Serotonin-elicited inhibition of Cl\(^{-}\) secretion in the rabbit conjunctival epithelium

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Alvarez, Lawrence J., Helen C. Turner, Aldo C. Zamudio, and Oscar A. Candia. Serotonin-elicited inhibition of Cl\(^{-}\) secretion in the rabbit conjunctival epithelium. Am J Physiol Cell Physiol 280: C581–C592, 2001.—The effects of serotonin [5-hydroxytryptamine (5-HT)] on the transepithelial electrical properties of the short-circuited rabbit conjunctiva were examined. With this epithelium, the short-circuit current \((I_{sc})\) measures Cl\(^{-}\) secretion plus an amiloride-resistant Na\(^{+}\) absorptive process. Apical addition of 5-HT (10 \(\mu\)M) elicited a prompt \(I_{sc}\) reduction from 14.2 ± 1.2 to 10.9 ± 1.2 \(\mu\)A/cm\(^2\) and increased transepithelial resistance from 0.89 ± 0.05 to 1.03 ± 0.06 k\(\Omega\)-cm\(^2\) (means ± SE, \(n = 21, \ P < 0.05\)). Similar changes were obtained with conjunctiva preexposed to bumetanide with the Cl\(^{-}\)-secreting epithelium in which 5-HT evokes Cl\(^{-}\) secretion (20, 33). In addition, recently identified Na\(^{+}\)/H\(^{+}\) and Cl\(^{-}\)/HCO\(_{3}^{-}\) exchangers that exist in parallel in the basolateral Na\(^{+}\)/H\(^{+}\) and Cl\(^{-}\)/HCO\(_{3}^{-}\) exchangers. In contrast, the 5-HT-evoked effects were attenuated by the absence of Cl\(^{-}\) \((\Delta I_{sc} = -0.5 ± 0.2, \ n = 5)\), suggesting that reduced Cl\(^{-}\) conductance is an effect of 5-HT exposure. In amphotericin B-treated conjunctiva and in the presence of a transepithelial K\(^{+}\) gradient, 5-HT addition reduced K\(^{+}\) diffusion across the preparation by 13% and increased transepithelial resistance by 4% \((n = 6, \ P < 0.05)\), indicating that an inhibition in K\(^{+}\) conductance was also detectable. Significant electrical responses also occurred under physiological conditions when 5-HT was introduced to epithelia pretreated with adrenergic agonists or protein kinase C, phospholipase C, phosphodiesterase, or adenyl cyclase inhibitors or after perturbation of Ca\(^{2+}\)-homeostasis. Briefly, the conjunctiva harbors the only known Cl\(^{-}\)-secreting epithilum in which 5-HT evokes Cl\(^{-}\) transport inhibition; receptor subtype and signal transduction mechanisms were not determined.

electrolyte transport; Ussing chamber; short-circuit current; serotonin receptors; chloride secretagogue

THE CONJUNCTIVA IS A THIN, transparent, mucus-secreting, vascularized epithelium that lines the inner surface of the eyelids (the palpebral conjunctiva) and covers the anterior sclera of the ocular globe (the bulbar conjunctiva). This epithelium is embryologically related to, and anatomically continuous with, that of the upper airway. It is stratified, with variations in cell layers from the tarsal portion of the eyelid to the corneoscleral junction (45), and manifests, as determined with rabbit specimens (20, 33), electrical features characteristic of “tight epithelia,” i.e., a relatively high transepithelial resistance \((R_i)\). Its electrolyte transport properties are similar to those of the mammalian intestinal epithelium (10) and the shark renal proximal tubule (3), in which mechanisms for Na\(^{+}\) absorption and Cl\(^{-}\) secretion coexist.

Identical to Cl\(^{-}\)-secreting epithelia, such as the extensively characterized frog corneal epithelium (5), the rabbit conjunctiva has a basolateral bumetanide-sensitive Cl\(^{-}\) secretion process (presumably Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransport) positioned in series with apical Cl\(^{-}\) channels (20, 33). In addition, recently identified Na\(^{+}\)/H\(^{+}\) and Cl\(^{-}\)/HCO\(_{3}^{-}\) exchangers that exist in parallel in the basolateral membrane (41) can also mediate Cl\(^{-}\) uptake. The contribution of the acid-base transporters to transepithelial Cl\(^{-}\) secretion is variable and dependent on individual rates of metabolic CO\(_{2}\) production, inasmuch as the transepithelial electrical parameters do not require extracellular HCO\(_{3}^{-}\) (unpublished observations).

Oppositely directed, electrogenic Na\(^{+}\) reabsorption is amiloride insensitive (33) and occurs at the apical surface via Na\(^{+}\)-dependent cotransporters such as those carrying glucose (14) and amino acids (21) in series with the basolaterally located Na\(^{+}\)-K\(^{+}\) pump, an arrangement similar to that found in the intestine, kidney, and liver (32, 39, 44). Furthermore, nonselective cation channels were identified in whole cell patch clamping of freshly isolated conjunctival epithelial cells (42), and the possibility that such channels reside at the apical surface has been suggested (40).

