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Atriopeptin, sodium azide and cyclic GMP reduce secretion of aqueous humour and inhibit intracellular calcium release in bovine cultured ciliary epithelium

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1 This study examined the involvement of cyclic GMP, protein kinase G and intracellular Ca^{2+} movements in the modulation of aqueous humour formation.

2 Using the bovine arterially-perfused eye preparation, drug effects on intraocular pressure and aqueous humour formation rate were measured by manometry and fluorescein dilution, respectively. Drug effects on intracellular [Ca^{2+}] were determined by fura-2 fluorescence ratio technique in non-transformed, cultured ciliary epithelium.

3 Intra-arterial injection of atriopeptin (50 pmol) or sodium azide (10 nmol) produced significant reduction in aqueous humour formation (>38%). This was blocked by selective inhibition (KT-5823) of protein kinase G, but not by selective inhibition (KT-5720) of protein kinase A. Reductions of intraocular pressure produced by atriopeptin or azide were almost completely blocked by KT-5823.

4 ATP (100 μM) caused rapid, transient increase in intracellular Ca^{2+} followed by a slow decline and prolonged plateau. This response showed concentration-dependent inhibition by atriopeptin, azide or 8-bromo cyclic GMP, and this inhibition of the rapid (peak) Ca^{2+} increase was enhanced by zaprinast (100 μM; phosphodiesterase inhibitor). KT-5823 blocked the suppression of the peak Ca^{2+} response but not suppression of the plateau.

5 Arterial perfusion of ATP (0.1–100 μM) produced a concentration-dependent decrease in aqueous humour formation.

6 Aqueous humour formation in the bovine eye can be manipulated through cyclic GMP, operating *via* protein kinase G. Close parallels appear when Ca^{2+} movements are modified by similar manipulations of cyclic GMP, suggesting that Ca^{2+} transients may play an important role in aqueous humour formation and that interplay occurs between cyclic GMP and Ca^{2+}.

Keywords: Aqueous humour; ATP; atriopeptin; azide; bovine ciliary epithelium; cyclic GMP; intracellular calcium; protein kinase G

Abbreviations: AH, aqueous humour; cAMP, cyclic AMP; CE, ciliary epithelium; cGMP, cyclic GMP; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FCS, foetal calf serum; HEPES, (N-[2-hydroxyethylpiperazine-N′-[4-butanesulphonic acid]); IOP, intraocular pressure; NCS, newborn calf serum; PKA, protein kinase A; PKG, protein kinase G

Introduction

Aqueous humour (AH) is secreted into the posterior chamber of the eye by the ciliary epithelium (CE), a double layer of cells located on the surface of the ciliary processes of the eye. Cyclic AMP (cAMP) has long been considered by many to be the most important regulator of aqueous humour (AH) formation and this area has been extensively studied (Neufeld *et al*., 1972; Nathanson, 1980; Mittag, 1989) although the evidence is still not entirely conclusive (Shahidullah *et al*., 1995). In many tissues, other intracellular second messengers have become firmly established including both cyclic GMP (cGMP) and Ca^{2+}. Results are now emerging which point to cGMP as an inhibitory modulator of AH production (Korenfeld & Becker, 1989; Becker, 1990; Millar *et al*., 1997).

Topical, intravitreal or intracameral administration of atriopeptin lowers intraocular pressure (IOP) in rabbits (Sugrue & Viader, 1986; Nathanson, 1987; Mittag *et al*., 1987; Korenfeld & Becker, 1989) and in glaucoma patients (Diestelhorst & Krieglstein, 1989). Involvement of cGMP in suppressing AH formation was postulated (Nathanson, 1987). Korenfeld & Becker (1989) reported a significant decrease in IOP and AH formation following intravitreal injection of atriopeptin in rabbit with a concomitant increase in cGMP in iris-ciliary body. Indeed 8-bromo cGMP lowers IOP when given topically in rabbits (Becker, 1990) and decreases AH flow when injected intravitreally in monkey (Kee *et al*., 1994). In the bovine eye, atriopeptin increases cGMP in both ciliary processes of the whole eye and in cultured CE cells (Millar *et al*., 1997); it also decreases IOP and AH formation.

Ca^{2+} has been established as a regulator of a number of cellular functions including secretion (Berridge, 1993; Tsunoda, 1993). Many workers have reported 'transients', or sudden changes in intracellular Ca^{2+} in cultured CE cells (Lee *et al*., 1989; Yoshimura *et al*., 1995; Suzuki *et al*., 1997; Farahbakhsh & Cilluffo, 1997). We have recently identified and characterized $P2Y_2$ receptors in cultured CE cells (Shahidullah & Wilson, 1997). Stimulation of these receptors with ATP causes intracellular Ca^{2+} release and thus a rise in intracellular free Ca^{2+}concentration or $[Ca^{2+}]_i$. Release of

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Ca^{2+} has also been reported in CE by stimulation of various other receptors (Crook & Polansky, 1992; Schütte *et al*., 1996; Suzuki *et al*., 1997). An important role is well known for cGMP and guanylyl cyclase-stimulating agents in the inhibition of cytosolic Ca^{2+} transients in vascular smooth muscle (Meisheri *et al*., 1986; Lincoln, 1989) and in platelets (Geiger *et al*., 1992). In ocular tissue, sodium nitroprusside, a nitric oxide donor, has been reported to relax bovine ciliary muscle (Azuma *et al*., 1997) and relaxation of carbachol pre-contracted muscle was caused through elevation of cGMP (Kamikawatoko *et al*., 1998). No information is presently available regarding the effects of cGMP, atriopeptin or other guanylyl cyclase-stimulating agents on intracellular Ca^{2+} in CE cells.

The present study investigated the action of atriopeptin and sodium azide on the ATP-induced increase in intracellular Ca^{2+} in bovine cultured CE and on the AH formation rate and IOP in the isolated arterially perfused bovine eye with a view to correlating its biochemical and physiological effects. Since ATP profoundly affects cytosolic Ca^{2+} in the CE, we studied its effect on AH formation in the whole eye. We have sought to bridge the gap in our knowledge between the effects of these agents on AH formation and the ion pumps which are the final biochemical step in AH secretion. Part of that mechanistic gap may well be filled by intracellular Ca^{2+}.

Methods

IOP and AH formation

IOP and AH formation rate were measured as described earlier (Wilson *et al*., 1993). In brief, bovine eyes obtained from the abattoir were perfused through a long posterior ciliary artery at 37°C with a modified Krebs' solution comprising (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose, 11.5; ascorbate, 0.05. The pH of the solution was adjusted to 7.4 by bubbling with O_2 containing 5% CO_2. Arterial flow was commenced at 0.2 ml min^{-1} and increased in 5–10 increments to 2.25 ml min^{-1}. After approximately 50 min, when aqueous humour secretion had started and the chamber was firm, it was cannulated with a 23G needle and connected *via* silicon rubber tubing (i.d., 0.8 mm) either with a water manometer alone for measuring IOP or *via* a T-piece with both a water manometer and the cuvette of a fluorimeter for measuring AH formation rate.

