Cl⁻ secretory effects of EBIO in the rabbit conjunctival epithelium

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Alvarez, Lawrence J., Aldo C. Zamudio, and Oscar A. Candia. Cl⁻ secretory effects of EBIO in the rabbit conjunctival epithelium. Am J Physiol Cell Physiol 289: C138–C147, 2005. First published February 9, 2005; doi:10.1152/ajpcell.00190.2004.—Experiments were conducted to determine whether the Cl⁻ secretagogue, 1-ethyl-2-benzimidazolinone (EBIO), stimulates Cl⁻ transport in the rabbit conjunctival epithelium. For this study, epithelia were isolated in an Ussing-type chamber under short-circuit conditions. The effects of EBIO on the short-circuit current (Isc) were measured under physiological conditions, as well as in experiments with altered electrolyte concentrations. Addition of 0.5 mM EBIO to the apical bath stimulated the control Isc by 64% and reduced Rl by 21% (P < 0.05; paired data). Under Cl⁻-free conditions, Isc stimulation using EBIO was markedly attenuated. In the presence of an apical-to-basolateral K⁺ gradient and permeabilization of the apical membrane, the majority of the Isc reflected the transcellular movement of K⁺ via basolateral K⁺ channels. Under these conditions, EBIO in combination with A23187 elicited nearly instantaneous 60–90% increases in Isc that were sensitive to the calmodulin antagonist calmidazolium and the K⁺ channel blocker tetraethyl ammonium. In the presence of an apical-to-basolateral Cl⁻ gradient and nystatin permeabilization of the basolateral aspect, EBIO increased the Cl⁻-dependent Isc, an effect prevented by the channel blocker glibenclamide (0.3 mM). The latter compound also was used to determine the proportion of EBIO-evoked unidirectional ³⁶Cl⁻ fluxes in the presence of the Cl⁻ gradient that traversed the epithelium transeccellularly. Overall, EBIO activated apical Cl⁻ channels and basolateral K⁺ channels (presumably those that are Ca²⁺ dependent), thereby suggesting that this compound, or related derivatives, may be suitable as topical agents to stimulate fluid transport across the tissue in individuals with lacrimal gland deficiencies.

Ussing chamber; short-circuit current; electrolyte transport; chloride secretagogue; potassium conductance; 1-ethyl-2-benzimidazolinone; 1,10-phenanthroline

After an early report of the electrolyte permeability of the conjunctiva and its electrogenic properties (21), more recent studies of the isolated rabbit conjunctival epithelium in Ussing-type arrangements have determined its active transport mechanisms (15, 29). It was demonstrated that the epithelium exhibits both Na⁺ absorption and Cl⁻ secretion, with the latter being more dominant. The underlying rationale for characterizing conjunctival transport was to understand the secretory activity of the epithelium under the premise that this knowledge might have utility in ameliorating complications from dry-eye diseases. Active transport by the conjunctiva with accompanying fluid secretion is now recognized as contributing significantly to the production of tears (18).

In an early concept, two types of secretion by the secretory epithelia of the ocular surface and the various orbital glands were proposed: basic secretion and reflex secretion. Basic secretion was regarded as a baseline rate of production, and reflex secretion was considered an increased rate caused by neuronal stimulation of the main lacrimal gland (12, 13). Overall, the conjunctival epithelium has adequate water permeability (2) and the transporters necessary to contribute significant fluid to the tear film (~50 μl·h⁻¹ on the basis of its total surface area) (18, 30). This level of fluid flow is sufficiently large that it may represent most of the baseline tear secretion not attributed to the lacrimal gland as suggested by Li et al. (18).

The major transporters of the rabbit conjunctival epithelium are identical to those in other Cl⁻-secreting epithelia; that is, it has a basolateral bumetanide-sensitive Cl⁻ uptake process (mediated by the Na⁺-K⁺-2Cl⁻ cotransporter, NKCC1) positioned in series with apical Cl⁻ channels, including CFTR (34, 35). In addition, Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers exist in parallel in the basolateral membrane and also can mediate Cl⁻ uptake (33).

Oppositely directed, electrogenic Na⁺ reabsorption is amiloride insensitive (29), indicating the absence of electrogenic Na⁺ channels at the apical surface, and occurs via Na⁺-dependent cotransporters such as those carrying glucose (9) and amino acids (16) in series with the basolaterally located Na⁺-K⁺ pump. Furthermore, nonselective cation channels (NSCC) were identified in whole cell patch clamping of freshly isolated conjunctival epithelial cells (39) and the possibility that such channels reside at the apical surface has been suggested (32). As commonly found among Cl⁻-secreting epithelia (4, 22), apical Cl⁻ and basolateral K⁺ conductances are simultaneously increased by cAMP-elevating agents (32).

Spontaneous fluid transport across the conjunctival epithelium engenders a net flow in the basolateral-to-apical direction (18, 30), a property consistent with the more dominant Cl⁻ secretory activity of the tissue. These studies demonstrated that the measured fluid secretion was dependent on transepithelial electrolyte transport, given its abolition by ouabain, sensitivity to K⁺ channel blockade, and Cl⁻ dependency. In addition, fluid transport was I Na⁺ markedly inhibited in experiments that increased the Na⁺ absorptive activity by raising the glucose concentration (to 25 mM) of the apical bath, an inhibition that did not occur with a similar concentration of mannitol (30); and 2 increased (~50–100%) by Cl⁻ secretagogues that included purinergic agonists acting via P2Y₂ receptors (17, 18, 30). Furthermore, on the basis of the stimulatory effects of purinergics on the short-circuited epithelium, these studies also found that the increase in the transepithelial transport current correlated with the increase in fluid transport. This latter observation implied that P2Y₂ receptor activation leads to a selective stimulation of only the Cl⁻ secretory activity of the epithelium (17, 30).

