decrease in Rₑ.

FIGURE 4. The effect of aqueous-side pH on the digitonin-induced I_sc in the ciliary body. A representative experiment is shown. All experimental details were as described for Figure 1B, except that the Hepes Ringer's of reduced buffer capacity (Methods) was used instead of Tyrodes. The pH was adjusted downward or upward by additions of small aliquots of 1 M Hepes acid or 1 M NaOH, respectively, and monitored with a standard combination glass electrode introduced in the aqueous-side hemichamber.
aqueous

serosa

![Diagram](http://example.com/diagram.png)

**FIGURE 5.** Schematic representation of the flow of Na\(^+\) established after permeabilization of the basolateral membrane of the NPE with either digitonin (*large circle*) or amphotericin B (*small circle*). Either membrane modification allows rapid entry of Na\(^+\) into the NPE. From this cell, the cation can diffuse through the interlayer gap junction into the pigmented epithelial cytosol. The Na\(^+-\)K\(^+\) - ATPase (*filled circle*) of this cell transfers the incoming Na\(^+\) into the serosal medium. This unidirectional process can be abolished by gap junction closure with heptanol or by ATPase inhibition with serosal-side ouabain, irrespective of the permeability agent applied. Additionally, in the case of digitonin, Ca\(^{2+}\) and H\(^+\) can enter rapidly from the aqueous-side to affect the junctional permeability. These inhibitory actions are indicated by dashed line arrows. Amphotericin B does not translocate Ca\(^{2+}\); thus, with this agent, the same I\(_{sc}\) is established whether the aqueous-side is Ca\(^{2+}\) rich or free. The junctional permeability, the PE pump, or both, may be subjected to regulation by second messengers induced by agonists via cell receptors (R). With the NPE cell permeabilized by digitonin, intracellular signal can only occur within the PE. The presence of a Na\(^+-\)K\(^+\) - ATPase (*filled circle*) in the NPE results, normally, in an effect of aqueous-side ouabain in the tissue I\(_{sc}\). However, after the NPE basolateral membrane is permeabilized, this ATP-consuming process is no longer rheogenic, i.e., inhibition of the ATPase by ouabain no longer affects the I\(_{sc}\) because all Na\(^+\) and K\(^+\) transported by this pump will originate and return to the aqueous.

Increasing resistance is a logical outcome of the blockade of an ionic path. The absence of such an effect in the ciliary body may reflect the existence of a large conductance in the apical membrane of the NPE, i.e., in parallel to the junctional path. Second, similar to its effect on the ciliary body, the addition of digitonin to the Ca\(^{2+}\)-free luminal side of the gallbladder produced a rapid I\(_{sc}\) increase to a level similar that generated by amphotericin B in this tissue (Fig. 3, dashed trace; \(n = 6\)). But this I\(_{sc}\) vanished rapidly, and it could not be recovered either by washing out the detergent or by adding amphotericin B, consistent with the expectation that digitonin will devitalize the cells of the single epithelium fully. In the ciliary body, the same devitalization presumably is undergone by the NPE, but in this case the loss of cell viability does not affect the I\(_{sc}\) because the Na\(^+\)-pumping function occurred within the contralateral PE cell and the path of entry, the NPE - PE gap junctions, remained open in the Ca\(^{2+}\)-free environment.

**Pharmacologic Modulation of the Induced I\(_{sc}\)**

We speculated that the simple two-step process demonstrated in the experiments above may prove useful in identifying putative pharmacologic controls of either the sequential NPE-PE junctional pathway in the PE cell, the PE Na\(^+\) pump, or both. All drugs were routinely added to the aqueous side. However, it should be noted that previous experience with this preparation indicates that, owing to the relatively leaky nature of the paracellular path of the ciliary body epithelium, hydrophilic compounds can reach and affect the pigmented epithelial cell layer with relative ease when applied at excess concentration, in spite of its "trans" location. In fact, pilot tests demonstrated that the drugs used had similar effects whether they were added to the aqueous side or the serosal side. However, in the latter case, the inhibitions developed at a slower pace, consistent with the presence of a bulky, tortuous serosal path.