Observations of IOP from the water manometer were made at 5 min intervals. Only eyes maintaining a stable IOP within the range of 95–165 mmH_2O (7–12 mmHg), and a stable arterial pressure within the range of 20–60 mmHg, after an equilibration period of a further 30–60 min, were accepted for study.

For measurement of AH flow rate, the anterior chamber was cannulated first with one 23G needle. This was connected by silicon rubber tubing through a peristaltic pump to the cuvette of a fluorimeter, returning to the anterior chamber *via* a second 23G needle. The AH formation rate was estimated by the fluorescein dilution technique, with the usual assumption that the marker leaves the anterior chamber only by bulk flow. The slope of the regression line constructed by plotting $\log_e$ of fluorescein concentration against time was taken as the rate constant for AH formation (K_{out} min^{-1}). In most experiments, bolus doses of drug or vehicle were injected into the perfusate, in volumes of 3–10 μl, immediately proximal to the arterial cannula. In the case of ATP, the drug was instead dissolved in the perfusate.

Isolation and culture of CE cells

Bovine CE cells were isolated and cultured as previously described (Shahidullah *et al*., 1995). Briefly, bovine eyes were dissected from the posterior pole. The vitreous was pushed aside and the lens along with its capsule were removed by cutting the zonular fibres. The tips of the ciliary processes lying on the iris were cut with fine scissors and collected in centrifuge tubes containing DMEM and gentamycin (200 μg ml^{-1}). The cut tips were then transferred to a large petri dish containing 20 ml of a calcium-free buffer (pH 7.4) comprising (mM): NaCl, 142; KCl, 13.41; HEPES, 4.82 and EDTA, 0.25 and incubated under constant, mild shaking (Luckham Rotatest, R100/WT) for 25–30 min. The tips were then transferred to another Petri dish containing 15 ml of sterile collagenase A (0.1%) prepared in a buffer (pH 7.6) comprising (mM): NaCl, 66.73; KCl, 13.41; HEPES, 3.84; $CaCl_2$, 4.82, then incubated under constant shaking for a further 25–30 min. After that 3 ml of a mixture (1:1) of NCS and FCS was added to neutralize the enzyme.

The partially digested tips were disrupted by squirting gently in DMEM. The dispersed cells were washed and resuspended in 2 ml of DMEM containing 10% each of NCS and FCS and gentamycin (200 μg ml^{-1}) 'complete medium'. The cells were seeded in 25 cm^2 culture flasks containing 5 ml of 'complete medium' and incubated at 37°C until confluence was achieved in 7–8 days. After growing to confluence, the cells were trypsinized with 5 ml of trypsin-EDTA solution in modified Puck's saline at 37°C for 2–4 min, then easily detached upon gentle shaking. Trypsin was immediately neutralized with 3 ml of a mixture of NCS and FCS (1:1). The cells were centrifuged, washed and resuspended in 5 ml of complete medium, seeded onto sterile coverslips (11 × 50 mm) and grown to confluence (4–5 days).

Measurement of $[Ca^{2+}]_i$ in the CE population

Loading of cells with fura-2 and measurement of $[Ca^{2+}]_i$ was done according to the method described earlier (Shahidullah & Wilson, 1997). Briefly, the confluent monolayer of first passage CE cells grown on coverslips was incubated for 45 min at 37°C with fura-2 AM (2 μM) in HEPES (25 mM)-buffered DMEM containing bovine serum albumin (1%) under constant mild shaking. The coverslips containing fura-2 loaded cells were transferred to HEPES (10 mM)-buffered Krebs' solution (pH 7.4) containing (mM): NaCl, 118; KCl, 4.8; $MgSO_4$, 1.0; $NaHCO_3$, 2.4; glucose, 11.0; HEPES 10.0 and $CaCl_2$, 1.8 and left at room temperature for 20 min, to allow for hydrolysis of fura-2 to the calcium-sensitive acid form.

Coverslips with fura-2 loaded cells were suspended diagonally across a plastic cuvette containing 3 ml of HEPES (10 mM)-buffered Krebs' solution in a Perkin Elmer LS-3B fluorescence spectrometer and maintained at 37°C with continuous stirring. The coverslip was suspended at an angle of 30° to the incident light with a custom-built plastic clip. Drugs were added either in the medium bathing the cells at appropriate concentrations or injected into it with a microsyringe to obtain the intended final concentration.

Measurement of $[Ca^{2+}]_i$ was done using the dual excitation method of Grynkiewicz *et al*., 1985). The excitation monochromator was computer-driven, alternating between 340 and 380 nm and fluorescence emission was collected at 509 nm. At the end of each experimental run, background autofluorescence was obtained according to Hallam *et al*. (1988). Ionomycin (2 μM) was added to permeabilize the cells to divalent cations and $MnCl_2$ (2 mM) was added to quench fura-

2 fluorescence. After subtraction of autofluorescence, the corrected fluorescence values obtained following excitation at 340 nm were divided by those obtained at 380 nm, giving a corrected ratio (R). $[Ca^{2+}]_i$ was then calculated by the computer using the equation of Grynkiewicz *et al*. (1985):

$$[Ca^{2+}]_i = K_d \times \frac{(R - R_{min})S_{f_2}}{(R_{max} - R)S_{b_2}} \quad (1)$$

and a resultant experimental trace of $[Ca^{2+}]_i$ versus time obtained. R_{max} and R_{min} are the maximal and minimal fluorescence ratios of fura-2 obtained in a saturating concentration of calcium and in calcium-free medium (with 40 mM EGTA), respectively. S_{f2} and S_{b2} are the fluorescence values obtained at 380 nm in the absence of calcium and in presence of saturating levels of calcium, respectively. The K_d for the fura-2-Ca^{2+} complex was assumed to be 224 nM at 37°C (Grynkiewicz *et al*., 1985).

It was important experimentally to obtain the values of R_{max}, R_{min}, S_{f2} and S_{b2} in fura-2 loaded CE cells, because the spectral characteristics of the dye in the cytoplasm may differ from those obtained in a simple buffer solution. The fluorescence properties of fura-2 were determined in CE cell suspension rather than in cells on coverslips as the low calcium concentrations required to obtain R_{min} would have resulted in detachment of cells. The cells were loaded with fura-2 AM (2 μm) at 37°C for 45 min as described above. Saturation of the fura-2 in the cytoplasm was obtained by addition of ionomycin (8 μM) which increased F_{340} to a maximum and reduced F_{380} to a minimum. The ratio of these values yielded the R_{max} for fura-2. Addition of EGTA (40 mM) stripped calcium from fura-2 binding sites, resulting in F_{340} falling to a minimum and F_{380} rising to a maximum. The ratios of these values yielded R_{min} for fura-2. $MnCl_2$ (200 mM) was then added to quench fura-2 fluorescence and yield autofluorescence values at 340 and 380 nm; these were then subtracted from the raw fluorescence data to yield corrected R_{max}, R_{min}, S_{f2} and S_{b2} values. The following calibration values were obtained and used throughout to calculate $[Ca^{2+}]_i$: R_{max}, 17.59 ± 0.34 ($n = 4$); R_{min}, 0.91 ± 0.04 ($n = 4$); S_{f2}/S_{b2}, 6.89 ± 0.02 ($n = 13$).

