Nucleotide-Induced Restoration of Conjunctival Chloride and Fluid Secretion in Adenovirus Type 5-Infected Pigmented Rabbit Eyes

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ABSTRACT

We evaluated the role of extracellular UTP and other nucleotides in the regulation of chloride secretion across the conjunctiva. Chloride secretion (J\textsubscript{sc}) and fluid secretion (J\textsubscript{v}) were determined in freshly excised conjunctival tissues mounted between two buffer reservoirs maintained in an enclosed environment at 37°C. Short circuit current (I\textsubscript{sc}) and 36Cl flux were measured using modified Ussing-type chambers. Fluid flux measurements were made with a pair of capacitance probes. After observing the baseline for 15 to 30 min, fluid flux was measured in the presence of mucosally applied nucleotides (10\textsuperscript{-6} M) for a period of 30 min. Mucosal application of 10\textsuperscript{-6} M each of UTP, UDP, ATP, ADP, AMP, adenosine, and ATP-γ-S transiently stimulated fluid secretion across the conjunctiva to a significant extent for 10 to 15 min. Other nucleotides did not show any significant effect. The stimulation of fluid secretion correlated well with the stimulation in I\textsubscript{sc} (r\textsuperscript{2} = 0.85). UTP (0.1–1000\textmu M) led to a maximal increase in fluid secretion by 11.72 ± 0.48 l/(h/cm\textsuperscript{2}) with an EC\textsubscript{50} value of 10.39 ± 1.08 \mu M. ATP (0.1–1000 \mu M) caused a maximal increase in fluid secretion by 11.89 ± 0.88 l/(h/cm\textsuperscript{2}) with an EC\textsubscript{50} value of 17.23 ± 2.63 \mu M. Adenovirus type 5 (Ad5) infection significantly decreased chloride secretion (J\textsubscript{sc}) and chloride movement across the conjunctiva to 6.5 ± 0.7 l/(h/cm\textsuperscript{2}) (Li et al., 2001). Mucosal application of 10 \mu M UTP, 10 \mu M INS365, and 1 mM 8-Br-cAMP, was able to elicit a normal stimulatory response in the Ad5-infected tissues. In conclusion, mucosal application of purinergic nucleotides may be therapeutically important in restoring ion and fluid secretion in the diseased conjunctiva.

Extracellular nucleosides and nucleotides regulate cellular ion transport and secretory activity in a variety of biological systems (Harden et al., 1995). These purinoceptor agonists have attracted a lot of attention recently for their ability to modulate mucin, chloride, and fluid secretion in a variety of epithelial cells, including those lining the rabbit conjunctiva (Jumblatt and Jumblatt, 1998; Hosoya et al., 1999; Shiue et al., 2000; Li et al., 2001). Specific subtypes of these purinergic receptors that act via a cAMP-independent and Ca\textsuperscript{2+}-dependent mechanism have been reported in intestinal, tracheal, pancreatic duct, and nasal epithelial cells (Harden et al., 1995). The subtypes include P1-purinergic receptors that are activated primarily by adenosine and P2-purinergic receptors that are activated primarily by ATP, ADP, and UTP.

Pigmented rabbit conjunctiva is capable of secreting fluid in the serosal-to-mucosal (s-to-m) direction, where the rate of fluid secretion (J\textsubscript{v}) ranges from 4.3 ± 0.2 l/(h/cm\textsuperscript{2}) (Shiue et al., 2000) to 6.5 ± 0.7 l/(h/cm\textsuperscript{2}) (Li et al., 2001). Mucosal application of 10 \mu M UTP, 10 \mu M INS365, and 1 mM 8-Br-cAMP stimulates the fluid secretion across the conjunctiva by 127, 66, and 95%, respectively (Shiue et al., 2000; Li et al., 2001), whereas serosal application of 0.5 mM ouabain and Cl\textsuperscript{-}free conditions abolishes it. There is a good correlation between cAMP-induced changes in fluid secretion (ΔJ\textsubscript{v}) and the changes in short circuit current (ΔI\textsubscript{sc}) stimulated by the same agent (Shiue et al., 2000). Because ~70% of I\textsubscript{sc} across the conjunctiva is accounted for by active chloride secretion.