Because of its frailty and the difficulty of the dissection needed to isolate the conjunctival epithelium intact, the above macroscopic electrolyte transport properties of this tissue, as measured in bicameral Ussing-type chambers, was characterized relatively recently (20). Hence, many fundamental aspects of the tissue have not been elucidated. The underlying rationale for characterizing conjunctival transport is a quest for elaborating the secretory functions of the epithelium

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under the premise that such efforts might have utility in ameliorating complications from dry-eye diseases. The surface area of the conjunctival epithelium is 9-fold larger in the rabbit and 17-fold larger in the human than that of the cornea (43). Thus, hypothetically, active transport by the conjunctiva with accompanying fluid secretion may contribute to a significant fraction of tear production, which is normally provided in healthy individuals by the lacrimal gland. On stimulation, the transepithelial conjunctival contribution could be greater.

Given that serotonin [5-hydroxytryptamine (5-HT)] is widely recognized as a Cl⁻ secretagogue in epithelial tissues (4, 7, 5, 12, 16, 19, 35–37, 49), it was posited a priori that 5-HT might serve as a stimulator of conjunctival Cl⁻ transport as well. The presence of mast cells within the conjunctival stroma (26) and the likelihood that these cells may release 5-HT, as well as the facts that trace amounts of 5-HT are found in tears (25, 46) and that 5-HT stimulates mucin secretion from goblet cells within the epithelium (17), suggested that the epithelium could harbor serotonergic receptors potentially linked to the regulation of transepithelial transport. However, it was unexpectedly found that 5-HT is an inhibitor of the Cl⁻-dependent short-circuit current (Isc) of the rabbit conjunctiva by a mechanism that requires further clarification.

METHODS

Adult albino rabbits of either sex weighing 2–3.6 kg were killed by CO₂ asphyxiation. The bulbar-palpebral conjunctiva was dissected as a cylinder and cut longitudinally to a parti- tiva was dissected as a cylinder and cut longitudinally to facilitate mounting as a preparation. The hemichambers included the necessary arrangements for electrical determinations and vigorous stirring. The transepithelial potential difference was short-circuited, with the current needed to maintain 0 mV across the tissue (the Isc) continuously recorded. Transmural electrical resistance (Rt) was determined by measuring the amount of current necessary to offset the short-circuited condition by 2 or 3 mV for a few seconds.

In general, it was observed that conjunctival preparations from heavier rabbits (≥3 kg) were less delicate and easier to handle and place in the chambers, given the larger areas of tissue that could be readily procured from such animals. The frailty of the preparation, however, appeared to contribute to a spontaneous, gradual decline in Rt that was commonly observed after a prolonged period in the chamber (10–30% reduction in Rt in control preparations during 3–4 h of observation). This decline is best explained as a loss in paracellular resistance, since it occurred in the presence of a steady Isc (unpublished data). Under the short-circuited conditions, increases in paracellular ion movement do not result in a net flow across this pathway, given the absence of a potential difference across the epithelium and identical electrolyte concentrations on each side of the preparation. Thus, although the Isc measured net transcellular flow in experiments with symmetrical solutions, conjunctival Rt changes elicited by the additions of various agents frequently underestimate changes in membrane resistance; i.e., because of the proportionally larger transcellular than paracellular resistance, large changes in transepithelial resistance are seen as smaller changes when Rt is measured. Nevertheless, unless indicated otherwise, the Rt changes given, although proportionally small in some sets of experiments, were statistically significant as paired data (P < 0.05) and reflect the ΔRt values elicited by experimental agents at the point of their introduction. Most illustrations of the electrical changes were acquired by scanning the chart recordings with a page scanner so that the background chart grids could be removed with commercial software.

The bathing medium used during the dissection and bathing of the tissue in the chambers in most experiments was a modified Tyrode solution with the following composition (in mM): 1.8 calcium gluconate, 1.2 MgCl₂, 4 KCl, 103 NaCl, 30 NaHCO₃, 1 NaH₂PO₄, 5.7 glucose, 0.3 glutathione, and 10 sucrose. The pH of this solution when bubbled with 5% CO₂-95% air was 7.5. It measured 280 mosmol/kgH₂O.

In some experiments, glucanone was used as a Cl⁻ substitute along with MgSO₄ replacing MgCl₂. For an Na⁺-free medium, the sodium salts of HCl and H₃PO₄ were replaced by their respective N-methyl-d-glucamine (NMDG) salts and choline was used as the counter ion for bicarbonate. In experiments measuring K⁺ diffusion, a Cl⁻-free, high-K⁺ solution with low Na⁺ was used to bathe the apical aspect of the conjunctiva, whereas the stromal side was bathed with a Cl⁻-free medium containing 4 mM K⁺ and low Na⁺. For the apical bath, sodium gluconate of the Cl⁻-free solution was replaced by potassium gluconate with the remaining components unaltered; for the stromal bath, the sodium gluconate was replaced by 103 mM NMDG plus 103 mM methanesulfonic acid.