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However, as observed with other Cl⁻-secreting epithelia (19), the reported stimulation of Cl⁻ secretion via purinergic receptors appears to be transitory. This led us to pose the question whether so-called Cl⁻ channel openers might prove useful in the conjunctival epithelium. To address this prospect, in the present study, we have examined the effects of 1-ethyl-2-benzimidazolone (EBIO), an agent previously shown to be an effective Cl⁻ secretagogue because of its activation of membrane channels (27, 31). The data reported herein, garnered from experiments with isolated rabbit conjunctivae in Ussing-type chambers under various conditions, demonstrate the effectiveness of the compound in activating conductances in the apical and basolateral aspects for Cl⁻ and K⁺, respectively. These results should encourage further examination of the effects of this agent on fluid transport, which might be of potential value in developing dry-eye therapies.

METHODS

Adult albino rabbits of either sex weighing between 2 and 3.6 kg were rapidly killed by administering CO₂ asphyxiation, a protocol approved by the Mount Sinai Animal Care and Use Committee. Barbitalures or other agents that might alter general physiological properties were not used. Before the rabbits were killed, their treatment conformed to the American Physiological Society’s “Guiding Principles for the Care and Use of Animals.” The bulbar-palpebral conjunctiva was dissected as a cylinder and cut longitudinally so that it could be converted to a flat epithelium that was mounted as a partition between Ussing-type hemichambers, exposing 0.38 cm² of cross-sectional area. The dissection procedure was presented in detail previously (29) and was based on one described by Kompella et al. (15).

The hemichambers included the necessary arrangements for electrical determinations and vigorous stirring. The transconjunctival potential difference (PD) was short-circuited, with the current needed to maintain 0 mV across the tissue (Isc) being continuously recorded (26). Transmural electrical resistance (Rt) was determined by measuring the amount of current necessary to offset the short-circuited condition by 3 mV for a few seconds.

In general, we observed that conjunctival preparations from heavier rabbits (at least 3 kg) were less delicate and easier to handle and place in the chambers, given the larger areas of tissue that could readily be procured from such animals. The frailty of the preparation, however, appeared to contribute to a spontaneous, gradual decline in procured from such animals. The frailty of the preparation, however, appeared to contribute to a spontaneous, gradual decline in

The medium used during the dissection and bathing of the tissue in the chambers in most experiments was a modified Tyrode solution composed of (in mM) 1.8 Ca²⁺ 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/kg H₂O.

In some experiments (see Fig. 3), gluconate was used as a Cl⁻ substitute along with MgSO₄ as a replacement for MgCl₂. In others in which transepithelial Cl⁻ gradients were established (see Figs. 5 and 6), the Cl⁻-rich side of the preparation contained 103 mM KCl in lieu of NaCl, with all other components unaltered, while SO₄²⁻ salts were used to replace Cl⁻ in the opposite-side bath, with the osmolality being maintained with sucrose.

For experiments measuring K⁺ diffusion (see Fig. 4), the apical bath contained a Cl⁻-free, high-K⁺ solution (107 mM supplied as a gluconate salt) with Na⁺ concentration ([Na⁺]) limited to 30 mM (with HCO₃⁻ used as counterion). The stromal side solution (also Cl⁻ free) contained 4 mM K⁺ gluconate, with 30 NaHCO₃ being the only source of Na⁺, as well as 103 mM N-methyl-d-glucamine plus 103 mM methanesulfonic acid.

Sulfate also was used instead of gluconate as a Cl⁻ substitute in a set of experiments to assess whether the latter affected the preparation as a Ca²⁺ chelator.

To measure unidirectional Cl⁻ fluxes, 5 µCi of ³⁶Cl⁻ was added to one chamber compartment (final concentration ~0.42 µCi·m⁻²), and periodic samples were obtained from the opposite compartment. The specific activity of the labeled solution remained constant throughout the experiment, and the activity of the opposite solution was always ~0.001 of the labeled side. Two-milliliter samples were taken every 15 min from the unlabeled side, the volume of which was kept constant by immediate addition of fresh medium. Twenty-five-microliter samples were taken from the labeled side and diluted up to 2 ml with bathing medium to determine the specific activity of the labeled solution. The samples were counted with a Wallac scintillation counter.

Chemicals. H³⁶Cl⁻ was obtained from PerkinElmer Life Sciences (Boston, MA). EBIO was purchased from Tocris Cookson (Ballwin, MO), stored at 5°C as a 1 M stock in dimethyl sulfoxide (DMSO), and consumed within 2 wk. Calbiochem (La Jolla, CA) was the source of calmidazolium, which was prepared as a 10 mM stock in DMSO, as well as the supplier of the isoquinolinesulfonamide H-89, which was stored at 5°C in aqueous solution (10 mM). All other chemicals were purchased from Sigma (St. Louis, MO). Agents solubilized in DMSO, stored at 5°C, and used within a few days included forskolin, glibenclamide, and A23187, each in 10 mM stock in DMSO, as well as the supplier of the isouquinolinesulfonamide H-89, which was stored at 5°C in aqueous solution (10 mM). All other chemicals were purchased from Sigma (St. Louis, MO). Agents solubilized in DMSO, stored at 5°C, and used within a few days included forskolin, glibenclamide, and A23187, each in ≈1,000-fold solutions. Also stored at refrigeration temperatures were aqueous solutions of amphotericin B (10 mM) and tetraethyl ammonium (TEA; 1 M). 1,10-Phenanthroline (0.2 M) was solubilized in methanol, kept at 5°C, and applied within a few days. Nystatin and UTP were freshly prepared immediately before dilution into the hemichambers, the latter as an aqueous 10 mM solution and the former as a 40 µg·ml⁻¹ DMSO suspension followed by 30 s of sonication.

RESULTS

Previous characterizations of isolated rabbit conjunctival epithelia in Ussing-type arrangements have demonstrated the coexistence of transport activities that mediate Na⁺ absorption and Cl⁻ secretion simultaneously. The relative proportions of these oppositely directed functions vary considerably from one individual preparation to another, but in general, Cl⁻ transport dominates and represents, on average, ~60% of the Isc (29). Given the possibility that EBIO could affect either or both of these transport processes, initial experiments were performed to determine the effects of the benzimidazolone on the control...
transepithelial electrical parameters, a topic that had not heretofore been examined.