ABBRVIATIONS: s-to-m, serosal-to-mucosal; J\textsubscript{sc}, rate of fluid secretion; INS365, P1,P4-di(urate 5’)-tetraphosphate, tetrasodium salt; I\textsubscript{sc}, short circuit current; 8-Br-cAMP, 8-bromoadenosine-3’,5’-cyclic monophosphate; Ad5, adenovirus type 5; BRS, bicarbonated Ringer’s solution; J\textsubscript{v,max}, maximal rate of fluid secretion; PD, potential difference; AQP, aquaporin; TEER, transepithelial electrical resistance.
(Kompella et al., 1993), this chloride secretion may play a principal role in fluid secretion across the conjunctiva.

The role of cystic fibrosis transmembrane conductance regulator in active Cl⁻ transport and concomitant fluid secretion in the presence of 8-Br-cAMP or forskolin in the conjunctiva has been suggested previously (Shiu et al., 2000, 2002). Purinergic receptor agonists prove to be very useful in defective cystic fibrosis transmembrane conductance regulator conditions by offering a cAMP-independent, Ca²⁺-dependent alternative mechanism of Cl⁻ and fluid secretion in the conjunctiva. Hosoya et al. (1999) demonstrated that nucleotides are capable of stimulating net chloride secretion across the pigmented rabbit conjunctiva up to 50%. At 10 μM, the potency order for stimulation of Iᵥₑₑₑ was UTP > ATP > ADP = AMP = adenosine > 2-MeSADP = 2-MeSATP = UDP > BzATP > UMP > α,β-methylene ATP.

Adenoviral ocular infections remain the most common external ocular viral infection worldwide. Ocular adenoviral infections are associated with significant patient morbidity, including symptomatic distress, with visual disturbances that can last months to years. Of the 47 serotypes of human adenovirus, about one-half of these are known to cause ocular disease in patients. It has been previously determined that one serotype of human adenovirus, adenovirus type 5 (Ad5), has the ability to extend its host range to permit replication in the eyes of New Zealand rabbits (Gordon et al., 1992).

The purpose of present study was to characterize the role of extracellular nucleotides in fluid secretion across the pigmented rabbit conjunctiva. Furthermore, we wanted to evaluate the effect of Ad5 infection on the fluid secretory properties of the conjunctiva and to study whether extracellular nucleotides can be used to restore its fluid-secreting properties. The focus was on UTP because it is one of the most potent agonists for the P₂Y₁₃ purinergic receptor.

Materials and Methods

Reagents. ATP, ADP, AMP, adenosine, UTP, UDP, UMP, adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S), benzoyl-benzoyl-ATP (BzATP), and α,β-methylene-adenosine 5'-triphosphate (α,β-MeATP) were all purchased from Sigma-Aldrich (St. Louis, MO). 2-(Methylthio)-adenosine-5'-diphosphate (2-MeSADP) were purchased from Sigma-RBI (Natick, MA). Chloride 36-isotope was purchased from Amersham Biosciences, Inc. (Piscataway, NJ) as sodium chloride in an aqueous solution with 3 mCi/g of Cl⁻ (6.13 mg of Cl⁻/ml). D-[¹⁴C]Mannitol (specific activity, 50 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA).

Buffers. All experiments were performed using bicarbonated Ringer’s solution (BRS) unless noted otherwise. BRS contains 115.5 mM NaCl, 4.82 mM KCl, 1.04 mM CaCl₂, 0.74 mM MgCl₂, 0.86 mM Na₂HPO₄, 29.2 mM NaHCO₃, and 5 mM D-glucose. The osmolality was 300 ± 10 mOsM. Cl⁻-free BRS was made by replacing chloride ions in solution with an equimolar amount of isethionate ions.

