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Nucleotide-induced restoration of conjunctival chloride and fluid secretion in adenovirus type 5 infected pigmented rabbit eyes.

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ABBREVIATIONS:

8-Br cAMP, 8-bromoadenosine-3', 5'-cyclic monophosphate; Ad5, adenovirus type 5; AQP, aquaporins; AQP3, aquaporin type 3; BRS, bicarbonated Ringer's solution; CFTR, cystic fibrosis transmembrane conductance regulator; I_{sc}, short circuit current; J_v, rate of fluid secretion; ΔJ_v, change in the rate of fluid secretion; EC_{50}, effective half-maximal concentration; $J_{v,}max$, maximal rate of fluid secretion; PFU, plaque forming units; PD, potential difference; PKC, protein kinase C; s-to-m, serosal-to-mucosal.

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ABSTRACT

We evaluated the role of extracellular UTP and other nucleotides in the regulation of chloride (J_{Cl}) and fluid secretion (J_v) across the pigmented rabbit conjunctiva. J_v was determined in freshly excised conjunctival tissues mounted between two buffer reservoirs maintained in an enclosed environment at 37°C. Short circuit current (I_{sc}) and ^{36}Cl flux were measured using modified Ussing-type chambers. Fluid flux measurements were made with a pair of capacitance probes. After observing the baseline for 15-30 minutes, fluid flux was measured in the presence of mucosally applied nucleotides (10 μM) for a period of 30 min. Mucosal application of 10 μM each of UTP, UDP, ATP, ADP, AMP, adenosine, and ATP-γ-S, transiently stimulated fluid secretion across the conjunctiva to a significant extent for 10-15 min. Other nucleotides did not show any significant effect. The stimulation of fluid secretion correlated well with the stimulation in I_{sc} ($r^2 = 0.85$). UTP (0.1-1000 μM) led to a maximal increase in fluid secretion by 11.72 ± 0.48 μl/(hr•cm^2) with an EC_{50} of 10.39 ± 1.08 μM. ATP (0.1-1000 μM) caused a maximal increase in fluid secretion by 11.89 ± 0.88 μl/(hr•cm^2) with an EC_{50} of 17.23 ± 2.63 μM. Ad5 infection significantly decreased both net ^{36}Cl secretion across the conjunctiva by ~56% and the rate of fluid secretion by ~56%. UTP (10 μM), but not 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^{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 secretion (J_v) ranges from 4.3 ± 0.2 µl/(hr•cm^2) (Shiue et al., 2000) to 6.5 ± 0.7 µl/(hr•cm^2) (Li et al., 2001). Mucosal application of 10 µM UTP, 10 µM 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), while serosal application of 0.5 mM ouabain and Cl^- free conditions abolishes it. There is a good correlation between cAMP induced changes in fluid secretion (ΔJ_v) and the changes in short circuit current (ΔI_{sc}) stimulated by the same agent (Shiue et al., 2000). Since ~70% of I_{sc} across the conjunctiva is accounted for by active chloride secretion (Kompella et al., 1993), this chloride secretion may play a principal role in fluid secretion across the conjunctiva.

The role of CFTR in active Cl^- transport and concomitant fluid secretion in the presence of 8-Br cAMP or forskolin in the conjunctiva has been previously suggested (Shiue et al., 2000; Shiue et al., 2002). Purinergic receptor agonists prove to be very useful in defective CFTR conditions by offering a cAMP independent, Ca^{2+} 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 upto 50%. At 10 μM, the potency order for stimulation of I_{sc} was UTP ≥ ATP > ATPγS = ADP = AMP = adenosine >2-MeSADP = 2-MeSATP = UDP > BzATP > UMP > α,β-methylene ATP.

Adenoviral (Ad) 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 study whether extracellular nucleotides can be utilized to restore its fluid secreting properties. The focus was on UTP because it is one of the most potent agonists for the $P2Y_2$ purinergic receptor.

MATERIALS AND METHODS

Reagents

Adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), adenosine, uridine 5'-triphosphate (UTP), uridine 5'-diphosphate (UDP), uridine 5'-monophosphate (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 Chemicals Co (St Louis MO). 2-(methylthio)-adenosine-5'-triphosphate (2-MeSATP) and 2-(methylthio)-adenosine-5'-diphosphate (2-MeSADP) were purchased from Research Biochemicals International (Natick, MA). Chloride-36 isotope was purchased from Amersham Biosciences (Piscataway, NJ) as sodium chloride in an aqueous solution with >3 mCi/g of Cl^- (6.13 mg Cl^-/ml). D-[^{14}C]Mannitol (specific activity = 50mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA).