Apical additions of EBIO elicited prompt $I_{sc}$ increases and reductions in $R_t$, changes that occurred in a concentration-dependent manner (Fig. 1). Although 1 mM concentration produced the largest stable change, for the purpose of economy, most experiments in this study restricted applications of EBIO to 0.5 mM. Additional observations indicated that adding this agent to the stromal side bathing solution resulted in slower and more variable responses than those obtained apically (data not shown). Presumably, EBIO does not readily traverse the stroma; it solely affected channels in the apical domain (data not shown). For comparative purposes, the effects of the Cl$^-$ channel opener 1,10-phenanthroline (7) were determined. This compound activates apical Cl$^-$ channels and basolateral K$^+$ channels in Cl$^-$-secreting epithelia (7), a property also ascribed to

An estimated EC$_{50}$ of 0.23 mM was predicted to be the concentration that would increase the $I_{sc}$ by 44% over the control level. In the presence of 2 mM EBIO, $I_{sc}$ was 29.9 ± 3.2 μA·cm$^{-2}$, or 88% over the baseline value, while $R_t$ was reduced to 0.57 ± 0.09 kΩ·cm$^{-2}$, a 33% decline. EBIO also was an effective stimulator when introduced after forskolin, a direct activator of adenylyl cyclase (Fig. 2A). The apical addition of 0.5 mM EBIO under control physiological conditions increased $I_{sc}$ from 21.3 ± 2.1 to 24.8 ± 1.8 μA·cm$^{-2}$ (means ± SE), a 16% stimulation, and reduced $R_t$ from 0.71 ± 0.06 to 0.64 ± 0.05 kΩ·cm$^{-2}$, a 10% decline (n = 6 conjunctivae) ($P < 0.05$; paired data). EBIO also was an effective stimulator when introduced after UTP (Fig. 2B). Yet, the UTP and EBIO prestimulated current could be increased further upon adding forskolin, a direct activator of adenylyl cyclase (Fig. 2B). In the presence of EBIO, the diterpenoid increased the $I_{sc}$ by 7.1 ± 2.8 μA·cm$^{-2}$,
a 29% enhancement, and reduced \( R_t \) by 0.06 ± 0.01 kΩ·cm², an 11% ebbing (\( n = 5 \) conjunctivae) (\( P < 0.05 \); paired data). When forskolin was used as the initial test compound (Fig. 2C), sequential stimulations with EBIO were not obtained and those elicited by UTP were transitory, suggesting that maximal elevation of intracellular cAMP levels may be the most effective approach for stimulating \( I_{sc} \).

**Effects of EBIO in the absence of Cl⁻.** To determine the effects of EBIO on the Na⁺-absorptive properties of the epithelium, conjunctivae were bathed bilaterally under Cl⁻-free conditions (gluconate substitution). In this situation, \( I_{sc} \) is dependent on the presence of Na⁺ in the apical bath (29) and reflects the movement of Na⁺ across the apical face balanced by basolateral K⁺ efflux and the electrogenic Na⁺-K⁺ pump current (32). Under these conditions, the \( I_{sc} \) stimulations evoked by EBIO addition were markedly smaller than those obtained in the presence of the halide (\( P < 0.05 \); unpaired data). This also was the case with preparations pretreated with the Ca²⁺ ionophore A23187 (Fig. 3). The introduction of EBIO increased the A23187-pretreated \( I_{sc} \) from 12.5 ± 3.8 to 16.7 ± 4.7 μA·cm⁻² (means ± SE; \( n = 4 \) conjunctivae), a 34% boost accompanied by a 9% decline in \( R_t \) from 0.79 ± 0.16 to 0.72 ± 0.15 kΩ·cm² (\( P < 0.05 \); paired data). As was true under more physiological conditions, the subsequent addition of forskolin increased the \( I_{sc} \) (to 20.7 ± 4.7 μA·cm⁻²; a 24% rise) and reduced \( R_t \) by ~10% to 0.65 ± 0.13 kΩ·cm² (\( P < 0.05 \); paired data) because of the activation of basolateral K⁺ channels gated by a PKA-dependent mechanism (32). The elevated current was returned to baseline by glibenclamide (27), which decreased the \( I_{sc} \) to 5.1 ± 0.9 μA·cm⁻² and increased \( R_t \) to 0.71 ± 0.13 kΩ·cm², changes of 75% and 8%, respectively. Under the present conditions, this inhibition apparently resulted from an effect on K⁺ channels in the lateral membranes, given the absence of a subsequent effect by the nonspecific K⁺ channel blocker Ba²⁺. Presumably, glibenclamide either directly blocked K⁺ channels in the conjunctiva or inhibited PKA activity (27).

Because of the possibility that gluconate, the Cl⁻ substitute used in the above experiments, might have influenced the electrical response of the preparation due to its weak Ca²⁺-chelating properties, the Cl⁻-free experiments were repeated with SO₄²⁻ used as a replacement anion. In these experiments, the introduction of A23187 did not evoke more substantial current changes than those observed with gluconate, suggesting that the cells had not been depleted of Ca²⁺ as a result of the combined presence of the ionophore and the chelator. More important, the subsequent addition of EBIO (0.5 mM) to conjunctivae bathed bilaterally with the SO₄²⁻ solution and A23187 pretreatment produced an \( I_{sc} \) increase from 13.1 ± 2.9 to 18.0 ± 3.3 μA·cm⁻², a 37% rise, and an 11% \( R_t \) decline from 0.98 ± 2.1 to 0.87 ± 1.9 kΩ·cm² (\( n = 5 \) conjunctivae; \( P < 0.05 \)). These changes were virtually identical to those obtained with gluconate as the Cl⁻ replacement (Fig. 3).