Animals and Tissue Preparation. The investigations using rabbits complied with the Guiding Principles in the Care and Use of Animals (Department of Health, Education, and Welfare Publication, NIH 80-23) and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Excision of the pigmented rabbit conjunctiva for Iᵥₑₑₑ and Jₑₑ measurements was carried out as described by Kompella et al. (1993). In brief, male Dutch-belted pigmented rabbits, weighing 2.5 to 3.0 kg (Irish Farms, Norco, CA), were euthanized by injection of an overdose of sodium pentobarbital (85 mg/kg) into the marginal vein of the ear. The eyeballs were then surgically removed from the socket, and the conjunctival tissues were carefully isolated and trimmed. For fluid secretion studies, the trimmed tissues were mounted on adapters with a circular aperture of 0.385 cm² and placed between two Lucite chambers, as described by Shiue et al. (2000). The tissue was bathed on both sides with BRS. The chambers were then placed in an enclosed environment maintained at 37°C and a relative humidity of 70%. In some experiments, fluid secretion was measured under chloride-free conditions in which the chloride ions in the buffer were replaced by isethionate ions. In Ussing chamber studies, the excised conjunctiva was mounted onto a tissue adapter with a circular aperture of 0.960 cm², which was then placed in a modified Ussing chamber housed in a circulating water bath maintained at 37°C. There was 5 ml of BRS on each side of the tissue, bubbled with 95% air to 5% CO₂ ratio, to maintain the pH at 7.4 and provide adequate agitation of the solution (Kompella et al., 1993).

Ad5 Inoculation of Pigmented Rabbit Conjunctiva. Virus inoculation was carried out as described by Wood et al. (1997). An intramuscular injection at 0.2 ml/kg of body weight of a mixture of ketamine and xylazine (4:1), each at a concentration of 100 mg/ml, was used to anesthetize the rabbits. After the rabbit was fully anesthetized, 0.5% proparacaine hydrochloride was applied topically to each eye for local anesthesia, and the eyes were then inoculated with Ad5 McEwen by injecting the virus intrastromally to form five focal blebs (10 μl/bleb) using a dice pattern. The corneal epithelium was then scarified around the blebs and was followed by topical application of an additional 50 μl of Ad5 McEwen (Gordon et al., 1992). Total volume of the inoculum was 10 μl plaque-forming units in 100 μl/eye. Sham-infected eyes received 100 μl of 0.1 M Tris-HCl buffer (pH 8.0). The viral titers of Ad5 on the ocular surface reached their peak 3 to 4 days post-inoculation (Gordon et al., 1994). Therefore, the conjunctival tissues were excised on day 3 post-inoculation for Cl⁻ and fluid transport studies as described above.

Bioelectric Parameter Measurements. All experiments were performed under open-circuit conditions. With the use of an automatic voltage-clamp device (558C-5; Department of Bioengineering, University of Iowa, Iowa City, IA), the Iᵥₑₑₑ, PD, and TEER were estimated at 15-min intervals to assess the tissue viability. The Iᵥₑₑₑ across conjunctival tissues was recorded with a strip chart recorder (Kipp and Zonen, Delft, The Netherlands). A 2 mV direct voltage pulse imposed for 3 s across the voltage-clamped tissues allowed to estimate the TEER as a surface area normalized ratio of applied voltage pulse to the resultant direct current response [TEER = (V/I)A, where A is the nominal surface area of the circular aperture on the tissue adapter]. Before each experiment, the solution resis-
of the probes. A relative humidity of 70% inside the enclosed environment helped reduce evaporation from the surface of the liquid. After recording the baseline value, an aliquot of BRS from the mucosal side was removed and simultaneously replaced with an equal volume of the appropriate nucleotide solution by a concurrent feed and drain method using a pair of 100-μl Hamilton syringes joined at the end of plungers in a back-to-back manner. The concentrations of nucleotides used in this study were based on their stimulatory effects on \( I_{sc} \) (Hosoya et al., 1999).