Drugs and chemicals

ATP, atriopeptin (rat, 5–27 sequence), sodium azide, zaprinast, 8-bromo cGMP, bovine serum albumin (fraction V), EDTA, EGTA, DMSO, acetoxymethyl ester of fura-2 (fura-2 AM), ionomycin, HEPES and gentamycin were all purchased from Sigma Chemical Co. KT-5823 and KT-5720 were obtained from Calbiochem. Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), new-born calf serum (NCS) and trypsin EDTA (1X) were obtained from Life Technologies and collagenase A from Boehringer Mannheim. $MnCl_2$ was obtained from BDH. All other reagents were of analytical grade. Drugs were dissolved in appropriate concentrations either in distilled water or in dimethyl sulphoxide (DMSO) according to their solubility.

Statistical analysis

All data were expressed as mean ± s.e.mean and a probability value of $P < 0.05$ was considered as significant. Data on AH formation were subjected to analysis of variance (one-way). Changes in IOP were analysed by comparing the slopes of the regression lines of IOP versus time, using Student's *t*-test. Concentration-response curves for the drug effects on peak $[Ca^{2+}]_i$ were generated by linear regression and compared by Bonferroni's multiple comparison test.

Results

Effects of atriopeptin and sodium azide on AH formation

The vasodilator drugs atriopeptin and sodium azide both caused a significant reduction of AH formation (Table 1). Estimation of AH formation rate was started 15 min after injection of drug or vehicle and continued for a further 60 min. Control A consisted of eyes treated exactly as drug-treated eyes, but injected with a similar volume of water instead of drug solution. Control B was a same-eye control in which the AH formation rate was estimated for 30 min before injection of drug solution, so that any variation among different eyes would be eliminated. AH formation rate estimation was restarted 15 min after drug injection and continued for a further 60 min. Timecourse analysis of these data (Table 1, last four columns) showed that suppression of AH formation by both these drugs was well maintained. There were no significant differences among the control values for different time intervals.

Effects of KT-5823 and KT-5720 on the AH-reducing action of atriopeptin and sodium azide

KT-5823, a specific inhibitor of cGMP-dependent protein kinase (PKG), abolished the AH-reducing effect of bolus doses of atriopeptin or sodium azide while KT-5720, the specific inhibitor of cAMP-dependent protein kinase (PKA) was ineffective (Table 2). KT-5823 or KT-5720 was injected immediately preceding the injection of atriopeptin or sodium azide. Timecourse analysis of these data (Table 2, last four

Table 1 Effects of atriopeptin (50 pmol) and sodium azide (10 nmol) on AH formation in the bovine perfused eye

			Aqueous humour formation rate: K_{out} ($min^{-1} \times 10^4$)			
Treatment	n	*Average*	*30–45 min*	*45–60 min*	*60–75 min*	*75–90 min*
Control A	8	48.0 ± 1.3	49.5 ± 4.7	51.7 ± 4.6	45.8 ± 3.8	48.5 ± 3.9
Control B	8	47.0 ± 3.0				
Atriopeptin	8	29.0 ± 1.9***	31.8 ± 3.4***	31.0 ± 2.0***	29.1 ± 2.2***	23.5 ± 2.0***
Control B	6	48.4 ± 2.0				
Sodium azide	6	25.7 ± 1.4***	30.0 ± 2.3***	24.4 ± 1.9***	26.7 ± 3.5***	24.6 ± 1.5***

Each value is a mean ± s.e.mean of the number (*n*) of experiments shown. Control A ('separate eye' control), injection of water, then AH formation rate measured for 90 min. Control B ('same eye' control), AH formation rate measured during 30 min prior to drug injection, then for 60 min after drug injection. Significance of differences from controls at appropriate time points; A and B, none; ***$P < 0.001$. The last four columns show the timecourse of changes in AH formation during the post-injection period of each experiment.

Table 2 Effects of KT-5823 and KT-5720 on the suppression of AH formation by atriopeptin (50 pmol) and sodium azide (10 nmol) in the bovine perfused eye

		Aqueous humour formation rate: K_{out} ($min^{-1} \times 10^4$)				
Treatment	n	*Average*	*30–45 min*	*45–60 min*	*60–75 min*	*75–90 min*
Control A + (KT-5823)	8	48.3 ± 2.1	51.5 ± 2.2	45.8 ± 3.9	46.9 ± 2.4	47.2 ± 4.3
KT-5823 + atriopeptin	8	43.2 ± 1.8	47.4 ± 3.1	38.5 ± 3.5	34.9 ± 2.2**	33.1 ± 1.8**
KT-5720 + atriopeptin	7	27.8 ± 3.5***				
Control A + (KT-5720)	8	48.4 ± 1.3				
KT-5823 + sodium azide	8	44.6 ± 1.2	49.2 ± 3.7	39.9 ± 3.5	35.6 ± 2.0**	35.4 ± 2.1*
KT-5823 × 4[a] + sodium azide	6	46.8 ± 4.1	48.6 ± 2.1	51.2 ± 3.7	48.8 ± 2.6	40.9 ± 10.7
KT-5720 + sodium azide	8	28.4 ± 4.0***				

AH formation rate (shown as K_{out} $min^{-1} \times 10^4$) was measured for 30 min (to obtain 'same eye' control values; not shown in Table) prior to drug injection, then for 60 min after drug injection. The PK inhibitors, KT-5823 and KT-5720 were dissolved in DMSO (3 μl) to give a dose of 10 nmol, which was injected immediately before atriopeptin or sodium azide. [a]KT-5823 × 4 indicates that the PKG inhibitor was injected four times, i.e. just before 0, 15, 30 and 45 min. Each value is a mean ± s.e.mean of the number (n) of experiments shown. Control A: injection of vehicle and PK inhibitor. The last four columns show the timecourse of changes in AH formation during the post-injection period of each experiment. Significance of difference from control at appropriate time points: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

columns), showed that, unlike the effects of atriopeptin and sodium azide, the abolition by KT-5823 was short-lived.

Effects of KT-5823 or KT-5720 on IOP-reducing effects of atriopeptin and sodium azide

KT-5823 also abolished the IOP-reducing effect of atriopeptin while KT-5720 was ineffective (Table 3). Drug or vehicle was injected after obtaining steady IOP for 15 min and the reading was recorded after another 15 min. Separate eyes were challenged with drug or vehicle to obtain treated or control values respectively. Likewise, KT-5823 abolished the IOP-reducing effect of sodium azide, while KT-5720 was ineffective (Table 4).