The isoquinolinesulfonamide H-89 was purchased from Calbiochem (La Jolla, CA) and stored at 5°C in aqueous solution (10 mM). All other chemicals were from Sigma Chemical (St. Louis, MO) and its Research Biochemicals affiliate. Solutions of bumetanide (20 mM) and A-23187 (5 mM) were prepared with ethanol (EtOH) and stored at 5°C. Also stored at refrigerator temperature as 10 mM stock solutions were forskolin, IBMX, rolipram, and stau- rosporine, all in DMSO, as well as aqueous preparations of MDL-12330A hydrochloride, ouabain octahydrate, and amphotericin B. Thapsigargin and U-73122 were stored at −20°C as 1 mM solutions in DMSO. All such stocks were used within 3 mo. Maleate salts of 5-HT and methyl analogs, as well as buspirone HCl, 8-hydroxydipropylamino- notetralin HBr (8-OH DPAT), isoproterenol HCl, propranolol HCl, epinephrine bitartrate, and dibutylryl cAMP (DPB) were dissolved in water (10 mM) immediately before dilution into the hemichambers. The serotoninergic antagonists WAY-100635 maleate and 4-[2’-(methoxyphen- yl)1-[(2’-N-(2’-pyridinyl)p-fluorobenzamido]ethyl]-piperazine (p-MPPF) dihydrochloride were prepared as aqueous 10 mM solutions, while such concentrations of ketanserin tartrate and spiroxatrine were used in DMSO. Spiroperone HCl was stored at 2 mM in EtOH. All stocks of the antagonists were consumed within 2–3 days. Acetazolamide and amiloride were prepared as suspensions in amounts equivalent to 10 mM (in EtOH) and 100 mM (in H₂O), respectively. The latter readily dissolved on warming (50°C) before dilution. In experiments involving multiple drug treatments, the vehicle concentrations in the chamber did not exceed 1% for EtOH and 0.1% for DMSO, levels not found to affect the control electrical parameters.
Fig. 1. Response of the transconjunctival electrical parameters to sequential additions of serotonin [5-hydroxytryptamine (5-HT)] to the apical-side bathing medium. Each point represents the succeeding change in the respective measurements [short-circuit current (I_{sc})] and transepithelial resistance (R_{t}, ■) at the doses indicated. Control values were 15.3 ± 1.9 μA/cm² for I_{sc} and 1.43 ± 0.38 kΩ·cm² for R_{t} (n = 6 epithelia).

Fig. 2. Effects of 5-HT on the transconjunctival electrical parameters when introduced to preparations bathed by media of various compositions. Photocopies of continuous recordings of the I_{sc} are shown. Upward deflections are the points at which R_{t} was recorded. All agents were present in the bathing media from the points indicated. Trace A: 5-HT was diluted into the apical solution of rolipram-prestimulated preparations bathed in the absence of Cl⁻ (n = 6). Trace B: conjunctivas were bathed by a medium containing 107 mM K⁺ and 30 mM Na⁺ in the apical-side hemichamber and by a 4 mM K⁺ solution with identical Na⁺ concentration on the stromal side; under these conditions, the I_{sc} is a measurement of transepithelial K⁺ diffusion. Trace C: apical medium lacked Na⁺, and the stromal bath comprised a complete physiological solution; inhibitors of carbonic anhydrase and Na⁺/H⁺ exchange were added before serotonin. Trace D: as in trace C, Na⁺ was excluded from the apical bath.
RESULTS

Previous characterizations of isolated rabbit conjunctival epithelia in a bicameral Ussing-type arrangement have demonstrated the coexistence of transport activities that simultaneously mediate Na+ absorption and Cl− secretion. The relative proportions of these oppositely directed functions vary considerably from one individual preparation to another for reasons that are unknown, but in general, Cl− transport dominates and represents on average ~60% of the Isc (33). Given the possibility that 5-HT could serve as a mediator of either (or both) of these transport processes, initial experiments determined the effects of the agonist on the control transepithelial electrical parameters, a topic that had not heretofore been examined.

Apical additions of 5-HT elicited prompt Isc reductions and increases in Rt, changes that occurred in a dose-dependent manner with a calculated EC50 of 7.9 nM (Fig. 1). Because maximal effects were obtained at 10 μM, this concentration was used in all subsequent experiments. Additional observations indicated that applying the agonist to the stromal-side bathing solution resulted in slower and more variable responses than those obtained apically (not shown). Presumably, 5-HT does not readily traverse the stroma; it either reaches receptors on lateral membranes when applied from the apical direction, and/or its receptors indeed reside at the apical surface. Virtually identical phenomena were reported earlier in the case of epinephrine (33). Nevertheless, all data were acquired from additions of various agents to the apical bathing medium.

