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Role of cyclic AMP-induced Cl conductance in aqueous humour formation by the dog ciliary epithelium

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1 The effects of isoprenaline, a forskolin derivative NKH-477, and dibutyryl cyclic AMP (db cyclic AMP) on the membrane potential, conductance and cell volume of the dog non-pigmented ciliary epithelium (NPE) were investigated by intracellular potential recording, nystatin-perforated patch clamp technique and videomicroscopic cytometry.

2 The resting membrane potential of NPE was about −70 mV in physiological saline and was depolarized by isoprenaline in a dose-dependent manner with an ED_{50} of about 3 nM. This depolarization was competitively antagonized by the β-adrenoceptor antagonist, timolol (pA_2 = ca. 9) and almost completely blocked by the Cl transport blocker, DIDS.

3 In single dissociated NPE cells, 10 μM isoprenaline induced an inward current and caused a concomitant decrease in cell volume. The reversal potential measurement indicated that this inward current was carried mainly by Cl ion. DIDS (10 μM) abolished both the current and cell volume decrease.

4 NKH-477 (10 μM) or db cyclic AMP (1 mM) also induced an inward current together with a cell volume decrease, the properties of which were similar to those caused by isoprenaline.

5 These results suggest that β-adrenoceptor stimulation in NPE leads to an increased rate of aqueous humour production by increasing Cl^- efflux via an elevation of cyclic AMP and this effect is efficiently blocked by timolol.

Keywords: β-Adrenoceptor; cyclic AMP; Cl current; aqueous humour; dog ciliary epithelium

Introduction

The intraocular pressure (IOP) is critically controlled by the balance between aqueous humour production in the ciliary epithelium and its outflow from the anterior chamber, and disorders disrupting this balance are believed to form the main aetiological basis for glaucoma. Although the physiology of these processes is not fully understood due to technical difficulties arising from the complex morphology of this epithelium, it is generally agreed that the non-pigmented layer of the ciliary epithelium (NPE) plays a central role in the formation of aqueous humour (Okisaka *et al.*, 1974; Caprioli, 1987; Bartels, 1989).

It has been suggested that production of aqueous humour involves primarily the active secretion of Na ion to the aqueous side, and this process is attributed largely to the Na^+,K^+-ATPase which is densely distributed on the basolateral membrane of NPE cells (Caprioli, 1987; Usukura *et al.*, 1988; Bartels, 1989; Sears, 1991). According to this hypothesis, the standing Na^+ gradient created by Na^+,K^+-ATPase osmotically drives water into the intercellular invaginated spaces of NPE cells and subsequently into the posterior chamber (Diamond & Bossert, 1967; Cole, 1961; Caprioli, 1987; Bartels, 1989). There is, however, evidence arguing that other ionic transport systems also participate in the production of aqueous humour. In several species including the dog, the transepithelial potential is found to be negative on the aqueous side and depends on chloride and bicarbonate concentrations (Holland & Gipson, 1970; Kishida *et al.*, 1981; Iizuka *et al.*, 1984). Furthermore, a net flux from the stromal to the epithelial side has been proved for Cl^- but not for Na^+ (Kishida *et al.*, 1983). These findings are not consistent with the dominance of Na^+ secretion, but suggest a possible role of Cl^- transport in aqueous humour production.

β-Adrenoceptor antagonists have recently been introduced in the treatment of glaucoma. Many clinical investigations have found that these drugs, especially timolol, are useful in lowering the IOP without affecting the outflow facility (Katz *et al.*, 1976; Zimmerman *et al.*, 1977). The mechanism underlying this effect is supposed to be related in part to the modulatory role of the sympathetic nervous system on the rate of humour production. There is however confounding controversy over the effects of β-adrenoceptor agonists such as adrenaline and isoprenaline, depending on the species and experimental conditions. For example, it has been reported that β-adrenoceptor stimulation or application of agents which increase adenosine 3′:5′-cyclic monophosphate (cyclic AMP) leads to the reduction in both the IOP and aqueous flow in the rabbit (Caprioli *et al.*, 1989; Sears, 1991), whereas the opposite evidence has been presented in the monkey and human being (Miichi & Nagataki, 1983; Schenker *et al.*, 1981). Further, a stimulatory role of adrenergic activity on humour production and IOP and its blockade by timolol have been demonstrated under conditions where the circadian rhythm of neural activities is preserved (Topper & Brubaker, 1985; Yoshitomi *et al.*, 1991). Some explanations have been attempted to resolve these discrepancies (Sears, 1991), but we feel that more simplified experimental conditions are preferable to gain more straightforward information about the modulatory role of β-adrenoceptor agents on the aqueous humour production. To this end, in the present experiments, we decided to measure both the net ionic and water movement across the NPE cell membrane of dogs using single isolated NPE cells, and to examine their causal relationship. As a result of the work, we have found that a net Cl^- efflux induced by an increase in the intracellular cyclic AMP is likely to produce a concomitant outflow of water from NPE cells.

Methods

Cell isolation

All procedures used here accord with the ARVO resolution. Mongrel dogs of either sex weighing 10∼15 kg were exsan-

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guinated under anaesthesia with intravenous administration of sodium pentobarbitone (30 mg kg^{-1}). Both eye balls were quickly enucleated and transferred to physiological salt solution (PSS). For isolating the ciliary epithelium, we adopted the method described by Sears *et al.* (1991) with a minor modification. In brief, the eye was cleaned of attached connective tissue under a binocular microscope so that the two posterior ciliary arteries became clearly visible. After soaking in 100% methanol for several minutes to reduce contaminating microorganisms, 5 ml phosphate buffered saline (PBS) containing dispase (Gohdoh Shusei, Tokyo, Japan; 1000 p.u. ml^{-1}) was injected into the ciliary artery at a rate of about 0.5–1 ml min^{-1} through a 27-gauge subcutaneous needle. Thereafter, the eye was opened posteriorly and the ciliary segment was excised. A ring of ciliary body corresponding to the pars plicata was excised and then incubated in the Ca,Mg-free PSS containing dispase (1000 p.u. ml^{-1}) for 30–45 min at 35°C equilibrated with 5% CO_2. At completion of chemical digestion, a thin double layer of ciliary epithelium was almost detached from the stroma; thus we could easily strip it off from the stroma with fine forceps. The resultant epithelial sheet was then fixed to the rubber of a dish with the non-pigmented epithelial side up. A stream of PSS was ejected through a 23-gauge needle along the epithelial surface, resulting in release of a sufficient number of cells. In some experiments, a digested sheet of epithelium was minced and triturated with a blunt tipped Pasteur pipette. Dispersed cells were stored in Dulbecco's modified Eagle medium complemented with 33 mM bicarbonate and 10% foetal bovine albumin. Cells dissociated from the non-pigmented epithelial layer were readily identified by the absence of black pigment inside. Staining with 0.3% trypan blue revealed that nearly 90% of yielded cells were viable.

Patch clamp experiments

The experimental set-up used for generation of voltage commands and acquisition of data was the same as described by Chen *et al.* (1993). The liquid junction potential arising at the interface between the external PSS and the Cs-rich, Cl-reduced internal solution was about 5 mV. This potential was corrected *a posteriori* by shifting the current-voltage relationship by 5 mV toward the hyperpolarizing direction. Details of the nystatin perforated and conventional whole cell recordings have been described by Chen *et al.* (1993).

Videomicroscopic cytometry

Changes in cell size were monitored under an inverted phase-contrast microscope (Diaphot TMD, Nikon, Japan) equipped with a CCD camera (CS3130, Teli, Japan) and a TV monitor (12M310, Teli, Japan). Images were stored on a video tape connected through a casette tape recorder (AG-7350, National, Japan) and replayed later for off-line analysis: two cell diameters along the horizontal (D1) and vertical (D2) axes on the TV monitor screen were measured at an interval of 10 s using a width analyser (C3161, Hamamatsu, Japan). During about the first 10 s after the start of solution change, video images were distorted by stirred surface of bath solution. The measurement was therefore started at a point of 10 s from the beginning of solution change. Measured values were calibrated using an objective micrometer (Nikon, Japan). The change in relative cell volume was then calculated according to the following equation:

$$V/V_0 = (D1*D2)^{3/2}/(D1_0*D2_0)^{3/2}$$

where V_0, $D1_0$ and $D2_0$ represent the initial volume, vertical and horizontal diameters of the cell respectively, and V represents the cell volume at subsequent measurements. During application of drugs tested, the cell size also changed in the direction perpendicular to the floor of the experimental chamber, which obscured the maximum diameter of the cell. We tried to minimize this error by refocussing manually throughout the recording. All cytometric measurements shown in the figures were carried out under electrically unclamped conditions, since the patch electrode blurred the image of NPE cells.