Effect of ATP on AH formation

ATP at concentrations of 100, 10 and 1 μM produced a concentration-dependent decrease in AH formation over a period of 60 min (Table 5). Timecourse analysis of the data (Table 5, last four columns) showed that ATP produced a sustained suppression of AH formation. There were no significant differences between controls A and B, nor among the control A values for different time intervals.

Effect of atriopeptin, sodium azide and 8-bromo cGMP on the $[Ca^{2+}]_i$ response to ATP

Addition of ATP (100 μM) to cultured CE caused a highly reproducible Ca^{2+} transient whose peak was followed by a slow decline and plateau (see typical trace in Figure 1). The peak of the Ca^{2+} transient was usually reached within 30 s and its magnitude was 612 ± 10 nM (mean ± s.e.mean; $n = 20$), from a baseline value of 129 ± 4 nM.

When the CE cells were incubated in a medium containing atriopeptin, the magnitude of both the peak and the plateau values of the Ca^{2+} transient were inhibited (see typical traces in Figure 2). The maximal degree of inhibition by atriopeptin (using 100 nM) of the ATP-induced peak of $[Ca^{2+}]_i$ was substantial but not complete (37 ± 2% of control response; mean ± s.e.mean). In contrast to the effects on the peak Ca^{2+} transient, the inhibitory effect on the slower, plateau phase of the response occurred at concentrations of atriopeptin as low as 100 pM while at higher concentrations this inhibition was absolute (Figure 2). Figure 3A illustrates the concentration-dependence of the inhibition over the timecourse of the response. This is further shown in Figure 3B, where the inhibition of the peak Ca^{2+} response is seen to be potentiated to a modest degree by preincubation of the cells with zaprinast (100 μM).

Table 3 Effects of KT-5823 (10 nmol) and KT-5720 (10 nmol) on the reduction of IOP by atriopeptin (50 pmol) in the bovine perfused eye

Treatment	n	*Slope*
Control (DMSO)	10	−0.0108 ± 0.0012
Atriopeptin	8	−0.2160 ± 0.0091***
KT-5823 + atriopeptin	7	−0.0122 ± 0.0051
KT-5720 + atriopeptin	7	−0.1399 ± 0.0071***

The IOP-reducing effect is shown as the mean slope of the regression line drawn on IOP versus time (90 min). Each value is a mean ± s.e.mean of the number (n) of experiments shown. Significance of differences from control: ***$P < 0.001$.

Table 4 Effects of KT-5823 and KT-5720 on the reduction of IOP by sodium azide (10 nmol) in the bovine perfused eye

Treatment	n	*Slope*
Control (DMSO)	7	0.0192 ± 0.0016
Sodium azide	7	−0.2702 ± 0.0223****
KT-5823 + sodium azide	7	−0.0167 ± 0.0032
KT-5720 + sodium azide	6	−0.2537 ± 0.0195****

KT-5823 and KT-5720 were dissolved in DMSO (3 μl) to give a dose of 10 nmol. The IOP-reducing effect is shown as the mean slope of the regression lines drawn on IOP versus time (90 min). Each value is a mean ± s.e.mean of the number (n) of experiments shown. Significance of difference from control: ****$P < 0.0001$.

Very similar results were obtained for inhibition of the ATP-induced Ca^{2+} transient by sodium azide (Figure 4) or 8-bromo cGMP (Figure 5).

Effect of KT-5823 on atriopeptin or sodium azide inhibition of the $[Ca^{2+}]_i$ response to ATP

KT-5823 (10 nM) substantially reversed the inhibitory effect of atriopeptin or sodium azide on the peak phase of the rise in $[Ca^{2+}]_i$ induced by ATP (Figure 6A shows a typical trace), but

Table 5 Effect of perfusion of ATP on AH formation in the bovine perfused eye

ATP concentration perfused	n	*Control B average*	*Aqueous humour formation rate: K_{out} ($min^{-1} \times 10^4$) ATP 30–90 min*	*30–45 min*	*45–60 min*	*60–75 min*	*75–90 min*
100 μM	6	48.8±3.2	22.9±1.1***	29.8±3.7*	26.6±4.7**	26.3±3.9**	23.1±1.4***
10 μM	6	47.0±2.0	29.9±1.8***	32.1±1.7*	29.4±1.7**	29.1±3.5**	25.9±5.7**
1 μM	6	45.6±2.1	35.5±2.6**	38.1±1.7	34.7±4.0	36.6±2.2	33.7±2.5*
100 nM	3	47.3±1.5	38.4±3.1	41.1±2.2	36.0±1.4	40.3±5.1	41.3±3.3
Control A	6	–	43.7±2.7	42.9±3.2	40.4±3.0	43.9±2.4	44.6±3.3

Each value is a mean±s.e.mean of the number of experiments shown (*n*). Control A ('separate eye' control) represents values (K_{out} $min^{-1} \times 10^4$) for eyes perfused with Krebs' solution without ATP. Control B ('same eye' control) represents values for the initial 30 min perfusion with Krebs' solution for each group of eyes subsequently perfused with ATP. The perfusing solution was then changed to Krebs' with the appropriate concentration of ATP and rate measurements continued for the next 60 min. No significant differences exist between controls A and B. Significance of differences from control A at appropriate time points: $*P<0.05$; $**P<0.01$, $***P<0.001$.

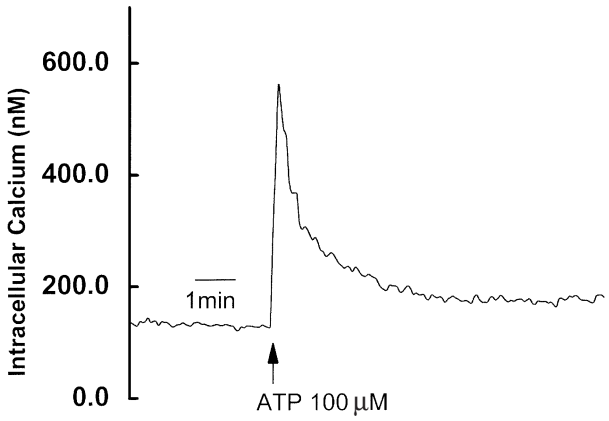

Figure 1 Release of intracellular Ca^{2+} in response to ATP (100 μM) in a population of bovine ciliary epithelial cells in culture. The record was generated by rapid, repetitive measurement of fluorescence ratios, converted to $[Ca^{2+}]_i$ using the calibration values described in Methods. The rapid phase of the increase in $[Ca^{2+}]_i$ is followed by a slower decline then a prolonged plateau.

it completely failed to reverse the plateau phase of the Ca^{2+} response (Figure 6B).