Characterization of the 5-HT effect on transepithelial Isc by electrolyte substitutions and transport inhibitors. The 5-HT-evoked electrical effects most likely resulted from reductions in conductances across apical Na+ and/or Cl− channels or basolateral K+ channels, given the measured increase in Rt. In addition, Isc reductions could also have been due to inhibitions of conjunctival transporters known to maintain intracellular K+ and Cl− above equilibrium, i.e., the Na+/K+ pump, the Na+/K+/2Cl− cotransporter, and the coupled activities of the Na+/H+ and Cl−/HCO3− exchangers. To discern the transport element(s) affected, three distinct experimental approaches were implemented. One quite simply entailed the bilateral exclusion of Cl− from the bathing solutions (gluconate substitution). Under these conditions, the Isc solely reflects the Na+ absorptive component of the transcellular current, which can be stimulated by cAMP-elevating maneuvers as a result of an increase in basolateral K+ conductance(s) (40). With Cl− absent, introduction of 5-HT reduced the Isc by 0.5 ± 0.2 (SE) μA/cm2 (an 11% decline from 4.7 ± 0.3 to 4.2 ± 0.5 μA/cm2, n = 5), saliently less than the inhibitions obtained with complete media (ΔIsc = −3.3 ± 0.5 μA/cm2, a 23% change, n = 21). The limited Isc reduction recorded in the absence of Cl− was accompanied by a statistically significant Rt increase of 0.08 ± 0.02 kOhm·cm2 (a 4% rise from 1.90 ± 0.23 to 1.98 ± 0.26 kOhm·cm2), suggesting finite contributions by other conductive pathways to the 5-HT response as well, with K+ channels the most likely participants.

To examine the latter, basolateral K+ conductance(s) was activated by the introduction of rolipram, a phosphodiesterase (PDE) inhibitor, under Cl−-free conditions (Fig. 2A). This maneuver increased the Isc (from 6.4 ± 0.9 μA/cm2 to a plateau level of 10.0 ± 1.0 μA/cm2 (n = 6), a change comparable to earlier results obtained with this agent in the absence of Cl− (40). Subsequent additions of 5-HT elicited prompt reductions in the stimulated current (ΔIsc = −2.1 ± 0.4 μA/cm2, n = 6) that were accompanied by an 8% increase in Rt (from 0.97 ± 0.39 to 1.05 ± 0.44 kOhm·cm2, n = 6). Consistent with a 5-HT-evoked inhibition of K+ conductance(s), succeeding introduction of the relatively nonselective K+ channel blocker Ba2+ did not produce salient effects (Fig. 2A). When the order of 5-HT and Ba2+ was reversed after rolipram stimulation, an effect by 5-HT in the presence of K+ channel blockade was barely discernable and insignificant (Table 1).

In contrast, the introduction of 5-HT to conjunctivae preexposed to rolipram plus Ba2+ under Cl−-rich conditions elicited pronounced Isc reductions (Table 1), suggesting that 5-HT also evokes an inhibition of apical Cl− conductance(s) in addition to such effect on basolateral K+ conductance(s).

To further examine the prospect of K+ channel involvement in the 5-HT response, a second experimen-

<table>
<thead>
<tr>
<th>n</th>
<th>Control Baseline</th>
<th>Rolipram (10 μM)</th>
<th>BaCl2 (5 mM)</th>
<th>5-HT (10 μM)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Isc</td>
<td>Rt</td>
<td>Isc</td>
<td>Rt</td>
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<tr>
<td>-----</td>
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</tr>
<tr>
<td>4</td>
<td>6.3 ± 0.8</td>
<td>1.26 ± 0.37</td>
<td>9.8 ± 0.9</td>
<td>1.07 ± 0.34</td>
</tr>
<tr>
<td>5</td>
<td>21.9 ± 1.9</td>
<td>0.90 ± 0.13</td>
<td>30.6 ± 0.9</td>
<td>0.72 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of conjunctivae assayed. Short-circuit currents (Isc) are expressed as μA/cm2 and resistances (Rt) as kOhm·cm2. Except for absence of an effect by serotonin (5-hydroxytryptamine (5-HT)) under Cl−-free conditions, all other changes are significant as paired data, P < 0.05.
tal approach (Fig. 2B), used earlier with the cornea (47) and conjunctiva (40) for demonstrating gating of basolateral K⁺ conductance(s) by Cl⁻ secretagogues, was employed. This involved bathing of the apical surface with a Cl⁻-free, high-K⁺ solution (107 mM) with Na⁺ limited to 30 mM (supplied with HCO₃⁻ as counter ion). The basolateral bath (also Cl⁻-free) contained typical physiological K⁺ concentration (i.e., 4 mM), with 30 mM NaHCO₃ the only source of Na⁺. Under these conditions, numerous empirical observations had earlier determined that the Na⁺-K⁺ pump is quiescent, as evidenced by the absence of an effect by ouabain, and the Iₘ reflects the diffusion of K⁺ from the apical to basolateral baths across transcellular and paracellular pathways. The apical introduction of amphotericin B eliminated a restriction to transcellular K⁺ pathways. The apical introduction of amphotericin B eliminated a restriction to transcellular K⁺ pathways. The apical introduction of amphotericin B eliminated a restriction to transcellular K⁺ pathways.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>n</th>
<th>Mean ± SE</th>
<th>%Change</th>
<th>Mean ± SE</th>
<th>%Change</th>
<th>Concentration Applied, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT</td>
<td>21</td>
<td>-3.3 ± 0.5</td>
<td>-23.2</td>
<td>0.14 ± 0.02</td>
<td>15.7</td>
<td>10</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>13</td>
<td>-4.7 ± 1.4</td>
<td>-27.6</td>
<td>0.16 ± 0.05</td>
<td>13.9</td>
<td>10</td>
</tr>
<tr>
<td>α-CH₃-5-HT</td>
<td>4</td>
<td>-4.0 ± 1.7</td>
<td>-24.9</td>
<td>0.11 ± 0.04</td>
<td>8.4</td>
<td>10</td>
</tr>
<tr>
<td>2-CH₃-5-HT</td>
<td>4</td>
<td>-1.7 ± 0.3</td>
<td>-12.8</td>
<td>0.07 ± 0.02</td>
<td>6.5</td>
<td>10</td>
</tr>
<tr>
<td>Buspirone</td>
<td>4</td>
<td>-2.6 ± 0.9</td>
<td>-15.3</td>
<td>0.06 ± 0.02</td>
<td>6.9</td>
<td>100</td>
</tr>
</tbody>
</table>