Buffers

All experiments were performed using bicarbonated Ringer's solution (BRS) unless noted otherwise. BRS contains 111.5 mM NaCl, 4.82 mM KCl, 1.04 mM $CaCl_2$, 0.74 mM $MgCl_2$, 0.86 mM NaH_2PO_4, 29.2 mM $NaHCO_3$, and 5 mM D-glucose. The osmolality was 300 ± 10 mOsmoles/L. 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 I_{sc} and J_v measurements was carried out as described by

Kompella et al. (1993). In brief, male Dutch-belted pigmented rabbits, weighing 2.5-3.0 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 adaptors with a circular aperture of 0.385 cm^2 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^oC 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 cm^2, which was then placed in a modified Ussing chamber housed in a circulating water bath maintained at 37^oC. There was 5 ml of BRS on each side of the tissue, bubbled with 95% air to 5% CO_2 ratio, to maintain the pH at 7.4 and provide adequate agitation of the solution (Kompella et al., 1993).

Adenovirus-5 (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 per 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 10^6 plaque-forming units in 100 µl per eye. Sham-

infected eyes received 100 µl of 0.01 M Tris-HCl buffer (pH 8.0). The viral titers of Ad5 on the ocular surface reached their peak 3-4 days post inoculation (Gordon et al., 1994). Therefore, the conjunctival tissues were excised on day 3 post-inoculation for Cl^- and fluid transport studies described above.

Bioelectric Parameter Measurements

All experiments were performed under open-circuit conditions. With the use of an automatic voltage clamp device (558C-5; Bioengineering Department, University of Iowa, Iowa City, IA), the I_{sc}, PD, and TEER were estimated at 15 min intervals to assess the tissue viability. The I_{sc} 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 sec 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 resistance (~100 $\Omega \cdot cm^2$) was compensated for by the automatic voltage clamp unit (Hosoya et al., 1999).

Cl^- Flux Measurements

Unidirectional Cl^- fluxes across the conjunctiva were determined using ^{36}Cl (0.5 µCi/ml). D-[^{14}C]Mannitol at 10 µCi/ml was used in tandem for monitoring the integrity of the paracellular pathway. At predetermined times, 500 µl samples were collected from the receiver fluid, and the aliquot removed was immediately replenished with an equal volume of fresh buffer. Sample radioactivity was assayed in a liquid scintillation counter (LS1801; Beckman, Fullerton, CA). Unidirectional flux (J) for ^{36}Cl or D-[^{14}C]mannitol was estimated from the steady-state rate of the respective radioactivity appearing in the receiver fluid as a function of time. The apparent

permeability coefficient (P_{app}) for mannitol transport was estimated from the steady-state slope of a plot of the cumulative amount of the radiolabeled tracer appearing in the contralateral fluid versus time. Apparent permeability is expressed as $P_{app}=(dQ/dt)\bullet(1/(C_0\bullet A))$, where the steady state flux (dQ/dt) of mannitol is normalized by both the effective surface area of the circular aperture of the tissue adapter ($A = 0.960\ cm^2$) and initial dosing concentration (C_0).

Fluid Flux Measurements

A pair of capacitance probes (ASP-10-CTA/SP; Mechanical Technology, Inc., Latham, NY) was used to measure the rate of fluid secretion (Shiue et al., 2000). The capacitance probes were equilibrated at 37^{o}C for at least one hour prior to the mounting of the tissues to prevent vapor condensation on the surface 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 fashion. 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 ΔJ_v and nucleotide concentrations using the Prism software (Graphpad Software Inc., San Diego, CA) and the following equation:

$$\Delta Jv = \Delta Jv_{min} + (\Delta Jv_{max} - \Delta Jv_{min}) / (1 + \log (EC_{50}/C)^n)$$

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where, C = nucleotide concentration, ΔJv_{max} = maximal value of ΔJv, ΔJv_{min} = minimal value of ΔJv, EC_{50} = effective half-maximal concentration of the nucleotide, and n = Hill coefficient. A similar approach was used to obtain the ΔI_{sc} values.

All results are presented as mean ± s.e.m. The stimulated fluid secretion rates after the mucosal application of nucleotides were compared to their own individual baselines in order to obtain the difference (ΔJ_v) in fluid secretion after application of nucleotide. Statistical significance among group (≥3) means were determined by one-way ANOVA, followed by modified Fisher's least-squared difference approaches. A value of $p<0.05$ was considered to be significant.

RESULTS

Effect of 10 mM adenosine and uridine analogs on J_v across the pigmented rabbit conjunctiva

Net fluid secretion rate (J_v) of 4.70 ± 0.25 μl/(hr•cm^2) (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 (ΔJ_v = 6.02 ± 0.02 μl/(hr•cm^2) (n=3, $p<0.05$)) during 10-15 minutes post-instillation. After instillation of 10 μM UDP on the mucosal side, ΔJ_v was 2.51 ± 0.38 μl/(hr•cm^2) (n=3, $p<0.05$), a significant increase in fluid secretion compared to pretreated value. Unlike UTP and UDP, UMP showed no significant effect on J_{vo}. ATP, ADP and adenosine, each at 10 μM afforded an increase in the fluid secretion (ΔJ_v) of 3.44 ± 0.38 μl/(hr•cm^2) (n=3, $p<0.05$), 3.43 ± 0.39 μl/(hr•cm^2) (n=3, $p<0.05$) and 3.44 ± 0.38 μl/(hr•cm^2) (n=3, $p< 0.05$) respectively. AMP on the other hand could elicit only a 38% increase in J_v (ΔJv = 1.78 ± 0.09 μl/(hr•cm^2) (n=3, $p<0.05$)) (Figure 1).