Discussion

The present study demonstrates that the vasodilator drugs atriopeptin and sodium azide both lower IOP and AH formation significantly in the bovine isolated perfused eye at very low doses. A decrease in AH formation by atriopeptin in the anaesthetized rabbit was reported by Korenfeld & Becker (1989), but quantitative comparison with the present result is impossible since the dose, route and species all differ. We have previously shown (Millar *et al*., 1997) that in bovine eye these effects occur at doses which are less than are required to produce vasodilatation in the ciliary vascular bed.

The involvement of cGMP in the mechanism of atriopeptin in the rabbit ciliary body was first proposed by Steardo & Nathanson (1987). Atriopeptin produces an increase in cGMP in ciliary processes of the bovine perfused eye at a dose which also decreases AH formation (Millar *et al*., 1997). Sodium azide increases cGMP production by a different mechanism from atriopeptin, yet it too decreases AH formation, lending weight to the theory that this nucleotide may mediate the effects of both drugs on AH formation. Our present evidence in the bovine eye that KT-5823 (a specific inhibitor of PKG) completely suppresses both the atriopeptin- and sodium azide-induced suppression of AH formation and IOP, strongly suggests that the increase in cGMP production by these drugs is the most likely mediator of their effects on AH formation.

When the data for AH formation over separate 15 min periods was analysed, it was clear that the effect of a bolus dose of atriopeptin or sodium azide was persistent. This suggests that the result of phosphorylation by PKG is relatively long-lasting, in contrast with the blocking effect of KT-5823, which was short-lived, perhaps due to wash-out of drug from the tissue. This was substantiated by repeated injection of the inhibitor (Table 2, last line but one).

The present study demonstrates that both atriopeptin and sodium azide are potent inhibitors of the Ca^{2+} transient that is induced by exogenous ATP in bovine CE cells. In an earlier study we have shown that in these cells, ATP mobilizes $[Ca^{2+}]_i$ mainly from internal stores (Shahidullah & Wilson, 1997). Extracellular ATP acts on G-protein coupled cell-surface $P2Y_2$ receptors to stimulate the membrane-bound enzyme phospholipase C, causing production of IP_3, which then binds to its receptor on the endoplasmic reticulum, thus mobilizing stored Ca^{2+}. Both atriopeptin and sodium azide produce a substantial and concentration-dependent inhibition of the resulting rise in $[Ca^{2+}]_i$. 8-bromo cGMP, a cell permeable and more stable analogue of cGMP, also produces similar effects on $[Ca^{2+}]_i$ and in a concentration-dependent manner.

Similar effects occur in other tissues: nitrovasodilators (of which azide is one) suppress platelet activation and inhibit (through cGMP and PKG) agonist-induced mobilization of stored Ca^{2+} (Geiger *et al*., 1992), although exceptions exist (Rooney *et al*., 1996). Contraction of vascular smooth muscle is reversed by atriopeptin which also inhibits Ca^{2+} release (Meisheri *et al*., 1986). Similarly, sodium nitroprusside or 8-bromo cGMP cause inhibition of ATP-induced contraction and Ca^{2+} release, both of which are blocked by a PKG inhibitor (Andriantsitohaina *et al*., 1995). Azula *et al*. (1996) suggested that both cGMP- and cAMP-dependent protein kinases are involved in the platelet inhibitory mechanisms and that PKA is the more important. In the CE, however, PKA seems to have little involvement in the action of atriopeptin and sodium azide, since the selective inhibitor KT-5720 did not alter IOP nor AH formation (Tables 2, 3 and 4).

In our system, the $[Ca^{2+}]_i$ response of CE to ATP occurs in three phases. The rapid initial rise is due to release of intracellular stored Ca^{2+} (Shahidullah & Wilson, 1997). The

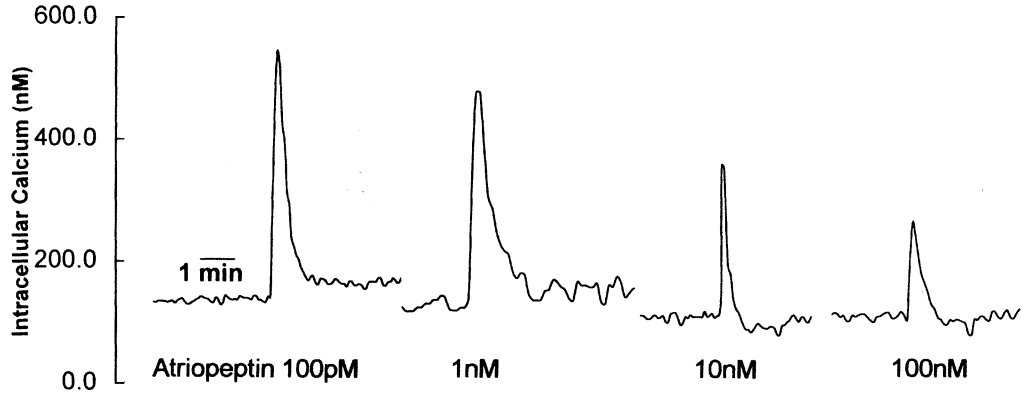

Figure 2 Typical traces showing inhibition of intracellular Ca^{2+} release in ciliary epithelial cells in response to ATP (100 μM), by increasing concentrations of atriopeptin. Each concentration was tested in a separate batch of cells. Inhibition of both the rapid peak and the slower plateau phases of Ca^{2+} release occurs in a concentration-dependent manner.