All changes are significantly different from zero as paired data (P < 0.05); n, number of freshly isolated conjunctivas to which the indicated compound was applied as an initial test substance.
It was further observed that the presence of the serotonergic agents did not affect the succeeding response of the system to the nonselective adrenergic agonist epinephrine (Fig. 3).

Absence of an attenuation of the 5-HT-evoked electrical effects by preexposure to various serotonergic antagonists. Receptor antagonists were surveyed to determine whether pretreatments with such agents could diminish the extent of the 5-HT-elicited $I_{sc}$ inhibitions (Table 3). None selected exhibited this property. Conversely, in the cases of ketanserin and spiperone, preexposure to these antagonists enhanced the 5-HT effect significantly as unpaired data. All the antagonists that were tested have reported activities against members of the 5-HT$_1$ receptor family (6, 15, 48), although ketanserin and spiperone have markedly lower binding affinities than the others and are generally regarded as more selective for 5-HT$_2$A receptors (15, 48).

Direct effects of various serotonergic antagonists on the control transepithelial electrical parameters. WAY-100635, regarded as a highly selective antagonist for 5-HT$_{1A}$ receptors (9, 15, 29), did not evoke significant electrical effects when applied under control conditions (Table 4). Spiperone elicited mixed results, with an $I_{sc}$ increase of 1 μA/cm$^2$ obtained in one experiment and
decrease of 1.6 μA/cm² in another; with five additional preparations, this agent did not generate remarkable $I_{sc}$ changes (i.e., $\Delta I_{sc} < 0.4 \mu A/cm^2$). In contrast, ketanserin and spiroxatrine produced statistically significant $I_{sc}$ rises. Conversely, p-MPPF, an agent shown to antagonize 5-HT$_1A$ receptors in some systems (23), elicited clear $I_{sc}$ decreases in the conjunctiva that were similar in magnitude to those evoked by 5-HT (Table 4), thereby suggesting that p-MPPF interacts with conjunctival receptors as if it were a serotonergic agonist. However, the addition of 5-HT after p-MPPF led to a further average $I_{sc}$ decrease of 3.4 μA/cm² in two cases in which this condition was attempted, a rather typical 5-HT effect that could indicate that these agents bind to distinct receptors. In two other experiments, ketanserin was applied after p-MPPF; this maneuver fully reverted the initial p-MPPF-elicited $I_{sc}$ decrease.

Ca$^{2+}$ independence of 5-HT-evoked electrical changes. The Cl$^-$ conductance(s) of the conjunctival epithelium can be increased by elevations of the intracellular concentrations of Ca$^{2+}$ or cAMP (22, 33, 34). Cellular levels of the former were augmented in two ways: 1) permeabilizing the apical surface to the divalent cation with the ionophore A-23187 and 2) inhibiting the Ca$^{2+}$-ATPase of the intracellular storage compartments with thapsigargin. Neither treatment averted the typical effects of 5-HT on the transepithelial electrical parameters (Fig. 5 illustrates use of the latter agent). Given that these maneuvers perturb Ca$^{2+}$ homeostasis irreversibly, the 5-HT-evoked $I_{sc}$ inhibitions presumably do not result from an increase in extrusion or sequestration of the cation. Furthermore, it is also unlikely that a Ca$^{2+}$ signal downstream from a G protein-linked 5-HT receptor is involved in the transduction mechanism.