Epinephrine, norepinephrine, phenylephrine, and acetylcholine all caused decreases in the I\(_{sc}\) amounting to approximately 50% of the decrease induced by the introduction of a high [Ca\(^{2+}\)] to the aqueous side, to elicit complete gap junctional closure (Fig. 6 and Table 1). Similar inhibitions were observed in tissues permeabilized to monovalent cations with amphotericin B. The adrenergic and cholinergic responses were essentially nonadditive; there was only a minimal effect of epinephrine after inhibition by acetylcholine (Fig. 6A, solid trace; \(n = 2\)) and, conversely, of carbachol after the inhibition by phenylephrine (Fig. 6A, dashed trace; \(n = 2\)). On the other hand, I\(_{sc}\) was totally insensitive to the \(\alpha\)-adrenergic agonists, UK-14304 (Fig. 6A, dashed trace; \(n = 4\)) and clonidine (not shown; \(n = 2\)). Consistent with this latter observation, adrenergic effects were blocked by the \(\alpha\)-adrenergic antagonist prazosin, (Fig. 6B, dashed trace; \(n = 4\)) but not by the \(\beta\)-adrenergic antagonist atenolol (not shown; \(n = 2\)). In addition, I\(_{sc}\) was not influenced by either the \(\beta\)-adrenergic agonist isoproterenol (\(n = 2\)), the adenylylcyclase activator forskolin (\(n = 4\)), the cyclic adenosine monophosphate membrane permeable analog 4Br cyclic adenosine monophosphate (\(n = 2\)) or, within the first 5 minutes of its addition, the protein kinase C activator phorbol myristyl acetate (\(n = 2\)), all applied at 10 \(\mu\)M (not shown). The agonists-induced decreases in I\(_{sc}\) occurred without measurable change in the R\(_{i}\).

Removal of extracellular Ca\(^{2+}\) from the serosa just before the introduction of either carbachol (not shown; \(n = 2\)) or epinephrine (Fig. 7; \(n = 6\)) prevented the inhibitory action of the receptor-mediated
agonists. Inhibition, however, was rescued by reintroduction of Ca\(^{2+}\) to the serosal side soon after the addition of the inhibitory agonists (Fig. 7, solid trace; \(n = 2\)). The extent of rescue decreased gradually with the increase in the time elapsed between agonist and Ca\(^{2+}\) addition (Fig. 7, dashed trace; \(n = 2\)).

Because the latter results pointed to an involvement of Ca\(^{2+}\) mobilization on the adrenergic and cholinergic inhibitions, the putative involvement of calmodulin mediation was investigated using two known inhibitors, trifluoperazine (TFP) and calmidazolium (Fig. 8). Five \(\mu\)M TFP, though having no visible effect on either the control (Fig. 8A) or the digitonin-induced \(I_{sc}\) (not shown), completely blocked the inhibitions induced by the adrenergic or cholinergic inputs (Fig. 8A, solid trace) but was unable to reverse them (Fig. 8A, dashed trace). Calmidazolium, at the same concentration, had no blocking effect, whether added before digitonin (not shown) or after it (Fig. 8B).

**Identification of the Pharmacologically Responsive Step**

The cholinergic and adrenergic effects could reflect changes at any of the two Na\(^{+}\) translocation steps, the
The inhibition was recovered with the addition of Ca\(^{2+}\) to saturating concentrations, 25 failed to interfere with the course of the inversion of the I\(_{sc}\) after the addition of ouabain (50 \(\mu\)M) and dihydrocytochalasin B; applied at under these conditions, the agonist failed to inhibit the current. The inhibition was recovered with the addition of Ca\(^{2+}\) to the serosa. The remaining inverted I\(_{sc}\) was abolished by 3 \(\mu\)M heptanol.