A good correlation (r^2 = 0.85) was obtained between ΔI_{sc} and ΔJ_v stimulated by 10 μM nucleotides (Figure 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/(hr•cm^2) to 0.94 ± 0.66 μl/(hr•cm^2) (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/(hr•cm^2) (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} of 17.23 ± 2.63 μM and $J_{v,max}$ of 11.89 ± 0.88 μl/(hr•cm^2) for ATP and an EC_{50} of 10.39 ± 1.08 μM and $J_{v,max}$ of 11.72 ± 0.49 μl/(hr•cm^2) for UTP (Figure 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 10-15 min after which fluid secretion returned to baseline value. In order to determine whether nucleotide metabolism was involved in this transient stimulation effect, we determined the effect of various concentrations of ATP-γ-S, a non-hydrolyzable analog of ATP, on J_v and I_{sc} across the conjunctiva. As seen in Figure 4, ATP-γ-S exhibited a dose dependent stimulation of ΔJ_v with an EC_{50} of 77.18 ± 1.89 μM and $J_{v,max}$ of 8.84 ± 0.80 μl/(hr•cm^2). The corresponding values for stimulation of I_{sc} were EC_{50} = 12.34 ± 1.13 μM and $I_{sc,max}$ = 8.91 ± 0.57 μA/cm^2.

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 Adenovirus type 5 (Ad5) infected rabbit eyes. The baseline PD of 15.6 ± 0.6 mV (tear-side negative) (n=7), I_{sc} of 10.4 ± 0.2 μA/cm^2 (n=7), and TEER of 1,500 ± 100 Ω • cm^2 (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/cm^2 (n=5) for I_{sc}, and 1,800 ± 250 Ω • cm^2 (n=5) for the TEER (n=5).

Unidirectional Cl^- flux in the serosal-to-mucosal (sm) direction (J_{sm}) was significantly decreased by Ad5 infection ($p < 0.05$, Table 2). Furthermore, J_{sm} of Cl^- in Ad5 infected conjunctivas was increased to 0.64 ± 0.07 μEq/cm^2 hr^{-1} by mucosal 10 μM UTP ($p < 0.05$, Table 2), whereas that in the mucosal-to-serosal (ms) direction ($J_{ms} = 0.31 \pm 0.18$ μEq/cm^2 hr^{-1}) was unaffected (Table 2). Two-fold stimulation of net Cl^- secretion (J_{net}), representing about 70% of the ΔI_{sc} elicited by 10 μM UTP (Table 2), was obtained in Ad5 infected tissues (Table 2). The integrity of conjunctival epithelial barrier was unaffected as can be seen from relatively constant P_{app}'s 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/(hr•cm^2) (n=4), corresponding to a 56% decrease from the baseline value of 4.69 ± 0.34 μl/(hr•cm^2) obtained from sham-infected tissues ($p<0.05$) that was not different from the J_v (4.70 ± 0.25 μl/(hr•cm^2)) observed for conjunctival tissues of normal rabbits. However, the J_v of 11.72 ± 0.90 μl/(hr•cm^2) (n=4), observed for 10 μM UTP treatment, was not different than that achieved in the sham-infected tissues ($J_v = 12.03 \pm 0.61$ μl/(hr•cm^2) (n=3)). The stimulatory effect of UTP lasted for 10-15 minutes 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 (Figure 1). UTP (128%), UDP (53%), ATP (73%), ADP (73%), AMP (38%) and adenosine (73%) were found to be potent stimulators of J_v. 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 J_v in chloride free conditions, upon mucosal application of 10μM UTP. Furthermore, to corroborate this idea, a good correlation ($r^2 = 0.85$) was observed between ΔI_{sc} and ΔJ_v, 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 I_{sc} 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 $P2Y_2/P2Y_4$, ATP activates $P2Y_2/P2Y_{11}$, ADP activates $P2Y_1/P2Y_{12}$, and UDP activates $P2Y_6$ type purinoceptors (Dubyak and el Moatassim, 1993; Boyer et al., 1997; Jumblatt and Jumblatt, 1998; Ralevic and Burnstock, 1998). The observed EC_{50} 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 EC_{50} of 10μM 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_v increase is also consistent with an agonist for UTP-sensitive P2Y-type purinergic receptors including $P2Y_2$ and $P2Y_4$. ΔJ_v afforded by UDP and UMP is much smaller as compared to UTP, suggesting that the contribution of $P2Y_6$ receptors to fluid secretion is rather insignificant, leaving $P2Y_2$ and $P2Y_4$ 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 A_1 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 (Gleeson 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_v was transient, lasting for 10-15 minutes 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 non-hydrolyzable 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 minutes as compared to ~9 minutes for UTP), significantly stimulates fluid secretion across rabbit conjunctiva in a transient manner lasting for about 60 min. The stimulation of I_{sc} by this analog is also transient, lasting for about 30 min (Li et al., 2001). Even though it appears 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 Ca^{2+} mobilization (Ko et al., 1997), followed by receptor desensitization, appears to be responsible for the transient stimulatory effect of nucleotides on conjunctival fluid secretion.