Differential effects of cAMP-elevating agents on 5-HT-elicited electrical changes. Conjunctival epithelia were preconditioned by three experimental procedures known to increase cellular cAMP concentration: 1) exposure to the $\beta_2$-selective agonist isoproterenol, 2) addition of DBcAMP, a cell-permeable form of the nucleotide, to the apical bath in combination with rolipram, a PDE inhibitor specific for cAMP-PDE IV (30), and 3) application of forskolin, a direct stimulator of adenylyl cyclase (Fig. 6). Use of the former condition significantly attenuated ($P < 0.05$, as unpaired data) the typical 5-HT-evoked $I_{sc}$ reduction normally recorded under control conditions (Fig. 6, trace C). In contrast, 5-HT-elicited electrical responses were not affected when 5-HT was applied to conjunctival currents stimulated by the other two approaches (Fig. 6, traces A and B). This phenomenon might be explained if one as-

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Table 3. Effect of pretreatments with various serotonergic antagonists on response of electrical parameters to 10 μM 5-HT

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>$\Delta I_{sc}$, μA/cm²</th>
<th>%Change</th>
<th>$\Delta R_{m}$, kΩ-cm²</th>
<th>%Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 5-HT response</td>
<td>21</td>
<td>-3.3 ± 0.5</td>
<td>-23.2</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>5-HT effect after exposure to</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ketanserin</td>
<td>7</td>
<td>-5.9 ± 1.6</td>
<td>-34.7*</td>
<td>0.22 ± 0.12</td>
</tr>
<tr>
<td>Spiperone</td>
<td>7</td>
<td>-5.6 ± 0.8</td>
<td>-40.8*</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Spiroxatrine</td>
<td>4</td>
<td>-10.0 ± 2.7</td>
<td>-30.0</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>WAY-100635</td>
<td>4</td>
<td>-3.2 ± 0.5</td>
<td>-17.7</td>
<td>0.13 ± 0.02</td>
</tr>
<tr>
<td>p-MPPF</td>
<td>2</td>
<td>-3.3 ± 1.8</td>
<td>-42.6</td>
<td>0.11 ± 0.03</td>
</tr>
</tbody>
</table>

All antagonists were present at 10 μM before introduction of 10 μM 5-HT. *5-HT effect on $I_{sc}$ is significantly increased in the presence of antagonist with $P < 0.05$ as unpaired data.

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Fig. 4. Representative trace of the effect of 5-HT addition on the tranconjunctival $I_{sc}$ in the presence of a commonly used antagonist. The presence of serotoninics did not affect the response of the conjunctiva to $\beta_2$-selective agents. MDL-12330A, an adenylyl cyclase inhibitor, was added before ouabain.
sumes that forskolin increased cAMP to levels beyond which a 5-HT-elicited inhibition could be effective. Other than forskolin, the only additional manipulation found to mitigate the 5-HT effect was pretreatment of the tissue with H-89 (Fig. 7), a kinase inhibitor with a high selectivity for protein kinase A (PKA) (13). Introduction of this agent as the initial test compound under control, Cl−-rich conditions reduced the Isc from 12.5 ± 1.9 to 8.3 ± 1.3 μA/cm² (a 34% decline) and increased Rt from 0.78 ± 0.18 to 0.86 ± 0.22 kΩ·cm² (a 10% rise, n = 5). When added to conjunctivae pretreated by cAMP-elevating maneuvers, the current inhibitions produced on H-89 addition were more pronounced (ΔIsc = −12.8 ± 1.7, a 44% change, n = 4; Fig. 7), an observation also made under Cl−-free conditions (40). Because H-89 reduced apical Cl− and basolateral K+ conductances (40) before 5-HT introduction, the mitigation of the 5-HT effect is merely consistent with the prospect that conjunctival 5-HT receptors are negatively linked to such PKA-gated conductances. It clearly does not demonstrate that activated 5-HT receptors lower cAMP concentration or close channels by an undefined mechanism, but PKA involvement appears a viable possibility.

Table 5 summarizes the changes in the electrical parameters produced by 5-HT when added after various agents known to affect signaling transduction. Yet to be mentioned compounds that were not effective on 5-HT-evoked electrical changes were the protein kinase C inhibitor staurosporine, the phospholipase C inhibitor U-73122, and the adenylyl cyclase inhibitor MDL-12330A. Although the latter decreased the Isc from 21.6 ± 2.7 to 17.1 ± 2.3 μA/cm² (n = 7) when it was applied as the initial test compound, succeeding 5-HT effects were not inhibited and were actually found to be enhanced statistically (Table 5) for reasons that are unclear. Presumably, MDL-12330A served as an inefficient cyclase inhibitor, as suggested by the fact that the subsequent addition of rolipram to MDL-12330A-pretreated preparations increased the Isc, thereby indicating the existence of a residual rate of cAMP generation (data not shown).