**Evidence for EBIO modulation of basolateral K⁺ conductance.** EBIO is recognized as an activator of Ca²⁺-dependent K⁺ channels (27), implying that the stimulatory effects of the compound under Cl⁻-free conditions most likely resulted from activation of such elements in the conjunctival basolateral membrane. To examine this prospect, experiments were conducted in the presence of a transepithelial K⁺ gradient in the apico-to-basolateral direction. This entailed bathing the mucosal aspect of the tissue with a Cl⁻-free, high-K⁺ solution with low [Na⁺], while the stromal side solution (also Cl⁻ free) contained physiological K⁺ levels with low Na⁺. Under these conditions, the Na⁺-K⁺ pump is quiescent (32, 40) and the \( I_{sc} \) reflects solely the diffusion of K⁺ from the apical-to-basolateral baths across both transcellular and paracellular pathways. The apical introduction of the ionophore amphotericin B, which increases the membrane permeability to monovalent cations, eliminated a restriction to transcellular K⁺ diffusion as shown by the marked \( I_{sc} \) increase that resulted (Fig. 4). In the presence of amphotericin B, the majority of the K⁺-dependent \( I_{sc} \) is transcellular as judged by the fraction of the current remaining in the presence of the nonspecific K⁺ channel blocker Ba²⁺, which was added at the end of these experiments (Fig. 4) to essentially reduce the \( I_{sc} \) to a representation of K⁺ diffusion across the paracellular pathway.

Five sets of experiments were conducted using the K⁺-dependent \( I_{sc} \) as a measure of basolateral K⁺ channel activity (Table 1), with representative traces of the first four conditions shown in Fig. 4. The introduction of EBIO (0.5 mM) as the initial test compound after amphotericin B permeabilization (Fig. 4A) enhanced the \( I_{sc} \) by 4.6 ± 1.3 μA·cm⁻² (\( n = 12 \) conjunctivae; Table 1, protocol A), an ~11% stimulation. In contrast, when the agent was added after A23187 (Fig. 4B), the \( I_{sc} \) rapidly increased by 29.6 ± 2.6 μA·cm⁻², a 90% enhancement (\( n = 16 \) conjunctivae; Table 1, protocol B), suggesting that a combination of high intracellular Ca²⁺ levels plus EBIO was required to produce a maximal change in basolateral K⁺ conductance. Consistent with this finding, the addition of A23187 to tissues preexposed to EBIO (Fig. 4A and Table 1, protocol A) also evoked salient \( I_{sc} \) stimulation. The \( I_{sc} \) increases that were obtained using the combination of A23187 and EBIO were statistically larger than those evoked using either agent alone (\( P < 0.01 \); unpaired data).

Pedersen et al. (25) noted that of the three major subtypes of Ca²⁺-activated K⁺ channels [i.e., large conductance (BK), intermediate conductance (IK), and small conductance (SK) channels], only the BK subtype appears to be capable of performing Ca²⁺-dependent gating, while the latter two forms combine constitutively with calmodulin to obtain Ca²⁺ dependency. As such, some experiments (Fig. 4A) determined the effects of the calmodulin antagonist calmidazolium on the increases of the K⁺-dependent \( I_{sc} \) elicited by the combination of A23187 plus EBIO. The application of calmidazolium led to a gradual halving of the stimulated \( I_{sc} \) concomitant with a detectable increase in \( R_t \) (Fig. 4A; Table 1, protocol A). However, pretreatment with calmidazolium did not preclude a subsequent stimulatory effect by EBIO (Fig. 4C; Table 1,
Although the antagonist in itself always evoked an inhibitory effect on the $K^+$-dependent $I_{sc}$ when applied directly after amphotericin B ($n = 4$ conjunctivae; data not shown) or in the presence of A23187 (Fig. 4C, which also demonstrates $K^+$ current oscillations as shown elsewhere with the epithelial membrane potential and the ion currents (37). When added after A23187 plus EBIO (Fig. 4B, Table 1, protocol C), TEA produced a $9.2 \pm 1.4 \mu A/cm^2$ current decline ($n = 16$ conjunctivae), an $\sim 15\%$ inhibition. This degree of $I_{sc}$ reduction was not markedly affected in the protocols in which TEA was added to tissues preexposed to calmidazolium, glibenclamide, and H-89, indicating that these other drugs may not have affected conductances sensitive to this channel blocker and that the TEA-sensitive channels contributed only a small amount to the $I_{sc}$ generated by the $K^+$ gradient (data not included for the sake of simplicity).

Evidence for EBIO modulation of apical $Cl^-$ conductance. To determine whether EBIO also affects channels in the conjunctival apical membrane, experiments analogous to those described above for $K^+$ currents were conducted, except that in this case, the isolated tissues were bathed in the presence of an apical-to-basolateral $Cl^-$ gradient (Fig. 5). For these experiments, the apical bath contained a high-$K^+$ medium (107 mM), while the basolateral side was $Cl^-$-free (solution prepared with 53.5 mM $K_2SO_4$). Under these conditions, a negative current ($-11.3 \pm 1.5 \mu A/cm^2$; $n = 27$ conjunctivae) representing transepithelial $Cl^-$ movement from the apical to basolateral hemichannels was recorded. The cholesterol-binding antibiotic nystatin was then introduced to increase the permeability of the basolateral aspect. Preliminary experiments determined that the effects of this agent varied considerably among isolated preparations, presumably because of variations in the thickness of the underlying stroma that was retained from the dissection. As such, a relatively high level of 400 $\mu g/mL$ was determined to produce the most consistent $I_{sc}$ increase in the negative current of $-8.5 \pm 1.0 \mu A/cm^2$ to $-19.8 \pm 2.2 \mu A/cm^2$; $n = 27$ conjunctivae).
Table 1. 