Intracellular recording

After opening the eye posteriorly, a thin small ridge of the pars plicata was dissected from the ciliary body and tightly pinned on the rubber bottom of the experimental chamber with the aqueous side up. In some experiments, an isolated sheet of double-layered ciliary epithelium was used. To equilibrate the tissue, the chamber was continously perfused with PSS (see below) which was aerated with 5% CO_2 and 95% O_2 and prewarmed at 32–34°C for 1 h. Microelectrodes of 40–100 MΩ resistance were fabricated with a puller (PG-1, Narishige, Japan) and filled with 3 M KCl, and a Ag/AgCl electrode was used as the ground. The microelectrode was connected to the head stage of a high-impedance preamplifier (MEZ-7101, Nihon Kohden, Japan) and voltage signals were monitored on a high gain oscilloscope (VC-9, Nihon Kohden, Japan) and a brush pen recorder (RJG-4024, Nihon Kohden, Japan). Impalement of the ciliary epithelium cells was achieved by use of a three-dimensional manipulator (Narishige, Japan) with the aid of a binocular microscope. Recordings were only started if the potential after impalement had stabilized.

Solutions

The composition of the solutions used in the present experiments was as follows (mM): PSS: Na^+ 142.2, K^+ 4.7, Mg^{2+} 1.2, Ca^{2+} 1.8, Cl^- 118.4, HCO_3^- 33.2, $H_2PO_4^-$ 1.2, glucose 6.9; PBS: Na^+ 153.1, K^+ 4.2, Cl^- 139.6, $H_2PO_4^-$ 1.5, HPO_4^{2-} 8.1; KCl internal solution for nystatin-perforated recording: K^+ 140, Mg^{2+} 2, Cl^- 44, EGTA 1 and HEPES 10 (titrated to pH 7.2–7.25 by Tris base); Cs-rich, Cl-reduced internal solution: Cs^+ 140, Mg^{2+} 2, Cl^- 24, aspartate$^-$ 110, Na_2ATP 2, EGTA 10/4~$5Ca^{2+}$ and HEPES 10 (titrated to pH 7.2–7.25 with Tris base). PSS was aerated with 5% CO_2 and 95% O_2, and prewarmed to 32–34°C, unless otherwise stated. The pH of PSS equilibrated with 5% CO_2 was about 7.5. For preparing Ca,Mg-free PSS, Ca^{2+} and Mg^{2+} were simply omitted from PSS.

Chemicals

Isoprenaline hydrochloride, acetylcholine chloride, phenylephrine hydrochloride, noradrenaline hydrochloride, timolol maleate and dibutyryl cyclic AMP (Na salt), were purchased from Wako (Osaka, Japan). Tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), $BaCl_2$, CsCl and 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) were purchased from Sigma (St. Louis, U.S.A.). 3-(Dimethylaminopropionyl)-forskolin (NKH-477) was kindly given by Dr Takeo Itoh, Department of Pharmacology, Kyushu University. All drugs used were freshly prepared from the powder for each experimental day.

Statistics

Results were expressed as mean ± standard deviation. Statistical difference was evaluated by Student's *t* test.

Results

Isoprenaline-induced depolarization of the non-pigmented epithelial membrane

The mean resting membrane potential of the non-pigmented layer of dog ciliary epithelium (Vm) was -71.0 ± 1.6 mV ($n = 20$) when superfused with physiological saline the com-

position of which was similar to the aqueous humour (e.g., Caprioli, 1987). Vm depended on the extracellular potassium concentration ($[K]_o$) (Figure 1). A ten fold increase in $[K]_o$ resulted in about 50 mV depolarization of the epithelium. Vm was also found to be sensitive to a number of drugs which are known to affect K and Cl transports (Table 1). Amongst the drugs listed in Table 1, quinine, Ba^{2+}, caesium and ouabain significantly depolarized the membrane, whereas very small or no effects were observed for tetraethylammonium, procaine, 4-AP and pinacidil even at high concentrations. DIDS caused no significant change in Vm at 10 μM (Table 1), but produced a small hyperpolarization at a concentration of 100 μM. This pharmacological profile suggests

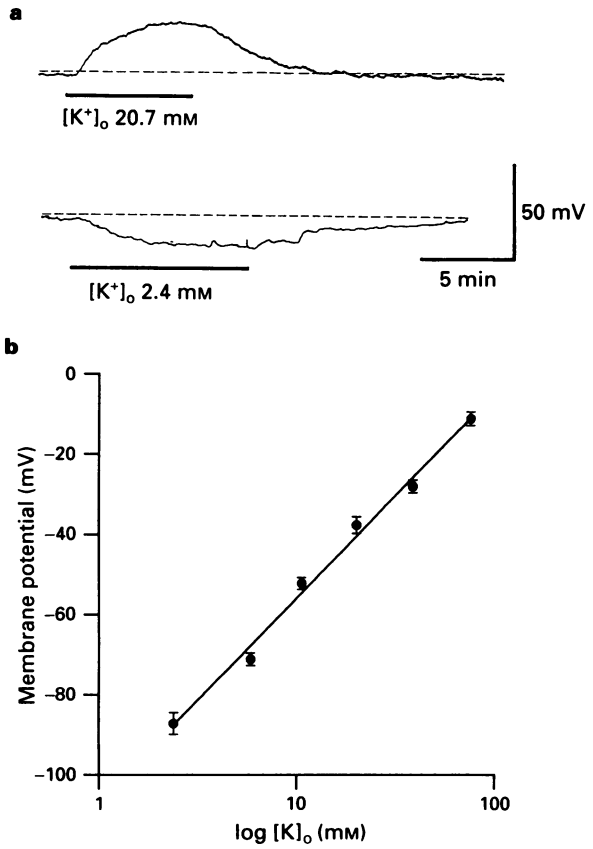

Figure 1 Effect of the external potassium concentration on the membrane potential (mV) of ciliary epithelium. The bath was continuously perfused with the gassed and prewarmed PSS. (a) Effect on Vm when the concentration of $[K]_o$ was changed from 4.7 mM. Dashed lines indicate − 70 mV. (b) The relationship between $[K]_o$ and Vm. Each datum point represents the mean with s.d. from 10–20 cells. The solid line is the best fit of data points according to the equation, $V_m = V_o{*}\log([K]_o/[K]_i)$, where V_o and $[K]_i$ = 50.8 mV and 127 mM, respectively ($r = 0.996$).

Table 1 Effects of various agents on the membrane potential of ciliary epithelium

Agent	*Membrane potential* (mV)
Control	− 71.0 ± 1.6 (20)
TEA 10 mM	− 71.4 ± 1.0 (9)
Procaine 10 mM	− 70.1 ± 1.0 (9)
4-AP 10 mM	− 70.7 ± 1.1 (7)
Quinine 0.1 mM	− 61.7 ± 1.4 (6)*
Ba^{2+} 0.1 mM	− 60.6 ± 0.9 (5)*
# Cs^+ 10 mM	− 41.5 ± 1.0 (4)*
DIDS 10 μM	− 70.1 ± 1.3 (10)
Ouabain 10 μM	− 60.8 ± 0.9 (10)*
Pinacidil 10 μM	− 71.6 ± 1.4 (7)

The values indicate mean ± s.d., and the number of experiments in parentheses.
Data obtained from isolated epithelial sheets. Statistically significant difference (*$P < 0.01$) from the value in the absence of drugs (control).