Data Analysis. The concentration-response parameters for nucleotide effects in the conjunctiva were estimated by nonlinear least-squares regression analysis of the data for \( \Delta J_v \) and nucleotide concentrations using the Prism software (GraphPad Software Inc., San Diego, CA) and the following equation:

\[
\Delta J_v = \Delta J_{v,\text{min}} + (\Delta J_{v,\text{max}} - \Delta J_{v,\text{min}})(1 + \log(\text{C}/C)^n)
\]

where \( C \) is nucleotide concentration, \( \Delta J_{v,\text{max}} \) is maximal value of \( \Delta J_v \), \( \Delta J_{v,\text{min}} \) is minimal value of \( \Delta J_v \), \( EC_{50} \) is effective half-maximal concentration of the nucleotide, and \( n \) is Hill coefficient. A similar approach was used to obtain the \( \Delta J_v \) values.

All results are presented as mean ± S.E.M. The stimulated fluid secretion rates after the mucosal application of nucleotides were compared with their own individual baselines to obtain the difference (\( \Delta J_v \)) in fluid secretion after application of nucleotide. Statistical significance among group (more than or equal to three) means was determined by one-way analysis of variance, followed by modified Fisher’s least-squared difference approaches. A value of \( p < 0.05 \) was considered significant.

Results

Effect of 10 μM Adenosine and Uridine Analogs on \( J_v \) across the Pigmented Rabbit Conjunctiva. Net fluid secretion rate (\( J_v \)) of 4.70 ± 0.25 μl/h/cm² (n = 45) in the s-to-m direction at baseline conditions was not statistically significant from the previously reported value (Shiue et al., 2000). Instillation of 10 μM UTP to the mucosal side of the excised conjunctiva resulted in a significant increase in net fluid secretion (\( \Delta J_v = 6.02 ± 0.02 \) μl/h/cm² (n = 3, \( p < 0.05 \))) during 10- to 15-min postinstillation. After instillation of 10 μM UDP to the mucosal side, \( \Delta J_v \) was 2.51 ± 0.38 μl/h/cm² (n = 3, \( p < 0.05 \)), a significant increase in fluid secretion compared with pretreated value. Unlike UTP and UDP, UMP showed no significant effect on \( J_v \), ATP, ADP, and adenosine, each at 10 μM afforded an increase in the fluid secretion (\( \Delta J_v \)) of 3.44 ± 0.38 μl/h/cm² (n = 3, \( p < 0.05 \)), 3.43 ± 0.39 μl/h/cm² (n = 3, \( p < 0.05 \)), and 3.44 ± 0.38 μl/h/cm² (n = 3, \( p < 0.05 \), respectively. AMP on the other hand could elicit only a 38% increase in \( J_v \) \( \Delta J_v = 1.78 ± 0.09 \) μl/h/cm² (n = 3, \( p < 0.05 \)) (Fig. 1).

A good correlation (\( r^2 = 0.85 \)) was obtained between \( \Delta J_v \) and \( \Delta J_v \) stimulated by 10 μM nucleotides (Fig. 2). Also, an 80% abolishment of the baseline fluid secretion was observed when chloride ions were replaced with the same concentration of isethionate ions in both the serosal and mucosal bathing fluids. \( J_v \) dropped from 4.7 ± 0.25 μl/h/cm² to 0.94 ± 0.66 μl/h/cm² (n = 4), which is not significantly different from zero (\( p > 0.2 \)). With chloride-free condition, application of 10 μM UTP increased the fluid secretion by 0.94 ± 0.01 μl/h/cm² (n = 4), which is not significantly different (\( p = 0.23 \)) from that observed under chloride-free and UTP-absent conditions (Table 1).

ATP and UTP stimulated the \( J_v \) in a dose-dependent manner with an \( EC_{50} \) value of 17.23 ± 2.63 μM and \( J_v,\text{max} \) of 11.89 ± 0.88 μl/h/cm² for ATP and an \( EC_{50} \) value of 10.39 ± 1.08 μM and \( J_v,\text{max} \) of 11.72 ± 0.49 μl/h/cm² for UTP (Fig. 3). These \( EC_{50} \) values were not significantly different from each other (\( p = 0.074 \)).