<table>
<thead>
<tr>
<th>Experimental Protocol</th>
<th>n</th>
<th>Amphotericin B Baseline</th>
<th>Drugs</th>
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<tbody>
<tr>
<td>A</td>
<td>12</td>
<td>I$_{sc}$ 42.6 ± 9.8</td>
<td>EBlO (0.5 mM)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>R$_i$ 0.58 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>16</td>
<td>I$_{sc}$ 30.2 ± 2.7</td>
<td>A23187 (10 μM)</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>R$_i$ 0.94 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>6</td>
<td>I$_{sc}$ 24.2 ± 3.8</td>
<td>A23187 (10 μM)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>R$_i$ 1.02 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>I$_{sc}$ 24.4 ± 4.5</td>
<td>H-89 (1 μM)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>R$_i$ 0.57 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>6</td>
<td>I$_{sc}$ 39.7 ± 3.0</td>
<td>Glibenclamide (0.3 mM)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>R$_i$ 0.86 ± 0.22</td>
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</table>

All values are means ± SE; n refers to no. of conjunctivae studied. I$_{sc}$, short-circuit current; R$_i$, transepithelial resistance; EBlO, 1-ethyl-2-benzimidazolone; TEA, tetraethyl ammonium; H-89, an isoquinolinesulfonamide. P < 0.05 (paired data), all values significantly greater or less than their immediate antecedents.

Under these conditions, treatment with A23187 (Fig. 5A) was ineffective (n = 12 conjunctivae), suggesting that intracellular Ca$^{2+}$ levels were not rate limiting to transepithelial Cl$^{-}$ movement. The addition of EBlO to the apical bath (Fig. 5A) induced an increase in the negative current of −4.6 ± 0.9 μA·cm$^{-2}$ (from −12.9 ± 2.2 to −17.5 ± 2.9 μA·cm$^{-2}$), a 35% change accompanied by a 17% R$_i$ decline (from 0.53 ± 0.10 to 0.44 ± 0.07 kΩ·cm$^{-2}$; n = 8 conjunctivae) (P < 0.05), effects consistent with activation of apical Cl$^{-}$ channels. When EBlO was added directly after nystatin (i.e., omitting the addition of A23187), the increase in the negative current (ΔI$_{sc}$ = −3.7 ± 1.0 μA·cm$^{-2}$; n = 9 conjunctivae) (data not shown) was comparable to that observed in the presence of the Ca$^{2+}$ ionophore A23187 (P > 0.5; unpaired data).

The negative, Cl$^{-}$-dependent I$_{sc}$ was increased 21% by forskolin (from −25.9 ± 7.3 to −31.3 ± 7.2 μA·cm$^{-2}$; n = 5 conjunctivae) (P < 0.05; paired data) when the diterpenoid was added as the initial test compound after nystatin (not shown). This cAMP-elevating agent was always effective when introduced after EBlO (by increasing I$_{sc}$ an additional 8%; n = 4 conjunctivae) (P < 0.05) (Fig. 5A). However, in experiments in which the order of addition was reversed, EBlO did not increase the negative current beyond that produced by forskolin (n = 5 conjunctivae; data not shown) as found with intact epithelial preparations under physiological conditions (Fig. 2C).

When applied in conjunctivae bathed with the transepithelial Cl$^{-}$ gradient, glibenclamide inhibited the negative current (Fig. 5, A and B), suggesting that under these conditions, the compound acted as a Cl$^{-}$ channel blocker. Consistent with this finding, Ba$^{2+}$ did not have a pronounced effect (n = 8 conjunctivae) when rapidly added in a sequential manner to one hemichamber and then the other.

In the presence of glibenclamide (Fig. 5B), the effects of EBlO and forskolin were markedly attenuated. When added as the initial compound after nystatin, glibenclamide decreased I$_{sc}$ from −12.6 ± 3.1 to −8.1 ± 3.7 μA·cm$^{-2}$, a 36% reduction, and increased R$_i$ by 24%, from 0.66 ± 0.05 to 0.82 ± 0.04 kΩ·cm$^{-2}$ (n = 4 conjunctivae; P < 0.05); an ensuing effect by EBlO was not discernible. The subsequent addition of forskolin (Fig. 5B) produced a transitory increase in negative current to −8.7 ± 1.2 μA·cm$^{-2}$ (n = 4 conjunctivae; P < 0.05), a 7% rise that was followed by a gradual current decline.

$^{36}$Cl$^{-}$ fluxes across conjunctivae in the direction of the Cl$^{-}$ gradient. To corroborate the electrical results with the transepithelial Cl$^{-}$ gradient, $^{36}$Cl$^{-}$ was added to the high-Cl$^{-}$ apical side bath and unidirectional fluxes were measured in the apical-to-basolateral direction for 1 h under baseline conditions, followed by three sequential, 1-h periods in which nystatin, EBlO, and glibenclamide were introduced to the bathing solutions (Table 2). Although the changes in the measured fluxes in response to these drugs mimicked those of the Cl$^{-}$-dependent I$_{sc}$ (see, e.g., Fig. 5), it was also clear that the absolute values for unidirectional flux were markedly larger.
converted to equivalent current values by the factor 26.8
of the radiolabeled side was measured every half hour during the 4-h protocol and did not change in experiments of this duration. Unidirectional fluxes were
Compared with this possibility, in the presence of glibenclamide,
consistent with the antecedent.
Unidirectional $J_{Clba}$ in the presence of a Cl$^-$ gradient in the same direction under short-circuit conditions:
flux data compared with simultaneously measured $I_{sc}$

Table 2. Unidirectional $J_{Clba}$ in the presence of a Cl$^-$ gradient in the same direction under short-circuit conditions: flux data compared with simultaneously measured $I_{sc}$