**FIGURE 9.** Effect of phenylephrine and serosal-side Ca\(^{2+}\) on the serosal-to-aqueous (inverted) I\(_{sc}\) induced by the addition of amphotericin B and ouabain to the serosal hemichamber. Representative experiments are shown. (solid trace) Full course of the inversion of the I\(_{sc}\), after the addition of ouabain (50 \(\mu\)M; \(n = 10\)) and amphotericin B (10 \(\mu\)M) to the serosa, the inhibition of the inverted current by phenylephrine when Ca\(^{2+}\) is present only in the serosal side (\(n = 4\)), and the subsequent complete elimination of I\(_{sc}\) by aqueous-side ouabain. (dashed trace) The last section of an experiment during which Ca\(^{2+}\) was not introduced into the serosal chamber before the addition of phenylephrine (\(n = 2\)). Under these conditions, the agonist failed to inhibit the current. The inhibition was recovered with the addition of Ca\(^{2+}\) to the serosa. The remaining inverted I\(_{sc}\) was abolished by 3 \(\mu\)M heptanol.

### DISCUSSION

Aqueous humor is formed through the cooperative interaction of the transporters and pharmacologic effectors present in both cell layers that form the ciliary body epithelium. Thus, regulation of the junctional path between the NPE and the PE may play a significant role in aqueous humor formation. Current models propose that ions secreted by the NPE into the aqueous enter the NPE from the PE rather than through the apical surface of the NPE. 1\(^{1,11,14,32}\) Because the two cell types exhibit marked differences in transporter distribution, hormonal receptors, and extracellular environments, the existence of biologic and/or pharmacologic regulatory mechanisms distinct from those governing the NPE – PE and PE – PE cell-to-cell communications may be necessary for the optimal function of the system.

A number of observations, including I\(_{sc}\) sensitivity to heptanol and to aqueous pH, suggest that under the experimental conditions used in this study, PE-to-NPE Na\(^{+}\) flux was partially or fully rate-limiting. Had this not been the case, a change in pH to 7, which is known to cause minimal inhibition of junctional permeability, should not have led to any measurable decrease of the I\(_{sc}\). This conclusion may appear to be inconsistent with recent studies\(^{3}\) suggesting a very fast NPE – PE transfer of fluorescent dyes microinjected into the NPE. However, it should be noted that the experiments with dye were performed at single locations at the very tips of ciliary processes, whereas our measurements average the NPE-to-PE permeability throughout the tissue. As shown for other tissue func-

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Regulation of Ciliary Body Gap Junctions

TABLE 1. Effect of Adrenergic and Cholinergic Agonists on the Digitonin- or Amphotericin B-induced Serosal to Aqueous I_{sc}

<table>
<thead>
<tr>
<th>Agonist</th>
<th>n</th>
<th>Before</th>
<th>After</th>
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<td>Digitonin</td>
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<td>Acetylcholine</td>
<td>15</td>
<td>9.3 ± 0.9</td>
<td>4.0 ± 0.4</td>
<td>54.4 ± 3.7</td>
</tr>
<tr>
<td>Carbachol</td>
<td>3</td>
<td>9.7 ± 1.2</td>
<td>5.0 ± 0.5</td>
<td>48.7 ± 1.8</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>3</td>
<td>7.1 ± 2.4</td>
<td>3.9 ± 1.8</td>
<td>49.3 ± 6.2</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>3</td>
<td>7.4 ± 0.8</td>
<td>3.5 ± 0.5</td>
<td>52.7 ± 1.9</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>4</td>
<td>9.7 ± 1.2</td>
<td>5.0 ± 0.5</td>
<td>55.3 ± 3.2</td>
</tr>
<tr>
<td>Amphotericin B</td>
<td></td>
<td></td>
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<tr>
<td>Acetylcholine</td>
<td>2</td>
<td>9.8 ± 1.3</td>
<td>4.3 ± 0.8</td>
<td>57 ± 2.0</td>
</tr>
<tr>
<td>Epinephrine</td>
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<td>10.0 ± 1.3</td>
<td>4.9 ± 0.6</td>
<td>52 ± 3.5</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>5</td>
<td>11.3 ± 0.4</td>
<td>7.9 ± 0.3</td>
<td>30 ± 3.5</td>
</tr>
</tbody>
</table>

Values are mean ± SD. The before values are the I_{sc} measured just before the introduction of the agonists. The after values were measured 5 minutes after agonist addition.

References

7. Helbig H, Korbmacher C, Berweck S, Kuhver D, Wiederholt M. Kinetic properties of Na+/H+ ex-