DISCUSSION

5-HT, a ubiquitous monoamine with roles in neurotransmission, paracrine signaling, and inflammatory responses, is widely recognized as a fluid and electrolyte secretagogue in epithelia. Throughout the gastrointestinal tract, as well as in airway epithelia, 5-HT promotes secretory activities by stimulating transcellular Cl− (or HCO3−) movements directly or by inhibiting absorptive processes such as neutral transapical Na+ and Cl− uptake or amiloride-sensitive Na+ con-
ductance (4, 7, 8, 12, 16, 24, 35–37, 49). The exact nature of the 5-HT receptors, signaling transduction mechanisms, and affected electrolyte transporters involved in 5-HT-elicited stimulation of net secretory processes differs depending on the species and anatomic region characterized. Among ocular tissues, 5-HT is a known stimulator of electrogenic Cl\(_2\) transport in the rabbit corneal epithelium (19). Thus the present results suggest that the rabbit conjunctiva may be unique among Cl\(_2\)-secreting and Na\(^+\)-absorbing epithelia, in that the indole elicited prompt and sustained inhibitions of transcellular Cl\(_2\) movement in the basolateral-to-apical direction because of the independent downregulation of apical Cl\(_2\) and basolateral K\(^+\) conductances.

The observed 5-HT-evoked \(I_{sc}\) reductions are likely manifestations of a downregulation in apical Cl\(^-\) conductance(s), as evidenced by 1) the marked 5-HT effect under conditions in which the apical bath lacked Na\(^+\), 2) such effect in the presence of bumetanide under conditions in which transcellular Cl\(^-\) movement is presumably mediated by the parallel activities of basolaterally located Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)^- exchangers (41), 3) a similar inhibition of the Cl\(^-\)-dependent \(I_{sc}\) after complete inhibition of the Na\(^+\)/H\(^+\) exchanger with amiloride, which in turn inhibits Cl\(^-\)/HCO\(_3\)^- exchange activity because of cell acidification, and 4) the extensive mitigation of 5-HT-elicited \(I_{sc}\) reductions with Cl\(^-\)-free media as well as with the PKA inhibitor H-89. Earlier studies on the effects of cAMP elevation (22) suggested that apical Cl\(^-\) conductance(s) in the conjunctiva is gated by PKA. A subset of conjunctival basolateral K\(^+\) conductance(s) is also modulated by PKA (40), and
the present study obtained evidence for an effect by 5-HT on K⁺ channels as well. The inhibitions of the Cl⁻-dependent \(I_{sc}\) could have solely arisen from a 5-HT-induced reduction in the driving force for apical Cl⁻ secretion secondary to K⁺ channel closure. However, the fact that an effect by 5-HT after K⁺ channel blockade was not observed in the absence of Cl⁻ but was indeed obtained with the anion present (Table 1) further supports the likelihood that 5-HT also directly regulates apical Cl⁻ channels in addition to basolateral K⁺ channels.

Given the Ca²⁺ independence of the 5-HT-elicited electrical effects and the fact that Ca²⁺ and cAMP are the intracellular messengers most commonly found to be involved in the gating of Cl⁻ and K⁺ channels, the present results are most plausibly explained as a consequence of a 5-HT-evoked reduction in cAMP concentration. If this is so, the conjunctiva expresses 5-HT receptors of the type 1 family, which are negatively linked to adenylyl cyclase activity.

Seven families of 5-HT receptors are recognized, with ≥15 subtypes identified (11). Furthermore, such subtypes can in some cases exist in several isoforms due to RNA editing or alternative splicing (18). One consequence of the extensive diversity of 5-HT receptors is that the degree of selectivity of ligands regarded as specific for a particular subtype appears to become questionable due to their newly realized affinities for recently discovered receptors. As such, a pharmacological characterization of the putative 5-HT receptors present in the conjunctiva based on functional criteria (i.e., agonist and antagonist rank order in affecting the electrical parameters) would not be efficient and was not attempted. Further studies in this regard using radioligand binding assays on membrane fractions to determine apparent affinity values of various ligands would be more appropriate (6). Measurements of second messengers, e.g., cAMP, and/or G protein activation assays (29, 38) may be similarly useful but are beyond the scope of this electrophysiological study.

The ligands that were screened in the present study were arbitrarily chosen from among several possibilities, but with the presumption in mind that the conjunctiva might harbor 5-HT₁A receptors. Such receptors had been identified in the ocular ciliary epithelium (2) and in cultured retinal pigmented epithelial cells

### Table 5. Summary of effects of 5-HT on transepithelial electrical parameters of rabbit conjunctiva after pretreatments with agents affecting signaling transduction