ATP-γ-S exhibited a dose dependent effect on I_{sc} as well as J_v. The half-maximal effective concentration (EC_{50}) of ATP-γ-S required to stimulate I_{sc} was 5-fold lower than that required to stimulate J_v. The cause of this difference in EC_{50} values is not clear. The possibility of ATP-γ-S affecting other ion transport processes to increase I_{sc} (e.g., active Na^+ absorption) is unlikely, because mucosal application of UTP does not affect conjunctival 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 (AQP). 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).

UTP 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 utilizing an ocular model of adenovirus type 5 infection (Gordon et al., 1992; Wood et al., 1997). This model developed in New Zeland 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 one. The ocular viral replication is associated with acute conjunctivitis and delayed-onset presumed immune-mediated clinical disease is associated with blepharoconjunctivitis, irititis, corneal edema and sub epithelial corneal infiltrates. A 56% decrease each in the net s-to-m chloride flux and fluid secretion was observed 3 days post-infection. 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 Figure 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 focussed on UTP because it is one of the most potent agonists for the $P2Y_2$ 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 ($Jv = 11.72 \pm 0.90$ μl/(hr•cm^2). A possible explanation for

the lack of 8-Br cAMP effect is the downregulation of cAMP dependent chloride secretory pathway in Ad5 infected conjunctival tissues. However, the cAMP independent PKC dependent purinergic pathway that acts via Ca^{2+} 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.

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FOOTNOTES

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LEGENDS

TABLE 1: Effect of 10μM UTP stimulation on the net fluid secretion across the pigmented rabbit conjunctiva under Cl^- replete and Cl^- free conditions. Each data entry represents mean ± s.e.m. (n = 3-4). Cl^- free BRS was prepared by equimolar replacement of Cl^- in regular BRS with isethionate. [a] significantly different from baseline, [b] not significantly different from each other, [c] significantly different from –UTP.

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. Each data entry represents mean ± s.e.m. Numbers in parentheses are the number of tissues used. Transport of ^{36}Cl was studied at 0.5 μCi/ml and D-[^{14}C] mannitol at 10 μCi/ml. J^{net} equals J^{sm} minus J^{ms}, where J is the chloride flux, sm and ms denote serosal-to-mucosal and mucosal-to-serosal directions, respectively. A positive value for J^{net} represents secretion and a negative value absorption. [a] significantly different from each other, [b] significantly different from each other

Figure 1: Changes in the rate of net fluid secretion (i.e. in the s-to-m direction) (ΔJ_v) elicited by various nucleotides at 10μM, applied mucosally to excised pigmented rabbit conjunctival tissues mounted on an adapter with a circular aperture of 0.385 cm^2 bathed on both sides with BRS maintained at 37^oC and pH 7.4. Each data point represents mean ± s.e.m of 3-4 tissues. * Significantly different from the baseline value.

Figure 2: Correlation between the changes in nucleotide-induced short circuit current (ΔI_{sc}) and those in nucleotide-induced fluid secretion (ΔJ_v) across pigmented rabbit conjunctiva. r^2, correlation coefficient. Each data point represents mean ± s.e.m of 3-4 tissues. Key: a, UTP; b, UDP; c, UMP; d, ATP; e, ADP; f, AMP; g, adenosine; h, α,β-methyleneATP; i, 2-MeSATP; j, 2-MeSADP; k, Bz-ATP; l, ATP-γ-S (0.1μM); m, ATP-γ-S (1μM); n, ATP-γ-S (10μM); and o, ATP-γ-S (100μM).

Figure 3: Dose-response curves for the changes in net fluid secretion in the pigmented rabbit conjunctiva stimulated by UTP (■) and ATP (?) (0.1μM – 1 mM). Each data point represents mean ± s.e.m of 3-4 tissues.

Figure 4: Dose-response curves for the changes in short circuit current (ΔI_{sc}) and net fluid secretion (ΔJ_v) in the pigmented rabbit conjunctiva as a function of ATP-γ-S concentration. The concentration range of ATP-γ-S used was 0.1 μM – 5 mM for ΔJv and 0.1 μM – 1 mM for ΔIsc. Each data point represents mean ± s.e.m of 3-4 tissues.

Figure 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 bars represent the sham-infected conjunctiva (?) and the closed bars represent the Ad-5 infected conjunctiva (¦). All values are represented as mean ± s.e.m of 3-4 tissues. * significantly different from baseline (sham infected conjunctiva). † significantly different from baseline (Ad5 infected conjunctiva).