<table>
<thead>
<tr>
<th>Conjunctival Preparation</th>
<th>Control, Baseline Conditions</th>
<th>Nystatin, 0.4 mg/ml</th>
<th>EBIO, 0.5 mM Apical Side</th>
<th>Glibenclamide, 0.3 mM Apical Side</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$J_{Clba}$, $\mu$eq h$^{-1}$ cm$^{-2}$</td>
<td>$I_{sc}$, $\mu$A cm$^{-2}$</td>
<td>$J_{Clba}$, $\mu$eq h$^{-1}$ cm$^{-2}$</td>
<td>$I_{sc}$, $\mu$A cm$^{-2}$</td>
</tr>
<tr>
<td></td>
<td>Calculated</td>
<td>Measured</td>
<td>Calculated</td>
<td>Measured</td>
</tr>
<tr>
<td>01/14/04a</td>
<td>1.46±0.28</td>
<td>±7.4 ± 2.7</td>
<td>1.60±0.45</td>
<td>±12.2 ± 3.0</td>
</tr>
<tr>
<td>01/14/04b</td>
<td>1.14±0.20</td>
<td>±3.1 ± 0.7</td>
<td>1.32±0.35</td>
<td>±13.8 ± 1.3</td>
</tr>
<tr>
<td>01/23/04a</td>
<td>1.57±0.28</td>
<td>±4.5 ± 1.5</td>
<td>1.89±0.50</td>
<td>±20.9 ± 1.3</td>
</tr>
<tr>
<td>01/23/04b</td>
<td>1.97±0.27</td>
<td>±3.0 ± 0.6</td>
<td>3.79±1.06</td>
<td>±7.8 ± 1.2</td>
</tr>
<tr>
<td>02/06/04a</td>
<td>2.72±0.28</td>
<td>±7.2 ± 1.0</td>
<td>3.81±1.02</td>
<td>±8.1 ± 1.2</td>
</tr>
<tr>
<td>02/06/04b</td>
<td>2.75±0.28</td>
<td>±7.3 ± 1.0</td>
<td>3.03±0.81</td>
<td>±23.0 ± 1.2</td>
</tr>
<tr>
<td>Means ± SE</td>
<td>1.94±0.28</td>
<td>±51.9 ± 10.2</td>
<td>2.57±0.45</td>
<td>±16.3 ± 3.0</td>
</tr>
<tr>
<td>Ratio of measured to calculated $I_{sc}$</td>
<td>0.20</td>
<td>0.24</td>
<td>0.23</td>
<td>0.07</td>
</tr>
</tbody>
</table>

$J_{Clba}$, unidirectional apical-to-basolateral $^{36}$Cl fluxes. Conjunctivae were isolated in divided chambers and bathed under short-circuit conditions using a solution containing 107 mM KCl in the apical side hemichamber and one containing 53.5 mM K$_2$SO$_4$ in the contralateral side. The concentrations of all other electrolytes were identical on both sides of the tissue, thereby creating a transepithelial gradient for Cl$^-$ in the basolateral-to-apical direction and for SO$_4^{2-}$ in the opposite direction. At time 0, $^{36}$Cl ($\sim 0.5$ $\mu$Ci/ml) was added to the apical bath, and samples were taken every 15 min from the basal side solution. The specific activity of the radiolabeled side was measured every half hour during the 4-h protocol and did not change in experiments of this duration. Unidirectional fluxes were converted to equivalent current values by the factor 26.8 $\mu$A cm$^{-2}$ = 1 $\mu$eq h$^{-1}$ cm$^{-2}$. * $P<0.05$ (paired data), significantly different from respective antecedent.

Table 3. Unidirectional $J_{Clba}$ in the presence of a Cl$^-$ gradient in the same direction under short-circuit conditions: flux data compared with simultaneously measured $I_{sc}$

<table>
<thead>
<tr>
<th>Conjunctival Preparation</th>
<th>Control, Baseline Conditions</th>
<th>Nystatin, 0.4 mg/ml</th>
<th>EBIO, 0.5 mM Apical Side</th>
<th>Glibenclamide, 0.3 mM Apical Side</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$J_{Clba}$, $\mu$eq h$^{-1}$ cm$^{-2}$</td>
<td>$I_{sc}$, $\mu$A cm$^{-2}$</td>
<td>$J_{Clba}$, $\mu$eq h$^{-1}$ cm$^{-2}$</td>
<td>$I_{sc}$, $\mu$A cm$^{-2}$</td>
</tr>
<tr>
<td></td>
<td>Calculated</td>
<td>Measured</td>
<td>Calculated</td>
<td>Measured</td>
</tr>
<tr>
<td>03/11/04a</td>
<td>0.85±0.12</td>
<td>±22.8 ± 18.8</td>
<td>1.33±0.25</td>
<td>±35.8 ± 21.9</td>
</tr>
<tr>
<td>03/11/04b</td>
<td>1.67±0.18</td>
<td>±44.8 ± 25.0</td>
<td>2.34±0.57</td>
<td>±64.7 ± 33.1</td>
</tr>
<tr>
<td>03/12/04a</td>
<td>1.11±0.14</td>
<td>±29.9 ± 6.9</td>
<td>1.37±0.37</td>
<td>±36.6 ± 14.4</td>
</tr>
<tr>
<td>03/12/04b</td>
<td>1.38±0.18</td>
<td>±37.1 ± 8.4</td>
<td>1.78±0.53</td>
<td>±47.7 ± 15.3</td>
</tr>
<tr>
<td>Means ± SE</td>
<td>1.25±0.18</td>
<td>±33.6 ± 14.8</td>
<td>1.72±0.45</td>
<td>±46.2 ± 21.1*</td>
</tr>
<tr>
<td>± 0.18 ± 4.7 ± 4.3</td>
<td>0.47±0.35</td>
<td>±12.6 ± 6.3</td>
<td>0.72±0.35</td>
<td>±19.1 ± 14.2</td>
</tr>
</tbody>
</table>