Transient Nature of Nucleotide-Induced Fluid Secretion in the Pigmented Rabbit Conjunctiva. The duration of stimulation of \( J_v \) observed with 10 μM concentrations of nucleotides ranged from 10 to 15 min after which fluid secretion returned to baseline value. To determine whether nucleotide metabolism was involved in this transient stimulation effect, we determined the effect of various concentrations of ATP-γ-S, a nonhydrolyzable analog of ATP, on \( J_v \) and \( I_{sc} \) across the conjunctiva. As seen in Fig. 4, ATP-γ-S exhibited a dose-dependent stimulation of \( \Delta J_v \) with an \( EC_{50} \) value of 77.18 ± 1.89 μM and \( J_v,\text{max} \) of 8.84 ± 0.80 μl/h/cm². The corresponding values for stimulation of \( I_{sc} \) were \( EC_{50} = 12.34 ± 1.13 \) μM and \( I_{sc,\text{max}} = 8.91 ± 0.57 \) μA/cm².

![Fig. 1. Changes in the rate of net fluid secretion (i.e., in the s-to-m direction) (\( \Delta J_v \)) elicited by various nucleotides at 10 μM applied mucosally to excised pigmented rabbit conjunctival tissues mounted on an adapter with a circular aperture of 0.385 cm² bathed on both sides with BRS maintained at 37°C and pH 7.4. Each data point represents mean ± S.E.M. of three to four tissues. * significantly different from the baseline value.](image)

![Fig. 2. Correlation between the changes in nucleotide-induced short circuit current (\( \Delta I_{sc} \)) and those in nucleotide-induced fluid secretion (\( \Delta J_v \)) across pigmented rabbit conjunctiva. Each data point represents mean ± S.E.M. of three to four tissues. a, ATP; b, UTP; c, UMP; d, ATP; e, ADP; f, AMP; g, adenosine; h, α,β-methylene-ATP; i, 2- MeSATP; j, 2-MeSADP; k, Be-ATP; 1, ATP-γ-S (0.1 μM); m, ATP-γ-S (1 μM); n, ATP-γ-S (10 μM); and o, ATP-γ-S (100 μM).](image)

![Fig. 3. Changes in net fluid secretion (\( J_v \)) across pigmented rabbit conjunctival tissues. Data were calculated from mean ± S.E.M. of three to four tissues and \( p < 0.05 \).](image)
TABLE 1
Effect of 10 μM UTP stimulation on the net fluid secretion across the pigmented rabbit conjunctiva under Cl⁻-replete and Cl⁻-free conditions. Each entry represents mean ± S.E.M. (n = 3–4). Cl⁻-free BRS was prepared by equimolar replacement of Cl⁻ in regular BRS with isethionate.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Fluid Secretion</th>
<th>μl/(h·cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular BRS</td>
<td>−UTP</td>
<td>4.7 ± 0.25⁴</td>
</tr>
<tr>
<td>Cl⁻-free BRS</td>
<td>−UTP</td>
<td>0.94 ± 0.66⁴</td>
</tr>
</tbody>
</table>

⁴ Significantly different from baseline.
⁵ Not significantly different from each other.
⁶ Significantly different from −UTP.

Fig. 3. Dose-response curves for the changes in net fluid secretion in the pigmented rabbit conjunctiva stimulated by UTP (●) and ATP (▲) (0.1 μM–1 mM). Each data point represents mean ± S.E.M. of three to four tissues.

**Effect of Ad5 Infection on Active Chloride Secretion and Fluid Secretion across the Conjunctiva.** We also tested the effect of UTP in excised conjunctival tissues using Ad5-infected rabbit eyes. The baseline PD of 15.6 ± 0.6 mV (tear-side negative) (n = 7), Isc of 10.4 ± 0.2 μA/cm² (n = 7), and TEER of 1500 ± 100 Oms/cm² (n = 7), taken from cumulative conjunctival tissue values used in these studies, were comparable with previously reported values (Kompella et al., 1993; Hosoya et al., 1996). With Ad5 infection, the respective values changed to 6.5 ± 0.4 mV for PD (n = 5), 3.6 ± 0.3 μA/cm² (n = 5) for Isc, and 1800 ± 250 Oms/cm² for the TEER (n = 5).