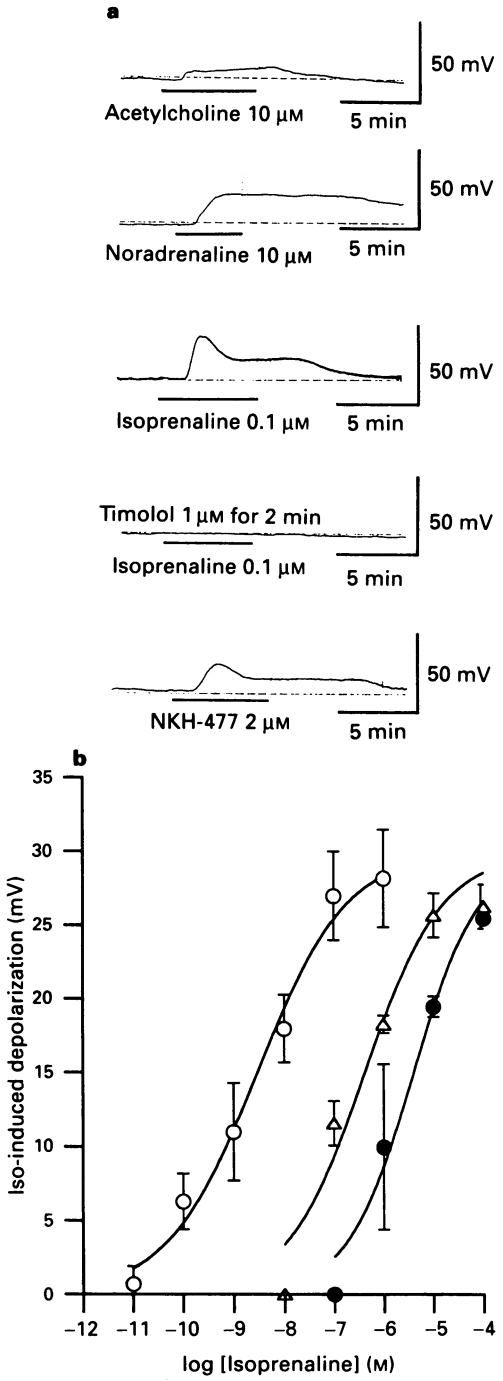

Figure 2 Effects of agents affecting intraocular pressure on the membrane potential of ciliary epithelium and the antagonism by timolol of isoprenaline. (a) Actual traces of membrane depolarization produced by 10 μM acetylcholine, 10 μM noradrenaline, 2 μM NKH-477 and 0.1 μM isoprenaline and antagonism of isoprenaline by 1 μM timolol. The drugs were present at the times indicated by the bars. Dashed lines indicate − 70 mV. (b) The peak of the isoprenaline-induced depolarization (measured from the resting membrane potential) plotted against the concentration of isoprenaline, in the absence (○) and presence of 0.1 (△) and 1 μM timolol (●). The curves are drawn according to the equation $V_o/(1 + (K_d/[Iso])^n)$, where V_o, K_d and n denote the maximum depolarization, the concentration of isoprenaline causing 50% of V_o and the Hill coefficient. The best fit values are 30 mV and 0.5 for V_o and n, and 3 nM (no timolol), 0.3 μM (0.1 μM timolol) and 4 μM (1 μM timolol) for the K_d values.

that K and Cl transporters and a Na^+/K^+ pump are functionally important in the control of Vm of ciliary epithelia.

It is well known that the intraocular pressure (IOP) is controlled by various neurotansmitters, autacoids and hormones (Caprioli, 1987). We therefore tested several representative drugs which are known to cause changes in IOP. As demonstrated in Figure 2a, acetylcholine, noradrenaline and isoprenaline all depolarized the ciliary epithelium, but the potency of isoprenaline was more than 100 times greater than the others. The depolarization induced by isoprenaline was blocked by β-adrenoceptor antagonists such as propranolol and timolol (Figure 2a). Figure 2b shows the relationship between the concentration of isoprenaline and the membrane potential of NPE. The threshold of depolarization was around 0.01 nM and half-maximum and maximum depolarization occurred at about 3 nM and 100 nM, respectively. Pretreatment with timolol shifted this dose-response curve to the right in a dose-dependent manner. The mode of this antagonism seems to be competitive with a pA_2 value of about 9. The effect of isoprenaline did not appear to result from stimulating nerve terminals on the stromal side of the epithelium, because quantitatively similar results were obtained with isolated sheets of ciliary epithelium (Table 2).

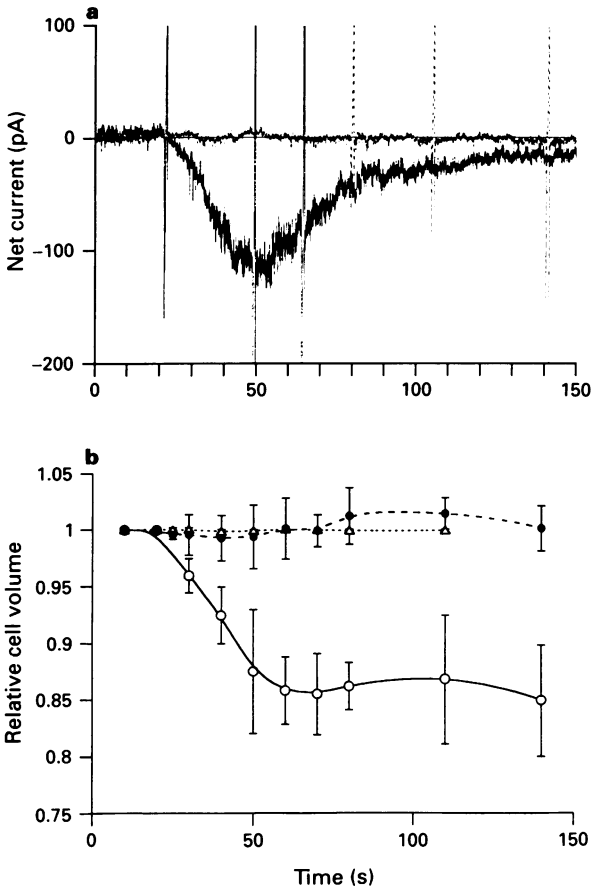

Figure 3 Time course of the isoprenaline-induced inward current and cell volume decrease. (a) Typical records of isoprenaline-induced inward currents in the absence (solid curve) and presence (dotted curve) of 10 μM DIDS, with nystatin-perforated recording (KCl internal solution in the pipette). 20 μM isoprenaline was introduced into the bath at time zero. The holding potential was −70 mV. Vertical deflections represent the currents responding to ramp voltages. (b) The time-dependent profile of cell volume change in the absence (○) and presence of 10 μM DIDS (●) or of 1 μM timolol (△). Isoprenaline (20 μM) was applied at time zero. Symbols represent the mean with s.d. from 5–11 cells. For the definition of relative cell volume, see the Methods and the legends to Table 3.

It has been reported that in the ciliary epithelium β_2-adrenoceptor stimulation elevates the intracellular cyclic AMP level by activating adenylate cyclase (Waitzman & Wood, 1971; Caprioli *et al.*, 1987). We therefore tested a newly synthesized derivative of forskolin, NKH-477, which is water-soluble and very potent at increasing intracellular cyclic AMP by directly activating the adenylate cyclase (Shafiq *et al.*, 1992). As shown in Figure 2a, superfusion with 2 μM NKH-477 produced a depolarization comparable in size to that induced by isoprenaline.

The pharmacological nature of the isoprenaline- or NKH-477-induced depolarization was investigated in the presence of various drugs listed in Table 2. Ouabain and Ba^{2+} reduced only partially but DIDS almost fully abolished the isoprenaline-induced depolarization. Furthermore, the extent of the depolarization was strongly attenuated by lowering the temperature of the superfusate to below 30°C.

These results suggest that isoprenaline applied at the aqueous side of non-pigmented epithelium is very efficacious in particular in producing a DIDS-sensitive ionic movement, presumably anions like Cl^-. It is however equivocal whether this is due solely to changes in the NPE cells or to combined actions of the two epithelial layers. To gain more insight, we explored the correlation between ionic and water movements by directly measuring the transmembrane current and cell volume change induced by isoprenaline, in single isolated NPE cells.