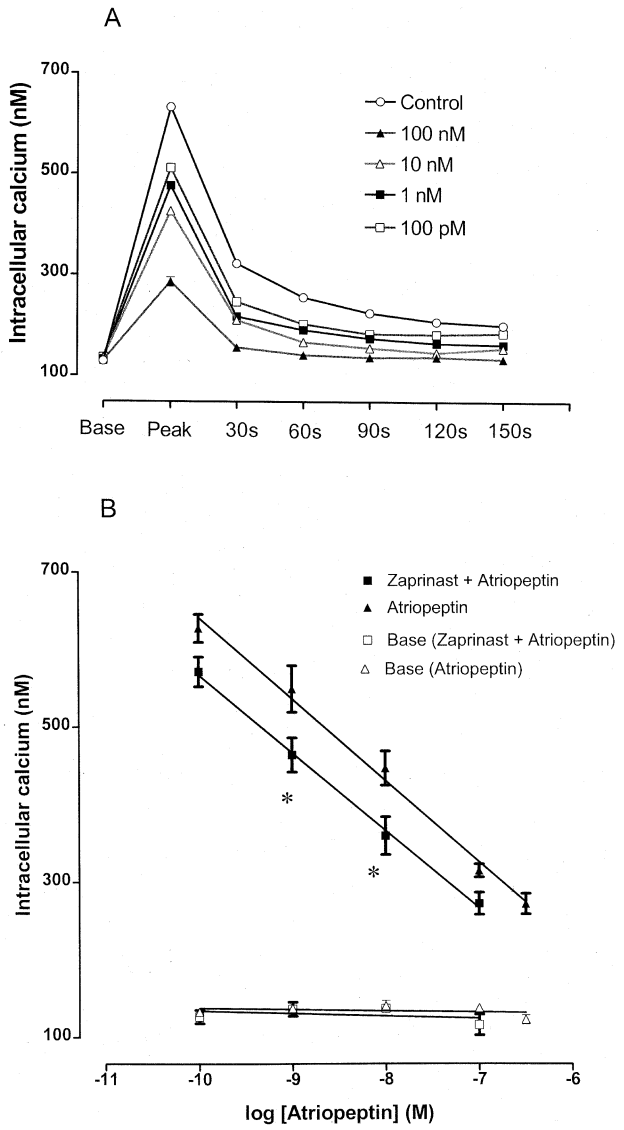

Figure 3 (A) Shows the family of curves representing the effect of increasing concentrations of atriopeptin on the Ca^{2+} mobilization response, both at the peak of the Ca^{2+} transient and at selected time intervals thereafter. (B) Shows the relationship between peak values for $[Ca^{2+}]_i$ and the concentration of atriopeptin, alone and in the presence of the phosphodiesterase inhibitor, zaprinast (100 μM, 30 min preincubation). Each point represents a mean$\pm$s.e.mean of 10–24 experiments. Significance of difference between incubations with and without zaprinast: $^*P<0.05$, Bonferroni's multiple comparison test. Base values indicate the $[Ca^{2+}]_i$ prior to addition of ATP.

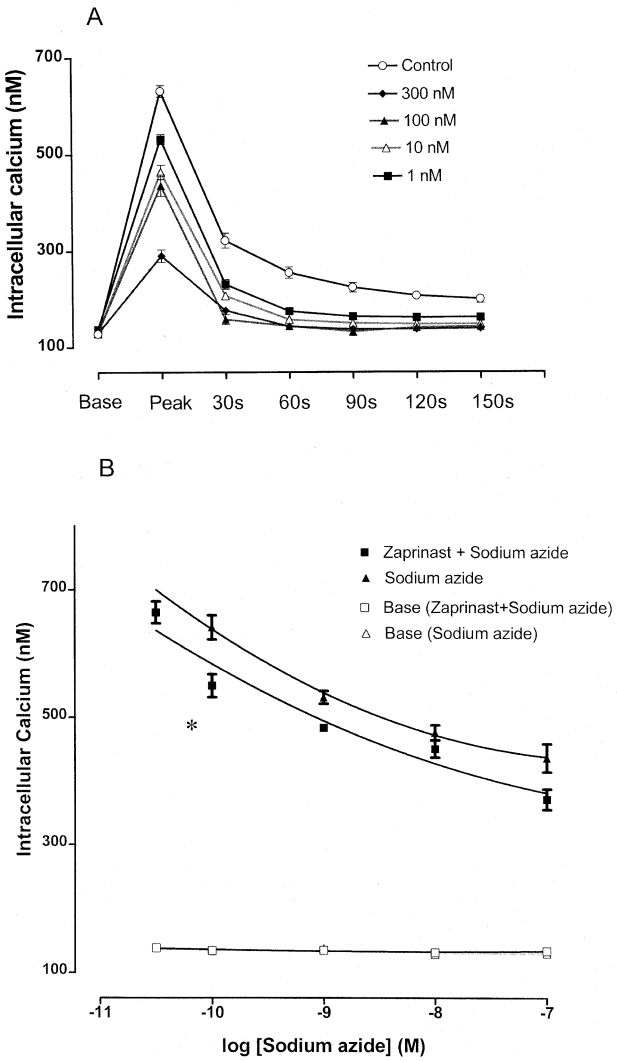

Figure 4 (A) Shows the family of curves representing the effect of increasing concentrations of sodium azide on the Ca^{2+}-mobilization response, both at the peak of the Ca^{2+} transient and at selected time intervals thereafter. (B) Shows the relationship between peak values for $[Ca^{2+}]_i$ and the concentration of sodium azide, alone and in the presence of the phosphodiesterase inhibitor, zaprinast (100 μM, 30 min preincubation). Each point represents a mean$\pm$s.e.mean of 10–24 experiments. Significance of difference between incubations with and without zaprinast: $^*P<0.05$, Bonferroni's multiple comparison test. Base values indicate the $[Ca^{2+}]_i$ prior to addition of ATP.

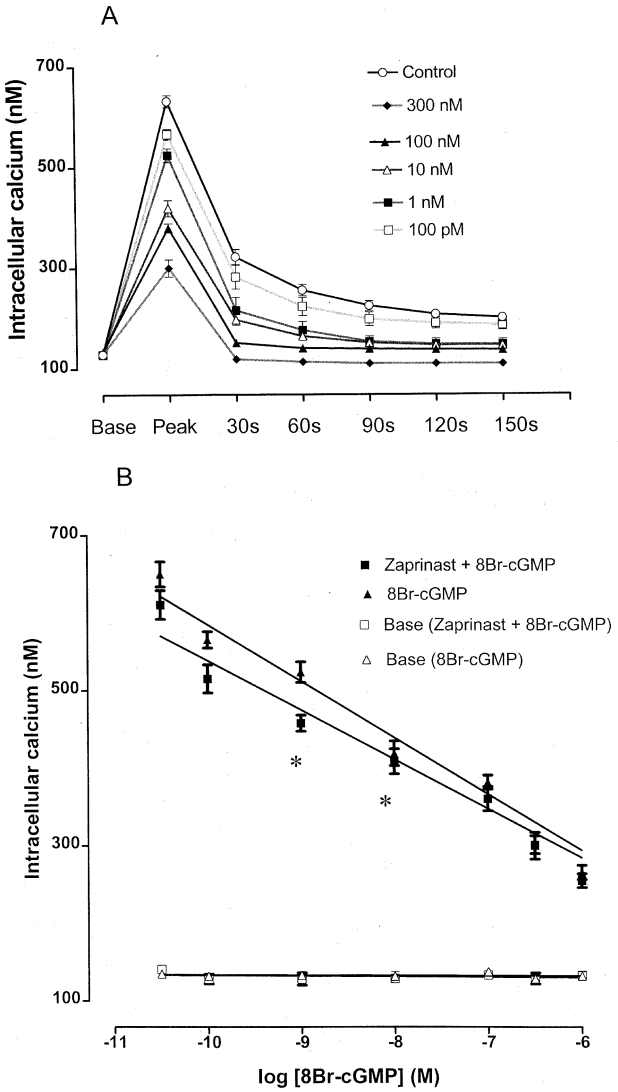

Figure 5 (A) Shows the family of curves representing the effect of increasing concentrations of 8-bromo cGMP on the Ca^{2+}-mobilization response, both at the peak of the Ca^{2+} transient and at selected time intervals thereafter. (B) Shows the relationship between peak values for $[Ca^{2+}]_i$ and the concentration of 8-bromo cGMP, alone and in the presence of the phosphodiesterase inhibitor, zaprinast (100 μM, 30 min preincubation). Each point represents a mean $\pm$ s.e.mean of 10–24 experiments. Significance of difference between incubations with and without zaprinast: $*P<0.05$, Bonferroni's multiple comparison test. Base values indicate the $[Ca^{2+}]_i$ prior to addition of ATP.