Unidirectional Cl⁻ flux in the s-to-m direction (Jsm) was significantly decreased by Ad5 infection (p < 0.05; Table 2). Furthermore, Jsm of Cl⁻ in Ad5-infected conjunctivae was increased to 0.64 ± 0.07 μEq/cm² h⁻¹ by mucosal 10 μM UTP (p < 0.05; Table 2), whereas that in the mucosal-to-serosal direction (Jms = 0.31 ± 0.18 μEq/cm² h⁻¹) was unaffected (Table 2). Two-fold stimulation of net Cl⁻ secretion (Jnet) by 73% was observed in Ad5-infected tissues (Table 2). The integrity of conjunctival epithelial barrier was unaffected as can be seen from relatively constant Papp values of D-mannitol under all conditions tested (Table 2).

Figure 5 shows that the baseline net fluid secretion in the Ad5-infected tissues was reduced to 2.03 ± 0.51 μl/h · cm² (n = 4), corresponding to a 56% decrease from the baseline value of 4.69 ± 0.34 μl/h · cm² obtained from sham-infected tissues (p < 0.05) that was not different from the Jv [4.70 ± 0.25 μl/h · cm²] observed for conjunctival tissues of normal rabbits. However, the Jv of 11.72 ± 0.90 μl/h · cm² (n = 4), observed for 10 μM UTP treatment, was not different than that achieved in the sham-infected tissues (Jv = 12.03 ± 0.61 μl/h · cm² (n = 3)). The stimulatory effect of UTP lasted for 10 to 15 min in both groups of tissues. 8-Br-cAMP (1 mM), on the other hand, did not elicit a normal stimulatory response on the net fluid secretion across the Ad5-infected conjunctiva.

**Discussion**

Purinergic Agonist-Induced Chloride Secretion Is the Driving Force for Nucleotide-Induced Net Fluid Secretion across the Pigmented Rabbit Conjunctiva. We have demonstrated that mucosal application of various adenine and uridine nucleotides is capable of stimulating net fluid secretion (i.e., s-to-m direction) in pigmented rabbit conjunctiva to a significant extent (Fig. 1). UTP (128%), UDP (53%), ATP (73%), ADP (73%), AMP (38%), and adenosine (73%) were found to be potent stimulators of Jv. The potency order for stimulation of Jv was UTP > ATP = ADP = adenosine > UDP > AMP. Other nucleotides (UMP, 2-MeSATP, 2-MeSADP, α,β-methylene ATP, and BzATP) did not exhibit any significant stimulation. Purinoceptor agonist-induced fluid secretion seems to be coupled to active chloride secretion. This is indicated by the lack of stimulation in Jv in
TABLE 2

Unidirectional and net Cl⁻ fluxes (J) and apparent permeability coefficient (P_app) of d-mannitol across the control and Ad5 infected conjunctival tissues in the presence and absence of 10 μM UTP added mucosally.

<table>
<thead>
<tr>
<th>Ad5</th>
<th>UTP</th>
<th>J⁺ (μEq/h · cm²)</th>
<th>J⁻ (μEq/h · cm²)</th>
<th>P_app (×10⁻⁷ cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>0.59 ± 0.05 (10)</td>
<td>0.34 ± 0.05 (7)</td>
<td>0.25 ± 0.06</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>0.97 ± 0.09 (10)</td>
<td>0.42 ± 0.10 (7)</td>
<td>0.55 ± 0.13</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>0.36 ± 0.10 (5)</td>
<td>0.25 ± 0.08 (4)</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.64 ± 0.07 (5)</td>
<td>0.31 ± 0.18 (4)</td>
<td>0.33 ± 0.19</td>
</tr>
</tbody>
</table>

a) Significantly different from each other.
b) Significantly different from each other.