Isoprenaline induced inward current associated with a cell volume decrease

Single NPE cells were cuboidal or spherical in shape with an approximate diameter of 9～18 μm (13.4 ± 3.3, $n = 20$) and had a capacitance of 6.2 ± 0.8 pF (4～8, $n = 21$) when

Table 2 Effect of various drugs on the isoprenaline- and NKH-477-induced depolarization

	Membrane potential (mV)	
Drugs	*Intact ciliary body*	*Epithelial sheet*
Control	− 71.0 ± 1.6 (13)	− 67.9 ± 2.7 (16)
Isoprenaline 10 μM	− 52.7 ± 2.3 (9)*	− 57.0 ± 1.0 (5)*
+ TEA 10 mM	− 53.0 ± 1.0 (2)*	− 58.0 ± 6.0 (2)*
+ Cs^+ 10 mM	–	− 31.0 ± 1.7 (3)*
+ Ba^{2+} 1 mM	− 55.5 ± 1.5 (2)*	–
+ Ouabain 10 μM	− 48.7 ± 1.3 (10)*	− 53.3 ± 1.2 (3)*
+ DIDS 10 μM	− 65.9 ± 1.3 (10)*	− 68.0 ± 1.0 (3)
28～30°C	− 70.8 ± 1.3 (4)	–
NKH-477 10 μM	− 55.8 ± 1.1 (5)*	− 53.0 ± 1.7 (3)*
+ Ouabain 10 μM	− 57.4 ± 0.9 (5)*	–
+ DIDS 10 μM	–	− 68.0 ± 3.5 (3)

The values indicate mean ± s.d., and the number of experiments in parentheses. Statistically significant difference (*$P < 0.01$) from the values of controls for the intact ciliary body and isolated epithelial sheet, respectively.

placed in PSS containing the HCO_3^-/CO_2 buffer. In the absence of HCO_3^- and at room temperature, however, they showed a progressive decrease in size and deterioration. To minimize this, it was necessary to perfuse the cells continuously with the bicarbonate-containing PSS gassed with 5% CO_2 and 95% O_2 and prewarmed at 32~34°C throughout the experiment.

Using the nystatin perforated whole-cell recording, administration of 20 μM isoprenaline into the bath evoked an inward current associated with an increased noise level at a holding potential of −70 mV (Figure 3a). The current reached a peak within a few tens of seconds and persisted after washout of isoprenaline. The peak amplitude of this inward current varied greatly from cell to cell (−5~−123 pA; −29.3 ± 14.4, $n = 35$), and this may have been due to the different extent of enzymic digestion of NPE cells. The isoprenaline-induced current was almost completely blocked by pretreatment with 1 μM timolol or 10 μM DIDS (Figure 3a) and was dependent on the temperature. When the temperature of the superfusate was lowered to 25–30°C, isoprenaline failed to evoke measurable inward currents (Table 3).

Administration of isoprenaline brought about a reduction in size of NPE cells and the time course appeared to parallel the development of the inward current (Figure 3). This cell volume decrease was sensitive to the same procedures used above. Namely, pretreatment with 1 μM timolol or 10 μM DIDS or lowering the temperature resulted in great attenuation of cell volume decrease (Figure 3b and Table 3).

Isoprenaline-induced current is a Cl current

Figure 4 shows the current-voltage relationships ($I-V$-curves) with the nystatin perforated recording. In most NPE cells examined, the $I-V$ curve was nearly ohmic or slightly inward rectifying at potentials between −150 and −50 mV, but outward-rectifying at more positive potentials. The leak conductance of NPE cells calculated from the linear portion of $I-V$ curve near the resting membrane potential (−70 mV) was 0.53 ± 0.24 nS ($n = 10$), a value similar to that reported elsewhere (Gooch *et al.*, 1992). TEA (10 mM) or quinine (0.1 mM) strongly suppressed the outward current at potentials positive to −50~−40 mV (Figure 4a), whereas Cs^+ (10 mM) (Figure 4b) or Ba^{2+} (1 mM) caused a substantial decrease in the membrane conductance near the resting membrane potential. Isoprenaline (10 μM) shifted this $I-V$ curve in the inward direction (Figure 5a). Figure 5b shows that for the current sensitive to 10 μM isoprenaline, there is no apparent reversal potential. Although at more negative potentials the difference current became more inward, it is difficult to

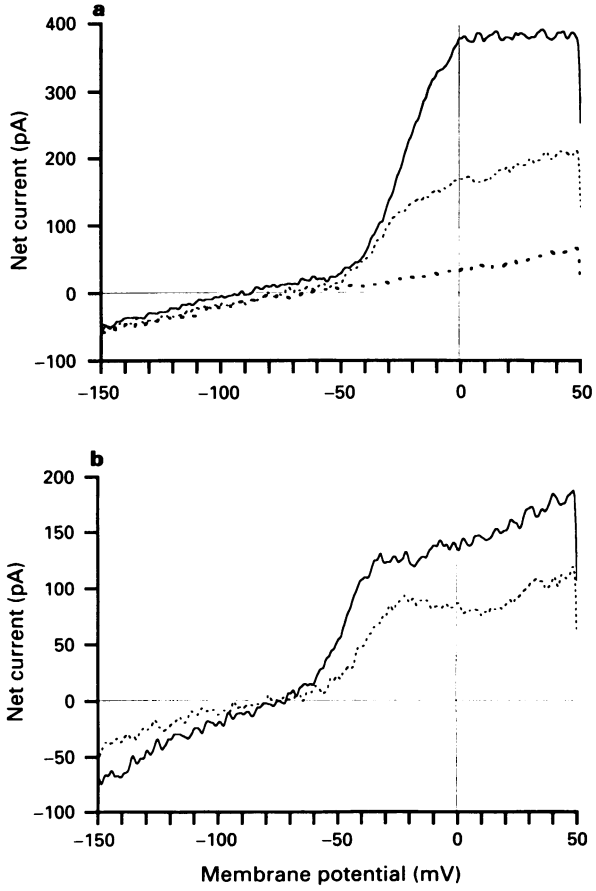

Figure 4 Current-voltage relationships (I-V curves) of non-pigmented epithelial (NPE) cells. Nystatin-perforated recordings with KCl internal solution. From a holding potential of −70 mV, ramp voltages (−150~+50 mV, 0.25 V/s) were applied for 800 ms. This minimized the change in NPE cell size due probably to perturbation of intracellular ionic content, which usually occurred on repeated application of voltage pulses. (a) I-V curves in the absence (solid curve) and presence of 10 mM TEA (thin dotted curve) or 0.1 mM quinine (thick dotted curve). (b) I-V curve in the absence (solid curve) and presence of 10 mM Cs^+ (dotted curve).

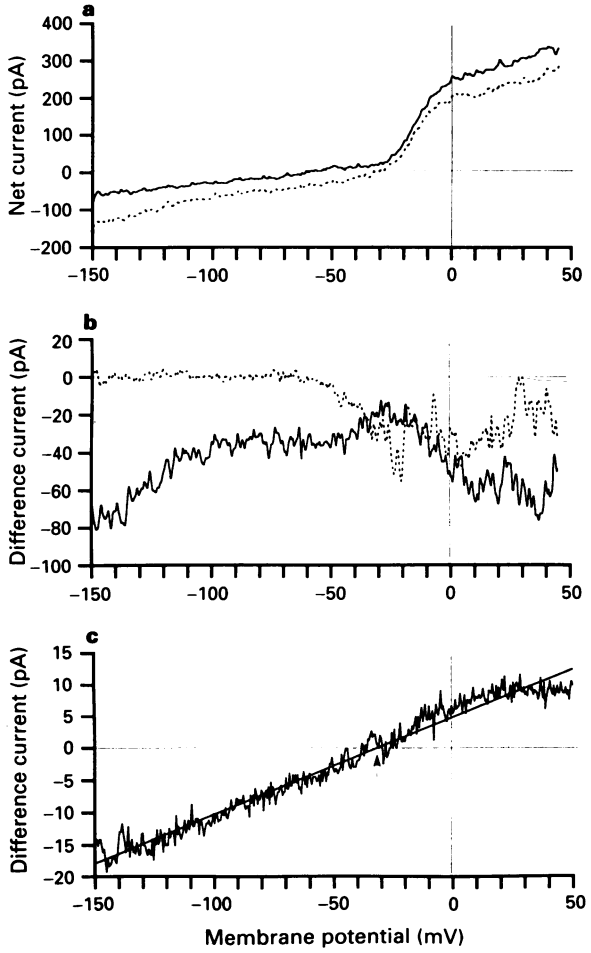

Figure 5 I-V curves of the isoprenaline-induced current. Nystatin-perforated recordings with KCl internal solution. The ramp protocol used was the same as in Figure 4. (a) Net currents in the absence (solid curve) and presence (dotted curve) of 10 μM isoprenaline are plotted against the membrane potential. (b) The difference in net currents recorded in the presence and absence of 10 μM isoprenaline (hereafter referred to as the isoprenaline-sensitive current) is plotted against membrane potential without (solid curve) and with (dotted curve) pretreatment of 10 μM DIDS. (c) The I-V curve for the isoprenaline (10 μM)-sensitive current in the presence of 10 mM TEA. An arrow indicates −31 mV.