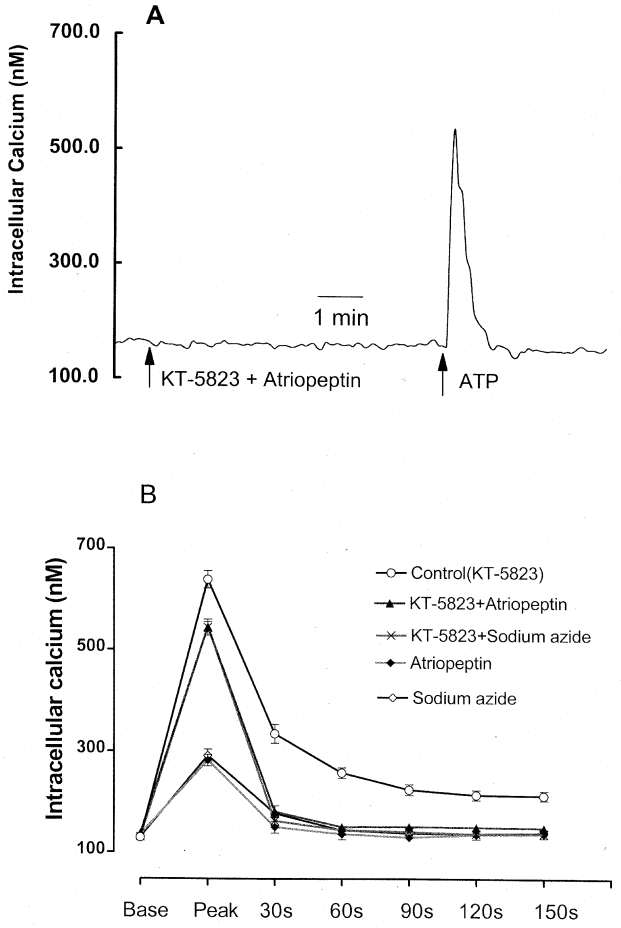

Figure 6 The effect of the PKG inhibitor, KT-5823 (10 nM), on the drug-induced inhibition of intracellular Ca^{2+} release by ATP (100 μM). (A) Shows a typical record of one experiment in which KT-5823 blocks the inhibition by atriopeptin (100 nM) of the rapid phase of the Ca^{2+} response, but suppression of the plateau phase persists. (B) Shows how KT-5823 largely reverses the inhibition by atriopeptin (100 nM) or sodium azide (300 nM) of the peak Ca^{2+} response, but fails to reverse the inhibition of the plateau response. Each point represents a mean $\pm$ s.e.mean of 7–21 experiments.

rapid decline which follows the peak is probably due to Ca-ATPase activity expelling Ca^{2+} through the plasma membrane (Lincoln *et al*., 1990) and/or to reuptake of Ca^{2+} by the intracellular stores. There follows the third (plateau) phase, a much slower decline in $[Ca^{2+}]_i$ which, in bovine CE cells, we previously reported was completely abolished in the absence of extracellular Ca^{2+} or when Ca^{2+} entry was prevented with $NiCl_2$ (Shahidullah & Wilson, 1997). It might be argued that when cGMP suppresses the plateau phase, it does so as a result of blocking the release of Ca^{2+} from stores since there is evidence that the plateau is sustained by Ca^{2+} influx across the plasma membrane which is dependent on the state of filling of the store (Pozzan *et al*., 1994). This seems unlikely in the light of our observation (Figure 6) that while the blockade of the store release is reversed by KT-5823, the suppression of the plateau phase is not. Thus the plateau phase appears to be due to an influx of extracellular Ca^{2+} (Low *et al*., 1993) which is sensitive to cGMP, but not *via* PKG.

Various mechanisms have been proposed for this inhibitory action of cGMP on Ca^{2+} signalling, including inhibition of influx of extracellular Ca^{2+} (Pozzan *et al*., 1994); activation of re-uptake by stores (Raeymakers *et al*., 1988); activation of pumps in the plasma membrane (Vrolix *et al*., 1988); and inhibition of IP_3-induced Ca^{2+} release (Komalavilas & Lincoln, 1994). Further work will be required to establish which mechanism(s) are involved in bovine CE.

Zaprinast, which selectively inhibits hydrolysis of cGMP by type V phosphodiesterase, enhanced the effect of atriopeptin, azide and 8-bromo cGMP, strengthening the argument that cGMP is involved, although a non-specific effect of zaprinast cannot be excluded.

A striking feature of the present data is the very low concentration of cGMP at which effects on $[Ca^{2+}]_i$ are evident. Previous reports suggest that ion transport in CE is altered by 1 mM cGMP (Carré & Civan, 1995) and oscillating Ca^{2+} signals are induced in hepatocytes by 50 μM cGMP (Rooney *et al*., 1996). We observed suppression of Ca^{2+} transients by sub-nanomolar concentrations of cGMP (Figure 5), compatible

with the concentration of cGMP, approx. 1–2 nM, known to occur in bovine CE (Millar *et al*., 1997).

Since all our evidence of drug modulation of Ca^{2+} movements is based on the use of ATP as a means of triggering Ca^{2+} transients, it was logical for us to test ATP for its effect on AH formation. We observed a concentration-dependent decrease. If Ca^{2+} spikes are responsible for driving AH formation, then at first sight, we might have expected that exogenous ATP applied in the whole eye would trigger vigorous Ca^{2+} transients which would serve to *increase* AH secretion. However, the nature of the stimulus delivered by exposure to exogenous ATP is continuous, while if Ca^{2+} transients are driving AH secretion, then they would be expected to be discontinuous and oscillatory. Very recently Hirata *et al*. (1998) have demonstrated oscillating Ca^{2+} spikes in isolated CE cells, suggesting that Ca^{2+} transients may indeed be responsible for driving AH secretion. These oscillations may be spontaneous or triggered by some endogenous signal. In the first case, Jacob & Civan (1996) have described the possible ionic/electrophysiological basis for such oscillations, including the involvement of Ca^{2+}-activated K^+ channels. In the latter case, the possible candidates for an endogenous signalling substance would include ATP, recently described by Mitchell *et al*. (1998) as stored in and released from CE. Continuous stimulation by ATP is unlikely, in fact, to produce vigorous repetitive Ca^{2+} transients, since rapid desensitization occurs when Ca^{2+} is repeatedly released by ATP *in vitro* (Shahidullah & Wilson, 1997). Hence exogenous ATP is more likely to disrupt the secretory process than to stimulate it.