Fig. 5. Effect of 10 μM UTP and 1 mM 8-Br-CAMP on net fluid secretion across conjunctival tissues obtained from pigmented rabbits whose eyes were infected with Ad5. The open columns represent the sham-infected conjunctiva (square) and the closed columns represent the Ad-5-infected conjunctiva (triangle). All values are represented as mean ± S.E.M. of three to four tissues. *, significantly different from baseline (sham-infected conjunctiva); †, significantly different from baseline (Ad5-infected conjunctiva).

chloride-free conditions, upon mucosal application of 10 μM UTP. Furthermore, to corroborate this idea, a good correlation (r² = 0.85) was observed between ΔJ⁺ and ΔJ⁻, suggesting the involvement of active chloride secretion as a driving force for net fluid secretion across the tissue.

Although conjunctival fluid flow is also sensitive to conditions that affect active Na⁺ absorption (Shiue et al., 2000) presumably due to active Na⁺ absorption (Hosoya et al., 1996), mucosal application of UTP does not stimulate Na⁺ transport (Hosoya et al., 1999), indicating that the change in I⁵⁺ measured after mucosal application of these nucleotides is solely due to their effect on active chloride secretion (Hosoya et al., 1999).

UTP-Sensitive P2Y-Type Purinoceptors Seem to be the Possible Candidates Mediating Nucleotide-Induced Net Fluid Secretion across the Pigmented Rabbit Conjunctiva. UTP activates P2Y₂/P2Y₄, ATP activates P2Y₆/P2Y₁₁, ADP activates P2Y₁/P2Y₁₂, and UDP activates P2Y₄-type purinoceptors (Dubayak and el Montassim, 1993; Boyer et al., 1997; Jumblatt and Jumblatt, 1998; Ralevic and Burnstock, 1998). The observed EC₅₀ values for the stimulatory effect of UTP and ATP on net fluid secretion are not significantly different from each other, suggesting the involvement of these purinoceptor subtypes in rabbit conjunctiva. Such a possibility was also suggested by the observations of Jumblatt and Jumblatt (1998) that conjunctival mucin secretion from the conjunctival goblet cells was stimulated by UTP and ATP with EC₅₀ value of 10 μM for ATP in the rabbit and 5 and 8 μM for UTP and ATP, respectively, in the human conjunctiva. The potency rank order for J⁺ increase is also consistent with an agonist for UTP-sensitive P2Y₂-type purinergic receptors, including P2Y₂ and P2Y₂₄, ΔJ⁺ afforded by UDP and UMP is much smaller compared with UTP, suggesting that the contribution of P2Y₂ receptors to fluid secretion is rather insignificant, leaving P2Y₁ and P2Y₄ as the dominant purinoceptors mediating net fluid secretion in the pigmented rabbit conjunctiva. Adenosine also elicited a 73% increase in the rate of fluid secretion, similar to ATP and ADP. Hence, the possible involvement of adenosine nucleoside-specific A₁ subtype receptor in the ATP effect, as suggested by Hosoya et al. (1999), cannot be ruled out.

Transient Effect of Extracellular Nucleotides on Net Fluid Secretion Is Not Due to Nucleotide Metabolism. Extracellular membrane-bound ectonucleotidases sequentially dephosphorylate nucleoside phosphates (Gleson et al., 1989; Guibert et al., 1998), leading to termination of nucleotide action (Westfall et al., 1996). Half-life of UTP on the mucosal surface of pigmented rabbit conjunctival epithelium was reported to be ~9 min (Gukasyan et al., 2002). The duration of the stimulatory effect of nucleotides on J⁺ was transient, lasting for 10–15 min after which the secretion rate returned to baseline. However, a transient increase in fluid secretion (73%) after mucosal application of 10 μM ATP-γ-S (a nonhydrolyzable analog of ATP) eliminated the possibility of nucleotide hydrolysis on the conjunctival cell surface. It has been reported that INS365, a metabolically resistant analog of UTP (half-life ~22 min compared with ~9 min for UTP), significantly stimulates fluid secretion across rabbit conjunctiva in a transient manner lasting for about 60 min. The stimulation of I⁵⁻ by this analog is also transient, lasting for about 30 min (Li et al., 2001). Even though it seems that INS365 has a longer duration of action than ATP-γ-S, it is quite clear that the stimulatory response is transient in nature. Signal transduction events comprising PKC activation and Ca²⁺ mobilization (Ko et al., 1997), followed by receptor desensitization, seem to be responsible for the transient stimulatory effect of nucleotides on conjunctival fluid secretion.