define the reversal potential. Pretreatment with 10 μM DIDS selectively blocked the portion below − 50 mV of the *I–V* curve (dotted curve in Figure 5b). We considered these results to be a consequence of a complex effect of isoprenaline on the membrane conductance of the NPE cell, i.e. induction of inward current and suppression of outward current. Therefore, we repeated the same experiment in the presence of 10 mM TEA or 10 mM CsCl to separate the two effects, because these drugs have been found to suppress the K outward current at positive potentials (TEA) and the inward rectifying K current at very negative potentials (CsCl), respectively (Gooch *et al.*, 1992). As shown in Figure 5c, in the presence of 10 mM TEA, the isoprenaline-sensitive current reversed near − 30 mV, mean − 23.3 ± 6.1 mV ($n = 5$). Similar values of reversal potential were obtained with 10 mM Cs^+ (− 20.4 ± 3.6 mV, $n = 5$). However, in this case, suppression of the remaining outward current by isoprenaline at very positive potentials was sometimes observed. Such data were excluded from the evaluation. It should be noted that the reversal potentials of about − 20 mV accords with the Cl^- equilibrium potential of intact ciliary epithelium that can be estimated from intracellular Cl^- concentration measurements (ca. 60 mM, Wiederholt & Zadunaisky, 1986).

In the next series of experiments, the cells were dialysed with the Cs-rich, Cl-reduced internal solution, in order to suppress K current and shift the theoretical equilibrium potential for Cl^- towards more negative potentials. Under these conditions, isoprenaline still evoked inward currents at a holding potential of − 70 mV (Figure 6a). The *I–V* curve of the isoprenaline-sensitive current is slightly outward-rectifying, and reversed polarity at − 42.3 ± 7.1 mV ($n = 5$) (solid line in Figure 6b). This reversal potential is in good agreement with the theoretical equilibrium potential of Cl^- under these experimental conditions (− 42.4 mV, at 34°C). Pretreatment with 10 μM DIDS almost completely abolished the ability of isoprenaline to induce the inward current (dotted line in Figure 6b). The chord conductance of isoprenaline-induced current at − 70 mV with the Cs pipette was 0.79 ± 0.29 nS ($n = 5$), which is somewhat smaller than that obtained with the nystatin-perforated recording (0.94 ± 0.47 nS; Table 3). This value, however, is reasonable to account for the maximum depolarizing effect of isoprenaline (ca. 30 mV, see Discussion). These results strongly indicate that the main conductance which contributes to the inward current induced by isoprenaline can be ascribed to Cl^-, thus suggesting that near the resting membrane potential isoprenaline produces efflux of Cl^- from NPE cells.

Pretreatment with 10 μM ouabain significantly reduced the conductance induced by isoprenaline recorded with the nystatin perforated patch electrode ($P < 0.01$; the values marked

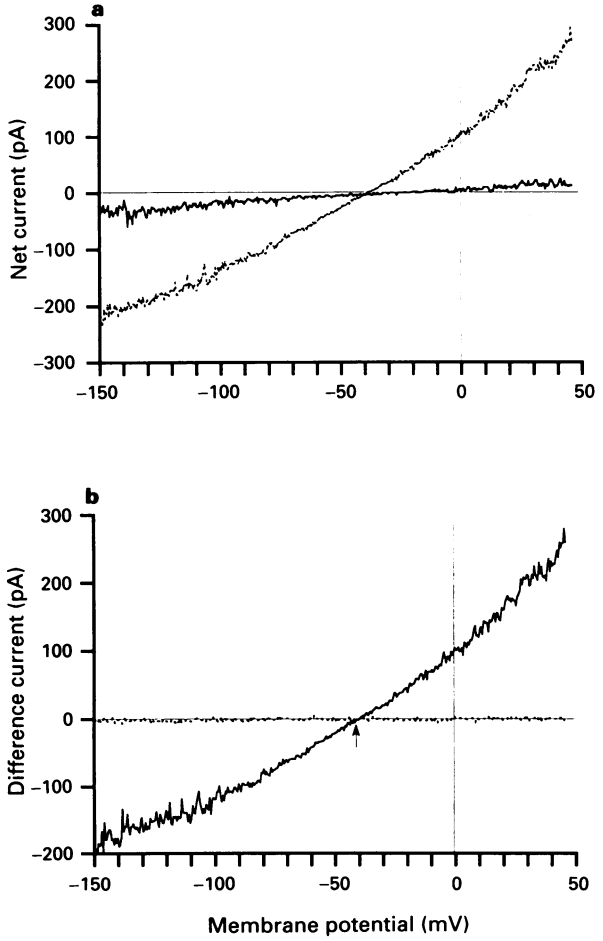

Figure 6 *I-V* curves of isoprenaline-induced currents recorded from Cs^+-loaded NPE cells. Experimental conditions were the same as in Figure 4 except that cells were dialysed with Cs-rich, Cl-reduced internal solution (see Methods) via a patch electrode. (a) Net currents recorded in the absence (solid curve) and presence (dotted curve) of 20 μM isoprenaline. (b) The *I-V* curve of the isoprenaline-sensitive current from (a) (solid curve) and that in the presence of 10 μM DIDS (dotted curve). An arrow indicates the equilibrium potential for Cl^- under these experimental conditions (− 42 mV at 34°C).

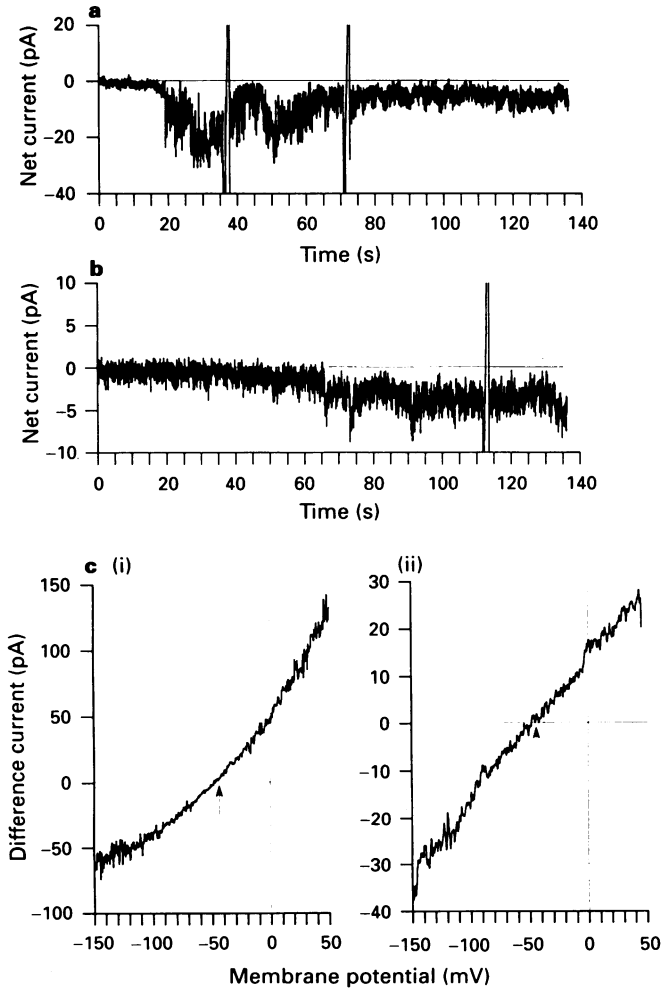

Figure 7 Membrane currents induced by NKH-477 and db cyclic AMP, and their *I-V* curves. The cell was dialysed with the Cs-rich, Cl-reduced internal solution. (a) and (b) At a holding potential of − 70 mV, 10 μM NKH-477 (a) or 1 mM db cyclic AMP (b) were applied at time zero. Small inward currents developed slowly. Vertical deflections represent ramp currents. (c) *I-V* curves for 10 μM NKH-477- (i) and 1 mM db cyclic AMP- (ii) sensitive currents. Arrows indicate the equilibrium potential for Cl^-.

with #, Table 3). However, this reduction became very small ($P > 0.05$) when the cell was dialysed with the Cs-rich, Cl-reduced internal solution. This may suggest a permissive role of the Na^+/K^+ pump in the control of isoprenaline-induced inward current.