Figure 1

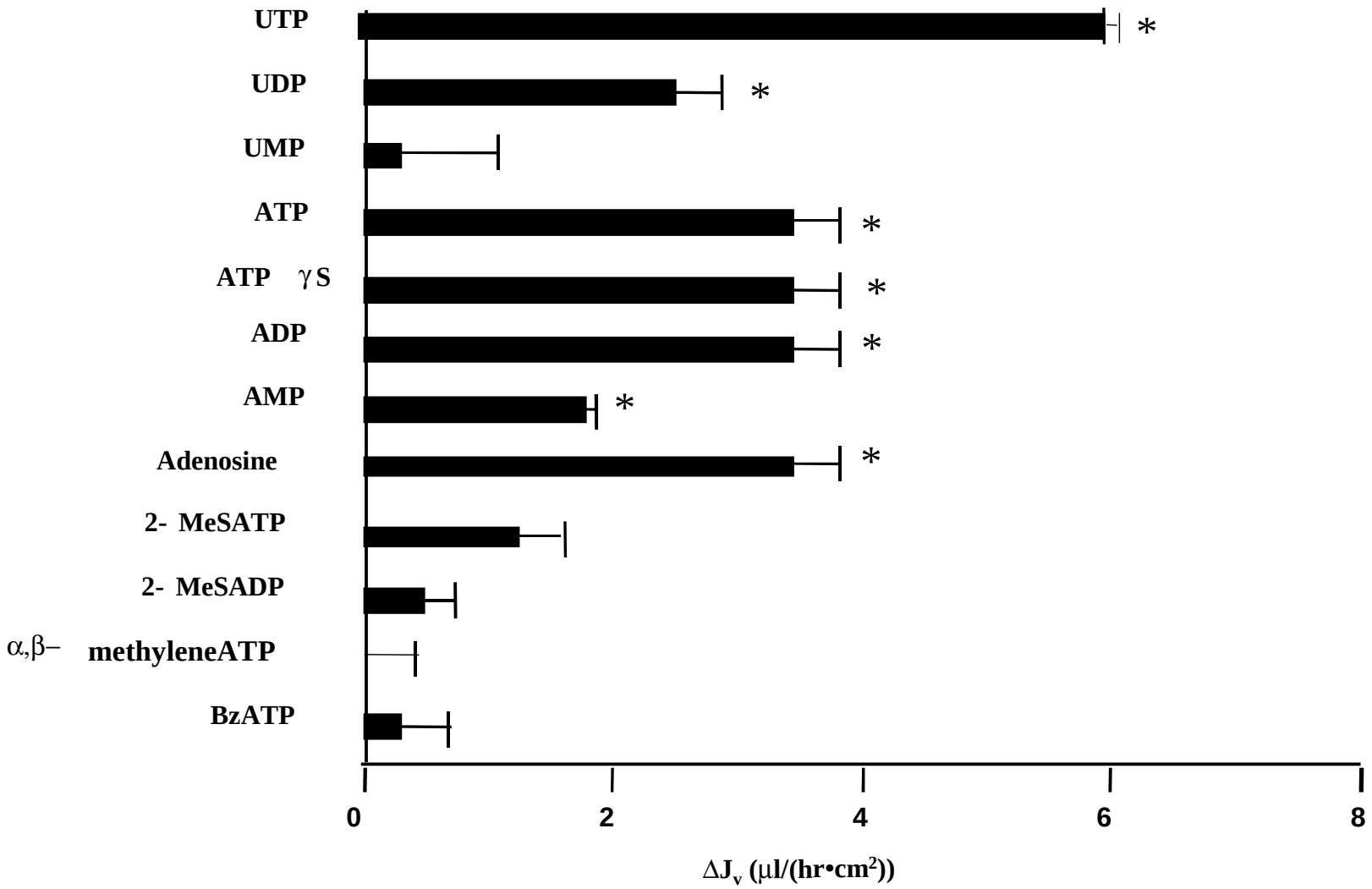

Figure 2

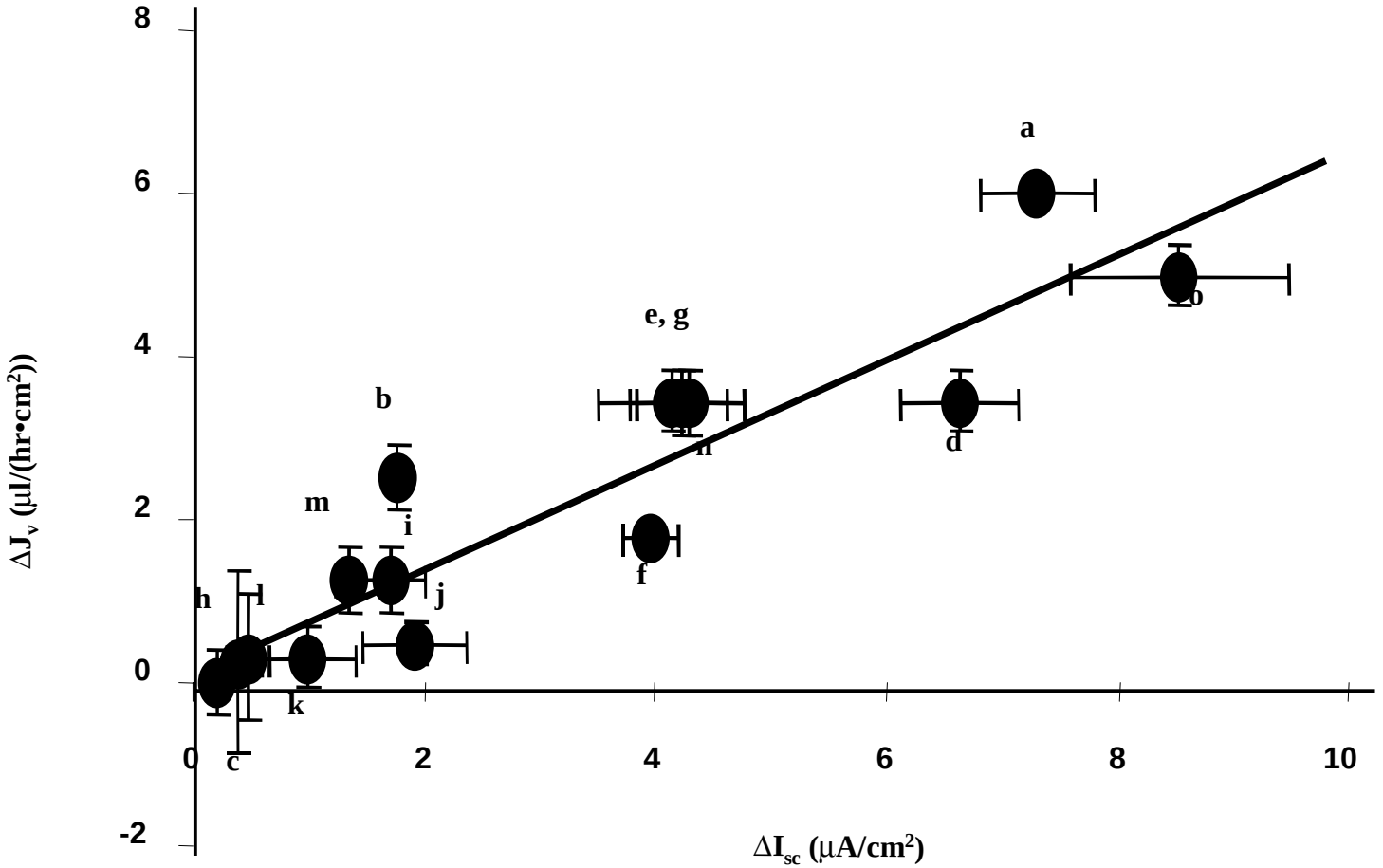

Figure 3

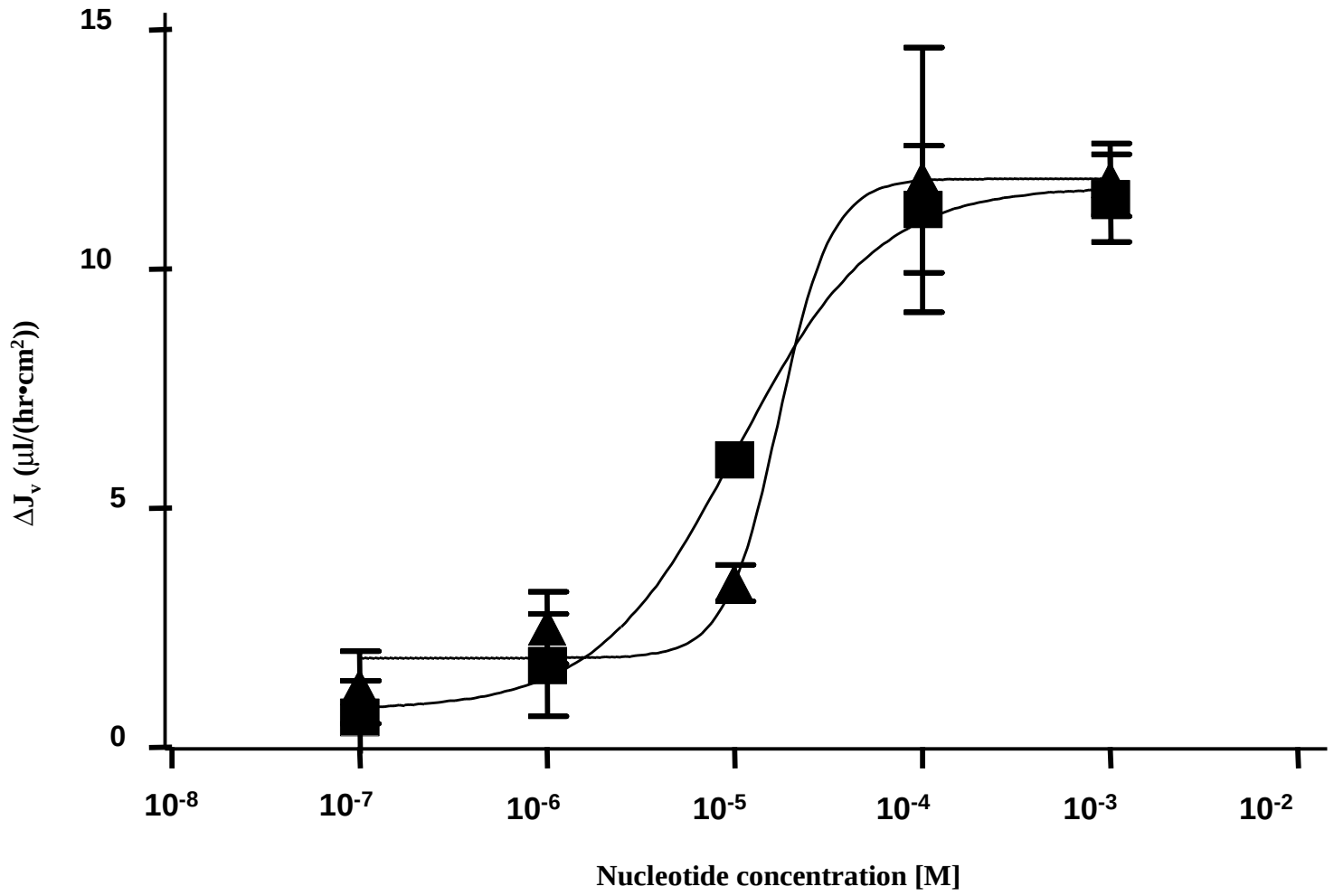

Figure 4

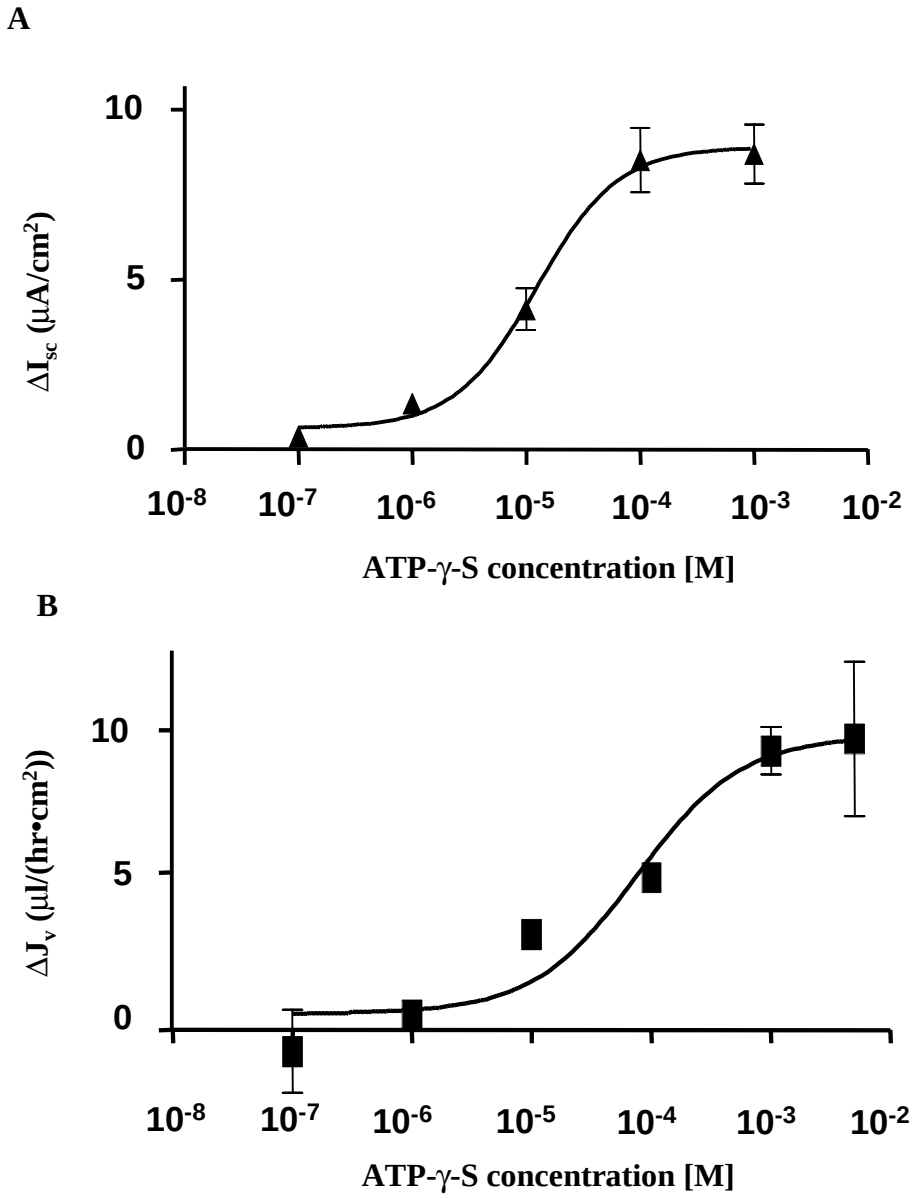

Figure 5