$J_{Clba}$, basolateral-to-apical $^{36}$Cl fluxes. Conjunctivae were isolated in divided chambers and bathed under short-circuit conditions using a solution containing 107 mM KCl in the basolateral hemichamber and one containing 53.5 mM K$_2$SO$_4$ in the contralateral side. The concentrations of all other electrolytes were identical on both sides of the tissue, thereby creating a transepithelial gradient for Cl$^-$ in the basolateral-to-apical direction and for SO$_4^{2-}$ in the opposite direction. At time 0, $^{36}$Cl ($\sim 0.5$ $\mu$Ci/ml) was added to the basolateral bath, and samples were taken every 15 min from the apical side solution. The specific activity of the radiolabeled side was measured every half hour during the 4-h protocol and did not change in experiments of this duration. Unidirectional fluxes were converted to equivalent current values using the factor 26.8 $\mu$A cm$^{-2}$ = 1 $\mu$eq h$^{-1}$ cm$^{-2}$. * $P<0.05$ (paired data), significantly different from respective antecedent.
fluxes were measured in the basolateral-to-apical direction in the presence of a Cl\(^-\) gradient in the same direction. Under these conditions, the diffusion of Cl\(^-\) produced a positive \(I_{sc}\) that was further stimulated by treatments with nystatin and EBIO [Table 3 and Fig. 6, which shows the measured \(I_{sc}\) from one of the four experiments in which basolateral-to-apical \(36^{\text{Cl}}\) fluxes \((J_{\text{Cl},\text{bas} \to \text{ap}})\) were measured]. As above, glibenclamide reduced the theoretical and empirical \(I_{sc}\) values identically as well as the ratio of measured-to-calculated \(I_{sc}\) values, results that are consistent with a proportionally larger paracellular flux of Cl\(^-\) in the presence of the blocker (Table 3). The relatively high values for the \(36^{\text{Cl}}\) fluxes in the presence of glibenclamide in either direction (Tables 2 and 3) suggest that the recorded current did not accurately report the magnitude of the unidirectional \(36^{\text{Cl}}\) flux, because of substantial paracellular fluxes that apparently included a backflux of SO\(_4^{2-}\). Nevertheless, it is clear from both sets of unidirectional \(36^{\text{Cl}}\) flux experiments that EBIO increased the apical permeability to the anion, an effect likely mediated by a glibenclamide-sensitive channel such as CFTR.

**DISCUSSION**

The benzimidazolone, EBIO, was an effective stimulator of the transepithelial \(I_{sc}\) across the rabbit conjunctiva because of its dual actions on apical Cl\(^-\) conductances and basolateral K\(^+\) conductances, a property initially identified in the colonic cell line, T84, and murine tracheal epithelial cells (5, 6). With intact conjunctival epithelial preparations (Figs. 1 and 2), the evoked \(I_{sc}\) stimulations represent a simultaneous increase in both Cl\(^-\)-secretory transport and Na\(^+\) reabsorption, with the latter being augmented as a result of the favorable electrical gradient provided by elevated K\(^+\) conductances that also promote apical Cl\(^-\) egress (32). Because Cl\(^-\) secretion dominates the electrolyte transport properties of this epithelium, cAMP-elevating maneuvers (30, 41) and purinergic receptor activation (18, 30) led to enhanced volumetric fluid movement across the tissue in the stromal-to-mucosal direction. It is posited a priori that EBIO might elicit a similar effect on fluid transport.

Yet, forskolin, which increases the baseline intracellular cAMP levels of the conjunctival epithelium ~3-fold (authors’ unpublished data), serves as a more potent agent than EBIO in increasing the \(I_{sc}\). This finding was observed in intact epithelial preparations (Fig. 2), epithelial preparations bathed bilaterally in solutions lacking Cl\(^-\) (Fig. 3), and putatively apical-membrane preparations bathed in the presence of a transepithelial Cl\(^-\) gradient plus nystatin treatment of the basolateral aspect (Fig. 5). In contrast, EBIO was markedly effective when combined with the Ca\(^{2+}\) ionophore A23187 in increasing the K\(^+\)-dependent \(I_{sc}\) of preparations bathed with a K\(^+\) gradient plus permeabilization of the apical membrane. Such current stimulations were salient for the nearly instantaneous response to the agents and for the magnitude of the current changes (Fig. 4, A and B), which superseded earlier descriptions of \(I_{sc}\) increases obtained with cAMP-elevating maneuvers under identical conditions (32). These observations could indicate that the more limited \(I_{sc}\) stimulations induced by EBIO with intact epithelia (Figs. 2 and 3) reflect rate-limiting conductances in the apical membrane.

Given the fact that gluconate was used as a Cl\(^-\) substitute for the experiments conducted in the absence of the halide with intact preparations (Fig. 3) and that gluconate is a weak Ca\(^{2+}\) chelator, it is possible that upon applying A23187, the cells may have been depleted of Ca\(^{2+}\) rather than loaded with it. If so, such conditions might explain the reduced effect of EBIO in the Cl\(^-\)-free experiments. Alternatively, substantial Ca\(^{2+}\) chelation by gluconate may not have been expected a priori, because 1) \(R_{t}\) was not atypical under these conditions and this parameter did not decay dramatically, suggesting that bath Ca\(^{2+}\) levels were sufficient to maintain epithelial integrity; and 2) gluconate was present bilaterally in the K\(^+\) gradient experiments, which were performed in Cl\(^-\)-free conditions (Fig. 4). In the latter case, EBIO elicited significantly larger \(I_{sc}\) stimulation when combined with A23187, suggesting that the ionophore indeed increased cellular Ca\(^{2+}\) levels in a gluconate-rich bath. Consistent with this finding, the EBIO stimulatory effects observed under Cl\(^-\)-free conditions were empirically identical to those shown in Fig. 3, when SO\(_4^{2-}\) was used as a Cl\(^-\) substitute, thereby inferring that transapical Na\(^+\) reabsorption limited the \(I_{sc}\) increase observed in the absence of Cl\(^-\) (Fig. 3) relative to that obtained with the halide present (Fig. 2).

The direct examination of the effect of EBIO on apical membrane preparations (Figs. 5 and 6) resulted in relatively high paracellular \(36^{\text{Cl}}\) fluxes as well as underestimated Cl\(^-\)-diffusional \(I_{sc}\) in the direction of the Cl\(^-\) gradient (Tables 2 and 3). Nevertheless, these experiments indicated an increase in unidirectional \(36^{\text{Cl}}\) fluxes in the presence of EBIO and a reduction with glibenclamide, changes that are consistent with the putative involvement of CFTR (27), which is expressed by the conjunctival epithelium (35).