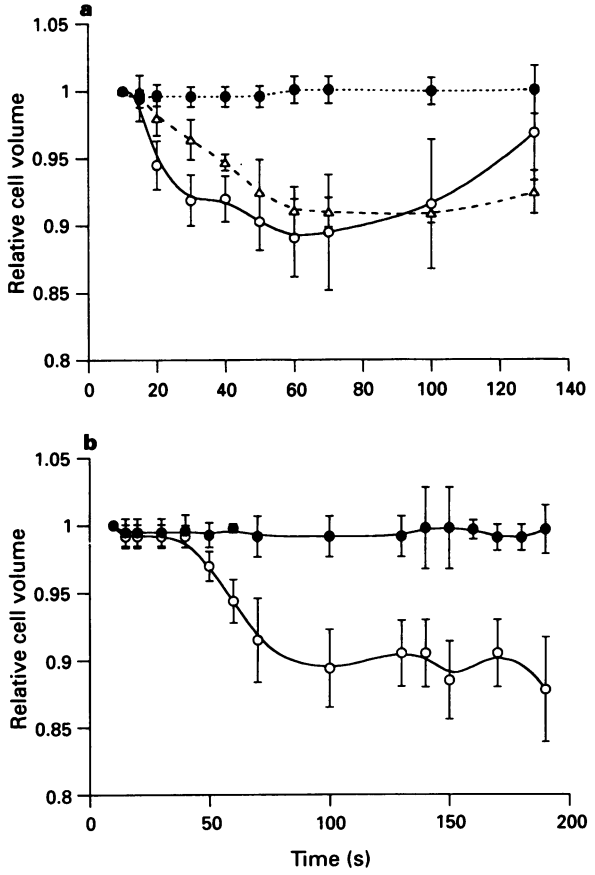

Figure 8 Cell volume decrease induced by NKH-477 and db cyclic AMP. NKH-477 or db cyclic AMP were applied at time zero. (a) Time course of cell volume decrease induced by 10 μM NKH-477, in the absence (○) and presence of 10 μM DIDS (●) or 1 μM timolol (△). (b) Time course of cell volume change induced by 1 mM db cyclic AMP, in the absence (○) and presence of 10 μM DIDS (●). Symbols represent the mean ± s.d. Data were obtained from 3–8 cells.

Induction by a forskolin derivative, NKH-477 and dibutyryl cyclic AMP of cell volume decrease and a current with similar properties

Similar results to the above were obtained when NKH-477 or a membrane permeable analogue of cyclic AMP, db cyclic AMP was administered. As shown in Figure 7, 10 μM NKH-477 or 1 mM db cyclic AMP induced an inward current which reversed near the Cl^- equilibrium potential (Figure 7c) and was blocked by 10 μM DIDS (Table 3). The reversal potentials of NKH- and db cyclic AMP induced currents with the Cs-rich, Cl-reduced internal solutions were -40.7 ± 6.7 mV ($n = 6$) and -46.2 ± 6.3 mV ($n = 4$), respectively. The cell volume decrease induced by 10 μM NKH or 1 mM db cyclic AMP was also greatly reduced by pretreatment with 10 μM DIDS, but not with 1 μM timolol (Figure 8).

Isoprenaline-induced cell volume decrease shows a similar pharmacological profile

Table 3 summarises the effects of various blockers and low temperature on the isoprenaline-induced inward current and cell volume decrease. Most noteworthy here is that DIDS and low temperatures are very efficacious in suppressing both the isoprenaline-induced conductance and cell volume decrease.

Discussion

The present study clearly shows that isoprenaline and other agents that increase intracellular cyclic AMP cause the cell volume to decrease along with activation of a Cl^- inward current in the dog NPE. These two cellular changes seem to be causally related because they occur with a similar time course and show similar sensitivities to the drugs tested (e.g. DIDS). The data obtained from the intact and isolated ciliary epithelia with intracellular potential recording also suggest that this could be extrapolated to the multicellular condition.

Historically, most of work regarding the aqueous humour production has emphasized the importance of a steady state standing Na^+ gradient existing between the interdigitated space of NPE cells and the posterior chamber. This gradient is thought to be maintained by the Na^+/K^+-ATPase localized on the basolateral membrane of NPE and to cause

Table 3 Summary of effects of isoprenaline, NKH-477 and db cyclic AMP on the membrane conductance and cell volume

	Relative cell volume (%)	*Conductance induced* (nS)
Isoprenaline 20 μM	85 ± 4 (11)	0.79 ± 0.29 (5) (#0.94 ± 0.47 (15))
+ TEA 10 mM	89 ± 1 (4)	0.42 ± 0.07 (5)**
+ Cs^+ 10 mM	92 ± 1 (3)**	0.28 ± 0.05 (5)*
+ Ouabain 10 μM	91 ± 2 (4)**	0.58 ± 0.15 (6) (#0.25 ± 0.1 (5))*
+ DIDS 10 μM	101 ± 1 (5)*	NC (4) (#NC(9))
25–30°C	97 ± 2 (6)*	NC (5)
NKH 10 μM	89 ± 3 (8)	0.41 ± 0.28 (5)
+ Ouabain 10 μM	95 ± 2 (5)*	–
+ DIDS 10 μM	95 ± 2 (5)*	NC (4)
db cyclic AMP 1 mM	88 ± 4 (3)	0.27 ± 0.13 (6)
+ DIDS 10 μM	99 ± 2 (4)*	NC (3)

The values indicate mean ± s.d., and the number of experiments (in parentheses). The relative cell volume is the percentage of the initial cell volume (just before drug application) 1 ~ 2 min after drug application.
#Data obtained with nystatin-perforated recording. Otherwise the conventional whole-cell recording with Cs-rich, Cl-reduced internal solution was used. The conductance induced by isoprenaline, NKH-477 or db cyclic AMP was measured as the chord conductance at − 70 mV for Cs-rich, Cl-reduced internal solution, and as the conductance estimated from the slope between − 150 ~ − 100 mV for the nystatin-perforated recording. NC: no measurable conductance change. Statistically significant difference (*$P < 0.01$ or **$P < 0.02$) from the values obtained in the presence of isoprenaline, NKH-477 or db cyclc AMP.

the osmotic outflow of water into the posterior chamber (Caprioli, 1987; Bartels, 1989). In line with this hypothesis, it has been postulated that the main target of β-adrenoceptor stimulation in humour production is the Na^+/K^+-ATPase *per se*, although the contribution of active and passive Cl^- transport to the aqueous humour production has not been entirely excluded (Sears, 1991). If Na^+/K^+ ATPase is the main and sole target of β-stimulation in NPE, the membrane depolarization induced by isoprenaline should reflect a reduced activity of the ATPase (Delamere *et al.*, 1990) and thus result in retention of water inside the NPE cell, i.e. swelling of the cell, since the net current carried by the ATPase is outward-going over a wide range of membrane potentials (DeWeer *et al.*, 1988; Guyton, 1991). However, the observed effect of isoprenaline is the opposite of this, a decrease in cell volume. The simplest explanation for this observation is that anions flow out from NPE cells along with the water in response to isoprenaline, as widely found in other types of epithelia (Stetson *et al.*, 1985; McCann & Welsh, 1990; Dawson, 1991; Reuss *et al.*, 1991). We have confirmed this by identifying a cyclic AMP-dependent and DIDS-sensitive Cl^- current in parallel with the cell volume decrease. Furthermore, the magnitude of this current seems almost to account fully for the extent of isoprenaline-induced depolarization observed in the multicellular preparation. By using a quantitative treatment proposed by Ginsborg (1967), the magnitude of agonist-induced depolarization can be estimated from the following equation:

$$\Delta E = (E_{agonist} - E_{rest})^*(\Delta G/(G + \Delta G)$$

where ΔE, E_{rest}, $E_{agonist}$, ΔG and G denote the magnitude of agonist-induced depolarization, the resting membrane potential, the reversal potential of the agonist-induced current, the conductance induced by the agonist and the resting (leak) conductance, respectively. If we use the values of -70 mV, 0.8 nS and 0.5 nS for E_{rest}, ΔG and G, respectively (taken from the present study), and assume $E_{agonist}$ as the equilibrium potential of Cl^- in the intact unstimulated NPE (ca. -20 mV, calculated from $[Cl^-]_i$ = ca. 60 mM; Wiederholt & Zadunaisky, 1986), then a value of about 30 mV for ΔE is obtained. This value is comparable to the maximum depolarization attained by isoprenaline (Figure 2b).