ATP-γ-S exhibited a dose-dependent effect on I⁵⁻ as well as J⁺. The half-maximal EC₅₀ value of ATP-γ-S required to stimulate I⁵⁻ was 5-fold lower than that required to stimulate J⁺. The cause of this difference in EC₅₀ values is not clear. The possibility of ATP-γ-S affecting other ion transport processes to increase I⁵⁻ (e.g., active Na⁺ absorption) is unlikely because mucosal application of UTP does not affect conjunc-
tival Na⁺ transport (Hosoya et al., 1999). We observed that 1 mM ATP-γ-S stimulated net fluid secretion to a much larger extent than the corresponding stimulation of short circuit current. This leads us to speculate that ATP-γ-S might be affecting the transcellular fluid flow mediated by aquaporins (AQPs). Aquaporin type 3 (AQP3) is expressed abundantly in the human and rat conjunctival epithelium as demonstrated by immunoblot analysis, high-resolution immunocytochemistry, and immunoelectron microscopy (Hamann et al., 1998).

**UPP Restores Net Fluid Secretion across the Pigmented Rabbit Conjunctiva under Ad5 Infection Conditions.** We evaluated the effect of Ad5 infection of the chloride and fluid-secreting properties of the pigmented rabbit conjunctiva by using an ocellar model of adenovirus type 5 infection (Gordon et al., 1992; Wood et al., 1997). This model developed in New Zealand rabbits has mean viral replication lasting for 8.3 days. Peak ocular viral titers are obtained on day 3 after inoculation and represent a 2-log increase over day 1. The ocular viral replication is associated with acute conjunctivitis and delayed onset presumed immune-mediated clinical disease is associated with blepharconjunctivitis, iritis, corneal edema, and subepithelial corneal infiltrates. A 56% decrease each in the net s-to-m chloride flux and fluid secretion was observed 3 days postinfection. The decrease in the expression of aquaporins (AQP3) might be responsible for the reduction in fluid secretion. Towne et al. (2000), using recombinant adenovirus, showed that acute adenovirus infection was responsible for a decrease in the expression of AQP1 and AQP5 in mouse lung.

As seen in Fig. 5, 1 mM 8-Br-cAMP was unable to fully restore net fluid secretion in the Ad5-infected conjunctiva. Ten micromolar UTP, on the other hand, was as effective in the Ad5-infected conjunctival tissues as in sham-infected tissues. We focused on UTP because it is one of the most potent agonists for the P2Y₂ purinergic receptor. The half-life of UTP at the mucosal surface of the pigmented rabbit conjunctival epithelium is ~9 min, as nucleotides are hydrolyzed by ectonucleotidases (Gukasyan et al., 2002). Mucosal application of 10 μM UTP restored the normal fluid secretion rate (Jₑ = 11.72 ± 0.90 μl/h·cm²). A possible explanation for the lack of 8-Br-cAMP effect is the down-regulation of cAMP-dependent chloride secretory pathway in Ad5-infected conjunctival tissues. However, the camp-independent protein kinase C-dependent purinergic pathway that acts via Ca²⁺ as the second messenger seems to remain intact (UTP effect).

In conclusion, we have shown in this study that certain nucleotides (such as UTP and ATP) are capable of restoring the rates of both net Cl⁻ and fluid secretion in Ad5-infected conjunctival tissues. UTP-sensitive P2Y-type purinergic receptors seem to be the possible candidates responsible for mediating the nucleotide-induced increases in net fluid secretion, as a result of increased active chloride secretion in the pigmented rabbit conjunctiva. Due to their stimulatory ability, nucleotides can be potential therapeutic modalities in the treatment of various transport defects encountered in the ocular tissues in diseased and/or inflamed states.

**References**


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