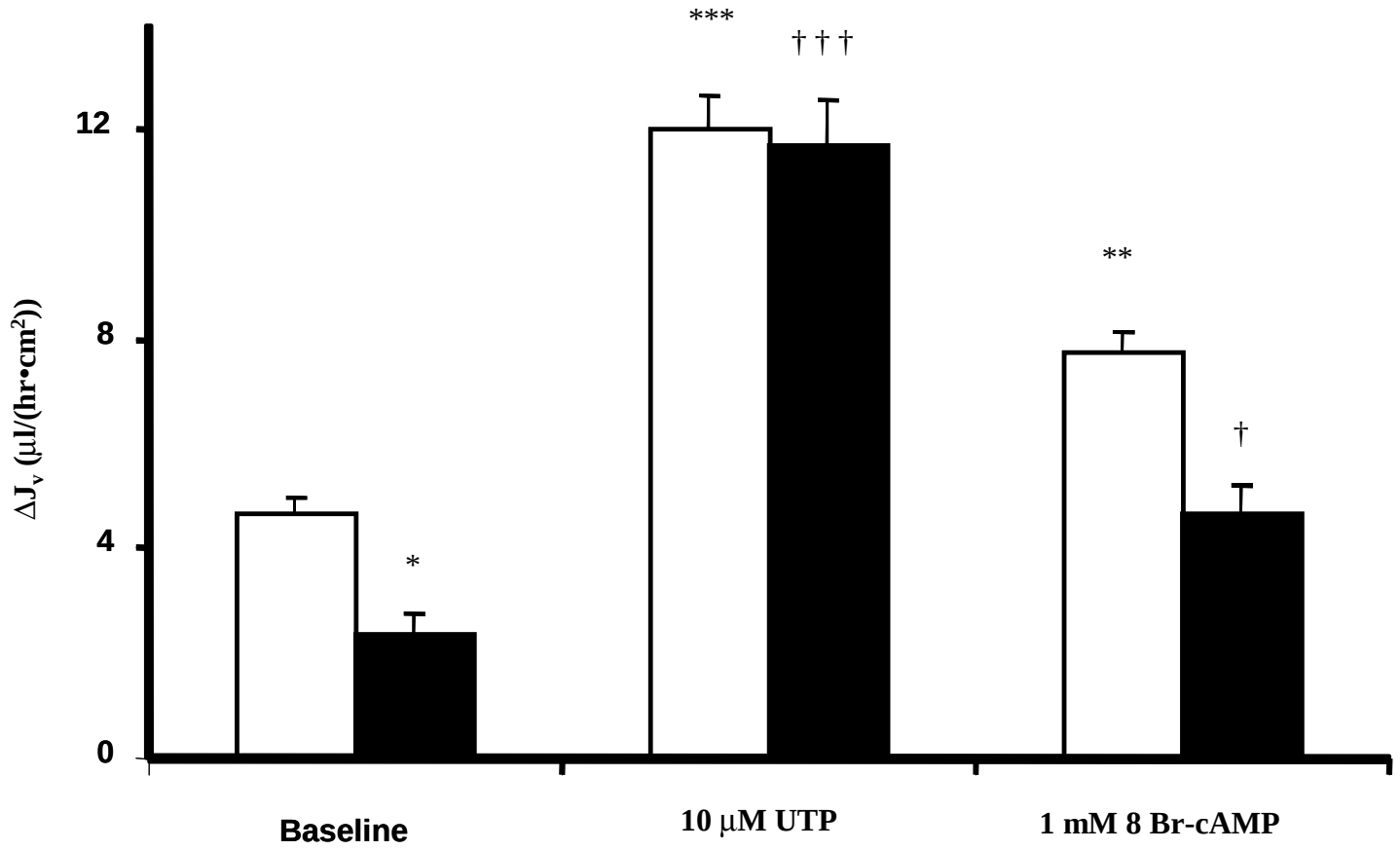

TABLE 1

BUFFER	Fluid secretion (μl/(hr•cm²))	
	- UTP	+ UTP
Regular BRS	4.7 ± 0.25^{a}	9.8 ± 1.06^{c}
Cl^- free BRS	$0.94 \pm 0.66^{a,b}$	$1.88 \pm 0.01^{b,c}$

TABLE 2

		Cl^- flux ($\mu Eq/(hr \cdot cm^2)$)			D-mannitol P_{app} (x 10^{-7} cm/s)		
Ad5	**UTP**	J^{sm}	J^{ms}	J^{net}	J^{sm}	J^{ms}	J^{net}
-	-	0.59 ± 0.05 (10)	0.34 ± 0.05 (7)	0.25 ± 0.06	1.82 ± 0.20 (10)	2.1 ± 0.29 (7)	-0.28 + 0.11
	+	0.97 ± 0.09 (10)	0.42 ± 0.10 (7)	0.55 ± 0.13[a]	2.01 ± 0.19 (10)	1.99 ± 0.30 (7)	0.02 ± 0.36
+	-	0.36 ± 0.10 (5)	0.25 ± 0.08 (4)	0.11 ± 0.10[a,b]	3.2 ± 0.89 (5)	2.33 ± 0.21 (4)	0.87 ± 0.91
	+	0.64 ± 0.07 (5)	0.31 ± 0.18 (4)	0.33 ± 0.19[b]	2.49 ± 0.33 (5)	2.25 ± 0.41 (4)	0.24 ± 0.53