The cyclic AMP-dependent Cl^- current has been found in a broad range of epithelia and is thought to be involved in secretory process (McCann & Welsh, 1990; Dawson, 1991; Hughes & Segawa, 1993). In general, Cl^- channels in epithelia are thought to be contained in the apical and/or basolateral membranes of epithelia and serve as electrodiffusional pathways: Cl ions exit passively via this route along the electrochemical gradient and are accompanied by an outflow of water. This pathway is often found to be tightly coupled to other Cl^- transport systems in a variety of complex ways. For example, in the tracheal epithelium (McCann & Welsh, 1990), a coordinated interplay of Na^+/K^+ pump, Na^+/Cl^- cotransporter and Cl^- channels has been envisaged: the Na^+,Cl^- or Na^+, K^+, Cl^- cotransporter accumulates Cl^- inside the cell by utilizing the Na^+ gradient which is maintained by a Na^+/K^+ pump. This increases the electrochemical force for Cl^- diffusion and drives Cl^- out of the cell through the Cl^- channels. The elevation of cyclic AMP facilitates these mechanisms, especially by inducing a cyclic AMP-dependent Cl^- conductance on the apical membrane of the epithelium. The ciliary epithelium is double-layered, so net movements of solutes and water must occur across the two layers of epithelium to produce the aqueous flow into the posterior chamber. However, very little is yet known about how the two layers are coordinated for this secretory process. The normal operation of the Na^+/K^+ pump is a prerequisite for the aqueous humour production (Bartels, 1989), but it is still unclear whether the pump controls the whole process or is involved merely as a primary initiator of aqueous secretion. We postulate that Cl^- channels, especially cyclic AMP-dependent Cl^- channels, may be localized predominantly on the aqueous side of NPE and serve as a final step of aqueous secretion from the NPE. Further investigation is required to explore the interaction of cyclic AMP-dependent Cl^- channels with other transport systems such as Cl^-/HCO_3^- exchange (Wolosin *et al.*, 1989; Saito, 1992), Na^+, K^+, Cl^- cotransport (Mito *et al.*, 1993) and Na^+/K^+ pump (Usukawa *et al.*, 1988; Martin-Vasallo *et al.*, 1989), in aqueous humour production. A close link of cyclic AMP-dependent Cl^- conductance to the Na^+/K^+ pump has been reported in rabbit corneal epithelium (Klyce & Wong, 1977).

Recently, Gooch *et al.* (1992) showed that in bovine NPE cells, isoprenaline did not change the membrane conductance despite its ability to increase the intracellular cyclic AMP level. However, this may not be simply a species difference. Firstly, they tested the effect of isoprenaline on the membrane conductance at 30°C or lower temperatures, whereas they measured the cyclic AMP level at 37°C. In our experiments, we have observed a clear dependence on temperature of the inward current as well as cell volume change induced by isoprenaline. At 30°C or less, the effects of isoprenaline on the intracelllar potential recording, patch clamp recording and cell volume measurement were almost undetectable. Secondly, in our preliminary experiments, we noticed that the use of unphysiological buffers such as HEPES and low temperatures caused progressive shrinkage of NPE cells. Normal cells could be restored by using a bicarbonate-CO_2 buffering system with oxygen supply and a temperature of 37°C. NPE cells are thought to contain many mitochondria and vigorously produce ATP (Caprioli, 1987). Provided that the induction of Cl^- current is mediated by phosphorylation of the channel proteins by cyclic AMP-dependent protein kinase as in other epithelia (McCann & Welsh, 1990), it is tenable to conceive the essential requirement of ATP to maintain normal function of NPE cells. In this sense, it is necessary to optimize the conditions for NPE cells when their functions are studied.

The rate of aqueous humour production stimulated by 10 μM isoprenaline in individual NPE cells can be estimated from the initial phase of cell volume decrease (Figure 3b). The maximum rate is approximately 0.19 pl min^{-1} per cell, assuming the initial diameter and the percentage rate of cell volume decrease to be on average 13.4 μm and 15% min^{-1}, respectively. This gives an estimated increased rate of aqueous flow by isoprenaline of 0.8 μl min^{-1}, by further assuming that the cross-sectional area of a single NPE cell is 1.41×10^{-6} cm^2 and the total surface area of the ciliary body is about 6 cm^2 (Farahbakhsh & Fain, 1987; Guyton, 1991). This implies that considerable elevation in IOP could result from β-adrenoceptor stimulation, considering that the basal rate of aqueous humour production is about 2.5 μl min^{-1} (Guyton, 1991). However, *in vivo*, the consequences of elevated cyclic AMP in the eye are not confined to modulation of the rate of aqueous humour production, but may include the decreased resistance to outflow (Bartels *et al.*, 1982) or the altered activity of the sympathetic nervous system (Potter *et al.*, 1985). Recent experiments with *in vivo* fluorophotometry suggest that the β-antagonist, timolol, produces a significant reduction in the rate of aqueous flow in the dark, in which the cyclic AMP level in the aqueous humour is increased due to the increased activity of sympathetic nerves (Yoshitomi & Gregory, 1991; Yoshitomi *et al.*, 1991). Since the threshold of the isoprenaline effect is as low as 0.01 nM (the concentration-depolarization curve in Figure 2b), a small alteration in the basal activity of the sympathetic nerves that occurs during the circadian rhythm might have considerable implications in the regulation of aqueous flow. This would partly provide a basic mechanism for the observation that at low sympathetic tone the aqueous

flow is stimulated while at high tone it is rather reduced, due presumably to antagonistic inhibition of adenylate cyclase by α_2-adrenoceptor activation (Sears, 1991).

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References

BARTELS, S.P. (1989). Aqueous humour formation. In *Glaucoma*, ed. Ritchshields, R., Bruce, M. & Krupin, J. pp. 199–218. St. Louis/Washington/Toronto: The C.V. Mosby Company.

BARTELS, S.P., LEE, S.R. & NEUFELD, A.H. (1982). Forskolin stimulates cyclic AMP synthesis, lowers intraocular pressure and increases outflow facility in rabbits. *Curr. Eye Res.*, **2**, 673–681.

CAPRIOLI, J. (1987). The ciliary epithelia and aqueous humour. In *Adler's Physiology of the Eye*. ed. Moses, R.A. & Hart, W.M. pp. 204–222. St. Louis/Washington/Toronto: The C.V. Mosby Company.

CAPRIOLI, J., SEARS, M., MEAD, A., LOSLEY, R.Jr., CHERILL, R.J., HUGER, F.P., ALLEN, R.C. & TRESSLER, C. (1989). Adenylate cyclase stimulation and intraocular pressure reduction by forskolin analogs. *J. Ocul. Pharmacol.*, **5**, 181–187.

CHEN, S., INOUE, R. & ITO, Y. (1993). Pharmacological characterization of muscarinic receptor-activated cation channels in guinea-pig ileum. *Br. J. Pharmacol.*, **109**, 793–801.

COLE, D.F. (1961). Electrochemical changes associated with the formation of the aqueous humour. *Br. J. Ophthalmol.*, **45**, 641–653.