<table>
<thead>
<tr>
<th></th>
<th>(I_{sc}), μA/cm²</th>
<th>(R_t), kΩ·cm²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>Mean ± SE</td>
</tr>
<tr>
<td>Control response to 5-HT</td>
<td>21</td>
<td>–3.3 ± 0.5</td>
</tr>
<tr>
<td>5-HT effect after exposure to</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forskolin</td>
<td>8</td>
<td>–1.0 ± 0.3</td>
</tr>
<tr>
<td>H-89</td>
<td>5</td>
<td>–0.8 ± 0.2</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>4</td>
<td>–5.5 ± 1.4</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>6</td>
<td>–2.5 ± 0.6</td>
</tr>
<tr>
<td>U-73122</td>
<td>2</td>
<td>–7.6 ± 1.1</td>
</tr>
<tr>
<td>MDL-12330A</td>
<td>7</td>
<td>–7.6 ± 1.0</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>7</td>
<td>–5.7 ± 0.8</td>
</tr>
<tr>
<td>DBcAMP + rolipram</td>
<td>7</td>
<td>–5.2 ± 1.2</td>
</tr>
<tr>
<td>A-23187</td>
<td>7</td>
<td>–7.1 ± 1.9</td>
</tr>
</tbody>
</table>

DBcAMP, dibutyryl-cAMP. *Delta and percent changes are significantly reduced in presence of agent \(P < 0.05\) as unpaired data. †Delta and percent change are significantly increased \(P < 0.05\) as unpaired data. ‡\(n\) value insufficient for unpaired comparison.
(28). It was posited a priori, for example, that WAY-
100635, given its high selectivity for this subtype (9), might attenuate the 5-HT-elicted current reduction and that ketanserin would be ineffective. However, none of the antagonists selected affected the evoked 5-HT effect, and all the agonists tested inhibited the $I_{sc}$ and increased $R_q$.

It is possible that the lack of a mitigation in the 5-HT effect subsequent to antagonist preexposure may have been due to the use of a supramaximal dose of 5-HT. However, pretreatment with an antagonist at the same dose as sequentially applied 5-HT should have resulted in a measurable attenuation in the 5-HT response if both agents were acting on the same site. Either 5-HT completely displaced the antagonist, or discrete receptors exist. Nevertheless, applying 5-HT at lower doses would result in smaller current changes and greater uncertainty (witness the magnitude of the standard errors obtained).

The $I_{sc}$ stimulations produced by the antagonists ketanserin, spiroxatrine, and, in one preparation, spiperone, suggest that these agents may be operating as “inverse” agonists (29). This phenomenon has been attributed to an inhibition of “constitutive” coupling between a receptor and a G protein. Alternatively, but perhaps less likely, the antagonists displaced endogenous 5-HT present at the receptors.

The notion that the conjunctival epithelium might express 5-HT$_1$ receptors rests on the assumption that reductions in cAMP levels are the most cogent manner in which K$^+$ and Cl$^-$ conductances could be downregulated. Hypothetically, one could also posit that phosphatase activity could have been augmented. Nevertheless, the present data uncovered the paradox that although 5-HT-evoked $I_{sc}$ reductions were not affected by pretreatments with isoproterenol or the combination of DBcAMP and rolipram, preexposure to forskolin significantly attenuated the 5-HT effect. These results illustrate complexities commonly encountered in functional measurements and are reminiscent of a phenomenon described with cultured human retinal pigmented epithelial cells (28), in which, conversely, 5-HT reduced cAMP levels stimulated by forskolin but not by isoproterenol or adenosine.

In regard to the present results with the conjunctiva, forskolin may have increased cAMP levels far beyond what is needed to fully stimulate epithelial conductances. Any putative reduction in cAMP concentration by a 5-HT-triggered mechanism could bring levels down to values still above a saturation level. This may not be the case for the other cAMP-elevating agents used in this study.

More problematic is the fact that a physiological role for the apparent 5-HT receptors that were discerned by this study is not clearly evident. Nor is the location of the disclosed receptor(s), i.e., apical vs. basolateral aspects of the epithelium, definitively stated from the experiments presented because of the limitations of the preparation. An apical presence would imply that such receptors are activated by changes in 5-HT levels in tears (25). Conversely, a basolateral location might suggest that 5-HT, possibly released by stromal mast cells, could serve as an inflammatory mediator under conditions such as allergic conjunctivitis and influence the secretory activity of the epithelium. This possibility is not inherently intuitive, given that mast cells also liberate histamine and prostaglandins, agents that might be expected to stimulate Cl$^-$ transport.

The central role of conjunctival goblet cells is to produce the mucus layer of the tear film, which protects the corneal and conjunctival epithelia from various environmental insults. However, in this case as well, there does not necessarily appear to be a connection between 5-HT-elicted goblet cell mucin secretion (17) and transepithelial Cl$^-$ transport. ACh also stimulates mucin secretion (31) without having an effect on the transepithelial electrical parameters (unpublished observations).

Briefly, a novel inhibitory effect by 5-HT on the Cl$^-$ secretory activity of the rabbit conjunctival epithelium was evinced, apparently rendering to this tissue a unique status among Cl$^-$-secreting epithelia. Further work is needed to characterize this unusual property, given the prospect that this tissue may provide an opportunity to study, via biochemical and molecular approaches, a previously unobserved effect of 5-HT.

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REFERENCES