Although glibenclamide is recognized as a blocker of Cl\(^-\) conductances mediated by CFTR, this agent also inhibits a variety of other Cl\(^-\) and K\(^+\) channels and affects numerous intracellular enzymes, including PKA (27). Its inhibition of the conjunctival \(I_{sc}\) under Cl\(^-\)-free conditions (Fig. 3), a situation in which only the Na\(^+\)-absorptive component of the \(I_{sc}\) is measured, could have resulted from K\(^+\) channel blockade as well as inhibition of PKA, given earlier evidence of the effectiveness of H-89 under these conditions (32).

Alternatively, one might suspect that glibenclamide could have inhibited a HCO\(_3^-\) current mediated by CFTR under Cl\(^-\)-free conditions (Fig. 3). However, a lack of evidence for
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such HCO₃⁻ transport by the conjunctival epithelium mitigates this possibility. It was observed earlier that in the bilateral absence of Cl⁻, the I_{sc} was reduced to zero upon unilaterally superfusing the apical side hemichamber with Na⁺—free solution, and it was completely restored upon reintroduction of the cation (29). In addition, parallel Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers on the basolateral side contribute to transepithelial Cl⁻ transport (indicating removal of HCO₃⁻ from the cell across the basolateral aspect) (33), and validation of a HCO₃⁻ pump such as a basolateral Na⁺-(n)H⁺-cotransporter was not successful with the rabbit conjunctiva (33). Moreover, introducing HCO₃⁻ plus CO₂ bubbling to tissues bathed with HEPES buffer and air bubbling under Cl⁻—free conditions did not result in an I_{sc} increase (authors’ unpublished data). As such, the most likely explanation for the finding of an inhibitory effect by glibenclamide under Cl⁻—free conditions (Fig. 3) is an effect on K⁺ channels, a possibility substantiated by demonstrating the negative influence of glibenclamide on the I_{sc} generated with a K⁺ gradient (Fig. 4 and Table 1). However, further work is needed to identify the specific K⁺ channels affected by glibenclamide in the conjunctival epithelium as well as to determine the potency of the drug as a PKA inhibitor in this system.

The mechanisms underlying the stimulatory effects of EBIO on epithelial transport are not well established. Benzimidazolone compounds (e.g., NS1619) were initially identified as openers of Ca²⁺-dependent K⁺ channels (K_{Ca}), exhibiting relatively large conductances (known as maxi-K or BK channels) (27). Another such analog, NS004, was the first described pharmacological opener of CFTR (27), but it did not stimulate transepithelial Cl⁻ transport in T84 cells, owing to its ineffectiveness on the K_{Ca} channels expressed by these cells (27, 29). Instead, it was serendipitously discovered that the EBIO analog served as a stimulator of Cl⁻ secretion in T84 cells because of its Cl⁻ channel-opening properties as well as its activation of the intermediate K_{Ca}, or IK, channels (27, 29).

It currently appears that EBIO increases the affinity for Ca²⁺ of both the small conductance K_{Ca}, known as SK channels, and the IK channels via a mechanism probably involving calmodulin (24, 25, 38). Although EBIO is ineffective in the absence of Ca²⁺+, it reportedly can stimulate SK and IK channels with intracellular Ca²⁺ levels in the 30–50 nM range (24, 25). In the presence of EBIO, current vs. Ca²⁺ activation curves were found to shift leftward and saturation occurred at lower Ca²⁺ concentration (25). On the basis of results obtained with macroscopic basolateral K⁺ conductance, we gained evidence consistent with a Ca²⁺-dependent mechanism in the conjunctival epithelium (Fig. 4).

Explanations of the mechanism by which EBIO activates CFTR are presently scant (31). It has been reported that EBIO increases intracellular cAMP levels in isolated colon crypts, presumably by affecting adenyl cyclase directly (3, 19), but the changes were relatively small compared with those induced by forskolin. Researchers in several studies have observed that EBIO does not increase intracellular Ca²⁺ levels (3, 19, 20, 28), while investigators in one study reported Ca²⁺ oscillations in the presence of EBIO (10).

Besides CFTR, the conjunctival epithelium expresses Ca²⁺-activated Cl⁻ channels such as CLCA2 (11), and these elements also may be involved in the EBIO-evoked stimulation of Cl⁻ conductance (Figs. 5 and 6). Moreover, in addition to these channels, very recent data derived using expression microarray assays (36) have indicated message for CLCA4 in the human conjunctiva, as well as mRNA for the human isoforms of the BK and SK4 K⁺ channels (Turner H and Wolosin M, personal communication). Assuming functional expression of these channels in the rabbit conjunctival membranes, such moieties could be responsive to EBIO. The inhibitory effects of TEA and calmidazolium on the K⁺-dependent I_{sc} (Fig. 4) seem consistent with this prospect. To date, patch-clamp studies of rabbit conjunctiva have defined only nonselective cation channels and inwardly rectifying K⁺ conductances in the epithelium (39); there have been no additional studies conducted in other species.

Regardless of such limited information, it is tempting to speculate that EBIO or EBIO-like compounds may alleviate dry-eye symptoms in individuals with lacrimal gland dysfunction by stimulating fluid transport across the conjunctival epithelium. This suggestion is analogous to the hypotheses of others who have assessed the potential therapeutic value of Cl⁻ channel openers in the treatment of patients with cystic fibrosis (7, 27, 31). Currently, the most suitable approach to palliate dry-eye complications seems to involve the administration of purinergics (23), because of not only the stimulatory effects of such agonists on conjunctival Cl⁻ secretion and fluid transport (17, 18) but also the fact that purinergics appear to have utility in conserving the composition of the tear film (23).

However, MacVinish et al. (19) noted that the stimulation of Cl⁻ transport in epithelia by P2Y₂ receptor activation is often transitory because of the nature of the Ca²⁺ signal itself and the fact that purinergic agonists produce receptor desensitization, from which recovery is slow. Such considerations might explain the need to administer synthetic P2Y₂ agonists four or five days in clinical trials as well as the time-dependent loss of efficacy that is observable in the data produced by such trials (23).

Currently, there is little information on the toxicity of EBIO. Clearly, an agent such as this one should be considered a potential adjunct to dry-eye therapies that are based on the administration of UTP or UTP derivatives.

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REFERENCES