DAWSON, D.C. (1991). Ion channels and colonic salt transport. *Annu. Rev. Physiol.*, **53**, 321–339.

DELAMERE, N.A., SOCCI, R.P. & KING, K.L. (1990). Alteration of sodium, potassium-adenosine triphosphate activity in rabbit ciliary processes by cyclic adenosine monophosphate-dependent protein kinase. *Invest. Ophthalmol. Vis. Sci.*, **31**, 2164–2170.

DEWEER, P., GADSBY, D.C. & RAKOWSKI, R.F. (1988). Voltage-dependence of the Na-K pump. *Annu. Rev. Physiol.*, **50**, 225–241.

DIAMOND, J.R. & BOSSERT, W.H. (1967). Standing-gradient osmotic flow: a mechanism for coupling of water and solute transport in epithelia. *J. Gen. Physiol.*, **50**, 2061–2083.

FARAHBAKHSH, N.A. & FAIN, G.L. (1987). Volume regulation of non-pigmented cells from ciliary epithelium. *Invest. Ophthalmol. Vis. Sci.*, **28**, 934–944.

GINSBORG, B.L. (1967). Ion movements in junctional transmission. *Pharmacol. Rev.*, **19**, 289–316.

GOOCH, A.J., MORGAN, J. & JACOB, T.J.C. (1992). Adrenergic stimulation of bovine non-pigmented ciliary epithelial cells raises cAMP but has no effect on K^+ or Cl^- currents. *Curr. Eye. Res.*, **11**, 1019–1029.

GUYTON, A.C. (1991). Transport of ions and molecules through the cell membrane, Chapter 4 & The Eye I, Chapter 49. In *Textbook of Medical Physiology*. 8th edition, Philadelphia, USA: W.B. Saunders Company.

HOLLAND, M.G. & GIPSON, C.C. (1970). Chloride ion transport in the isolated ciliary body. *Invest. Ophthalmol.*, **9**, 20–29.

HUGHES, B.A. & SEGAWA, Y. (1993). cAMP-activated chloride currents in amphibian retinal pigment epithelial cells. *J. Physiol.*, **466**, 749–766.

IIZUKA, S., KISHIDA, K., TUBOI, S., EMI, K. & MANABE, R. (1984). Electrical characteristics of the isolated dog ciliary body. *Curr. Eye Res.*, **3**, 417–421.

KATZ, I.M., HUBBARD, W.A., GESTON, A.L. & GOULD, A.L. (1976). Intraocular pressure decrease in normal volunteers following timolol ophthalmic solution. *Invest. Ophthalmol.*, **15**, 489–492.

KISHIDA, K., SASABE, T., IIZUKA, S., MANABE, R. & OTORI, T. (1981). Electric characteristics of the isolated rabbit ciliary body. *Jpn. J. Ophthalmol.*, **25**, 407–416.

KISHIDA, K., SASABE, T., IIZUKA, S., MANABE, R. & OTORI, T. (1983). Sodium and chloride transport across the isolated rabbit ciliary body. *Curr. Eye Res.*, **2**, 149–152.

KLYCE, S.D. & WONG, R.K.S. (1977). Site and mode of adrenaline action on chloride transport across the rabbit corneal epithelium. *J. Physiol.*, **266**, 777–799.

MARTIN-VASALLO, P., GHOSH, S. & COCA-PRADOS, M. (1989). Expression of Na,K-ATPase alpha subunit isoforms in the human ciliary body and cultured ciliary epithelial cells. *J. Cell. Physiol.*, **141**, 243–252.

MCCANN, J.D. & WELSH, M.J. (1990). Regulation of Cl− and K+ channels in airway epithelium. *Annu. Rev. Physiol.*, **52**, 115–135.

MICHII, H. & NAGATAKI, S. (1983). Effects of pilocarpine, salbutamol and timolol on aqueous humour formation in cynomolgus monkeys. *Invest. Ophthalmol. Vis. Sci.*, **24**, 1269–1275.

MITO, T., DELAMERE, N.A. & COCA-PRADOS, M. (1993). Calcium-dependent regulation of cation transport in cultured human non-pigmented ciliary epithelial cells. *Am. J. Physiol.*, **264**, C519–C526.

OKISAKA, S., KUWABARA, T. & RAPOPPORT, S.T. (1974). Selective destruction of the pigmented epithelium in the ciliary body of the eye. *Nature*, **184**, 1298–1299.

POTTER, D.E., BURKE, J.A. & TEMPLE, J.R. (1985). Forskolin suppresses sympathetic neuron function and causes ocular hypotension. *Curr. Eye Res.*, **4**, 87–96.

REUSS, L., SEGAL, Y. & ALTENBERG, G. (1991). Regulation of ion transport across gallbladder epithelium. *Annu. Rev. Physiol.*, **53**, 361–373.

SAITO, Y. (1992). Na^+-H^+ and HCO_3^--Cl^- antiport mechanisms in the isolated ciliary body epithelium and their role in aqueous humour secretion in the rat eye. *J. Physiol.*, **446**, 253P.

SCHENKER, H.I., YABLONSKI, M.E., PODOS, S.M. & LINDER, L. (1981). Fluorophotometric study of adrenaline and timolol in human subjects. *Arch. Ophthalmol.*, **99**, 1212–1216.

SEARS, M.L. (1991). Physiology and pharmacology of aqueous humour formation. Implications with respect to treatment. *Ophthalmol. Clinics of North America*, **4**, 767–780.

SEARS, M.L., YAMADA, E., CUMMINS, D., MORI, N., MEAD, A. & MURAKAMI, M. (1991). The isolated ciliary bilayer is useful for studies of aqueous humour formation. *Trans. Am. Ophthalmol. Soc.*, **89**, 132–154.

SHAFIQ, J., SUZUKI, S., ITOH, T. & KURIYAMA, H. (1992). Mechanisms of vasodilation induced by NKH477, a water-soluble forskolin derivative, in smooth muscle of the porcine coronary artery. *Circ. Res.*, **71**, 70–81.

STETSON, D.L., BEAUWENS, R., PALMISANO, J., MITCHELL, P.P. & STEINMETZ, P.R. (1985). A double-membrane model for urinary bicarbonate secretion. *Am. J. Physiol.*, **249**, F546–F552.

TOPPER, J.E. & BRUBAKER, R.F. (1985). Effects of timolol, adrenaline and acetazolamide on aqueous flow during sleep. *Invest. Ophthalmol. Vis. Sci.*, **26**, 1315–1319.

USUKURA, J., FAIN, G.L. & BOK, D. (1988). [^{3}H] ouabain localization of Na-K ATPase in the epithelium of rabbit ciliary body pars plicata. *Invest. Ophthalmol. Vis. Sci.*, **29**, 606–614.

WAITZMAN, M.B. & WOOD, W.D. (1971). Some characteristics of an adenyl cyclase preparation from rabbit ciliary process tissue. *Exp. Eye. Res.*, **12**, 99–111.

WIEDERHOLT, M. & ZADUNAISKY, J.A. (1986). Membrane potentials and intracellular chloride activity in the ciliary body of the shark. *Pflügers Arch.*, **407**, S112–S115.

WOLOSIN, J.M., BONANNO, J.A. & MACHEN, T.E. (1989). Na-dependent HCO_3^- transport and Cl-/HCO3- exchange in ciliary epithelium. *Ann. New York Acad. Sci.*, **574**, 131–133.

YOSHITOMI, T. & GREGORY, D.S. (1991). Ocular adrenergic nerves contribute to control of the circadian rhythm of aqueous flow in rabbits. *Invest. Ophthalmol. Vis. Sci.*, **32**, 523–528.

YOSHITOMI, T., HORIO, B. & GREGORY, D.S. (1991). Changes in aqueous norepinephrine and cyclic adenosine monophosphate during the circadian cycle in rabbits. *Invest. Ophthalmol. Vis. Sci.*, **32**, 1609–1613.

ZIMMERMAN, T.J., HARBIN, R., PETT, M. & KAUFMAN, H.E. (1977). Timolol and facility of outflow. *Invest. Ophthalmol. Vis. Sci.*, **16**, 623–624.

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