Atriopeptin and azide stimulate cGMP production through different mechanisms, the former acting on particulate guanylyl cyclase by binding with a cell surface receptor, while azide releases nitric oxide to act on a soluble guanylyl cyclase. These drugs and 8-bromo cGMP all cause a rise in cGMP in CE cells. All these drugs reduce AH formation and IOP. All three drugs also show an inhibitory effect on peak Ca^{2+} over a similarly wide range of concentrations. This lends weight to the argument that all are acting by the same mechanism, i.e. through cGMP. This argument is further strengthened by our observation that the ability of either atriopeptin or azide to suppress IOP and AH formation in the isolated eye was blocked by selective inhibition of PKG, whereas inhibition of PKA was entirely ineffective. Thus the action of cGMP on AH formation is mediated through PKG. KT-5823 also reversed atriopeptin inhibition of ATP-induced Ca^{2+} release in cultured CE, suggesting that Ca^{2+} transients may participate in control of secretion in the CE. This also indicates that, as in many other tissues, interplay occurs between cGMP and Ca^{2+} in the modulation of AH formation.

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References

ANDRIANTSITOHAINA, R., LAGAUD, G.J., ANDRE, A., MULLER, B. & STOCLET, J. (1995). Effects of cGMP on calcium handling in ATP-stimulated rat resistance arteries. *Am. J. Physiol.,* **268,** H1223–H1231.

AZULA, F.J., ALZOLA, E.S., CONDE, M., TRUEBA, M., MACARULLA, J.M. & MARINO, A. (1996). Thrombin-stimulated phospholipase C activity is inhibited without visible delay by a rapid increase in cyclic GMP levels induced by sodium nitroprusside. *Mol. Pharmacol.,* **50,** 367–379.

AZUMA, H., MASUDA, H., SATO, J., NIWA, K. & TOKORO, T. (1997). A possible role of endogenous inhibitor for nitric oxide synthesis in the bovine ciliary muscle. *Exp. Eye Res.,* **64,** 823–830.

BECKER, B. (1990). Topical 8-bromo-cyclic GMP lowers intraocular pressure in rabbits. *Invest. Ophthalmol. Vis. Sci.,* **31,** 1647–1649.

BERRIDGE, M.J. (1993). Inositol trisphosphate and calcium signalling. *Nature,* **361,** 315–325.

CARRÉ, D.A. & CIVAN, M.M. (1995). cGMP modulates transport across the ciliary epithelium. *J. Membr. Biol.,* **146,** 293–305.

CROOK, R.B. & POLANSKY, J.R. (1992). Neurotransmitters and neuropeptides stimulate inositol phosphates and intracellular calcium in cultured human non-pigmented ciliary epithelium. *Invest. Ophthalmol. Vis. Sci.,* **33,** 1706–1716.

DIESTELHORST, M. & KRIEGLSTEIN, G.K. (1989). The intraocular pressure response of human atrial natriuretic factor in glaucoma. *Int'l. Ophthalmol.,* **13,** 99–101.

FARAHBAKHSH, N.A. & CILLUFFO, M.C. (1997). Synergistic increase in Ca^{2+} produced by A1 adenosine and muscarinic receptor activation via a pertussis toxin sensitive pathway in epithelial cells of the rabbit ciliary body. *Exp. Eye Res.,* **64,** 173–179.

GEIGER, J., NOLTE, C., BUTT, E., SAGE, S. & WALTER, U. (1992). Role of cGMP and cGMP-dependent protein kinase in nitrovasodilator inhibition of agonist-evoked calcium elevation in human platelets. *Proc. Natl. Acad. Sci. U.S.A.,* **89,** 1031–1035.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440–3450.

HALLAM, T.J., PEARSON, J.D. & NEEDHAM, L.A. (1988). Thrombin stimulated elevation of endothelial cell cytoplasmin free calcium concentration causes prostacyclin production. *Biochem. J.,* **251,** 243–249.

HIRATA, K., NATHANSON, M.H. & SEARS, M.L. (1998). Novel paracrine signaling mechanism in the ocular ciliary epithelium. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 8381–8386.

JACOB, T.J.C. & CIVAN, M.M. (1996). Role of ion channels in aqueous humour formation. *Am. J. Physiol.,* **271,** C703–C720.

KAMIKAWATOKO, S., TOKORO, T., ISHIDA, A., MASUDA, H., HAMASAKI, H., SATO, J. & AZUMA, H. (1998). Nitric oxide relaxes bovine ciliary muscle contracted by carbachol through elevation of cGMP. *Exp. Eye Res.,* **66,** 1–7.

KEE, C., KAUFMAN, P.L. & GABELT, B.T. (1994). Effect of 8-Br cGMP on aqueous humour dynamics in monkeys. *Invest. Ophthalmol. Vis. Sci.,* **35,** 2769–2773.

KOMALAVILAS, P. & LINCOLN, T.M. (1994). Phosphorylation of inositol 1,4,5-triphosphate receptor by cyclic GMP-dependent protein kinase. *J. Biol. Chem.,* **269,** 8701–8707.

KORENFELD, M.S. & BECKER, B. (1989). Atrial natriuretic peptides–Effects on intraocular pressure, cGMP and aqueous flow. *Invest. Ophthalmol. Vis. Sci.,* **30,** 2385–2392.

LEE, C.H., REISINE, T.D. & WAX, M.B. (1989). Alteration of intracellular calcium in human non-pigmented ciliary epithelial cells of the eye. *Exp. Eye Res.,* **48,** 735–743.

LINCOLN, T.M. (1989). Cyclic GMP and mechanisms of vasodilatation. *Pharmacol. Ther.,* **41,** 479–502.

LINCOLN, T.M., CORNWELL, T.L. & TAYLOR, A.E. (1990). cGMP-dependent protein kinase mediates the reduction of Ca^{2+} by cAMP in vascular smooth muscle cells. *Am. J. Physiol.,* **258,** C399–C407.

LOW, A.M., DARBY, P.J., KWAN, C.Y. & DANIEL, E.E. (1993). Effects of thapsigargin and ryanodine on vascular contractility: cross-talk between sarcoplasmic reticulum and plasmalemma. *Eur. J. Pharmacol.,* **230,** 53–62.

MEISHERI, K.D., TAYLOR, C.J. & SANEII, H. (1986). Synthetic atrial peptide inhibits intracellular calcium release in smooth muscle. *Am. J. Physiol.,* **250,** C171–C174.

MILLAR, J.C., SHAHIDULLAH, M. & WILSON, W.S. (1997). Atriopeptin lowers aqueous humour formation and intraocular pressure and elevates cyclic GMP but lacks uveal vascular effects in the bovine perfused eye. *J. Oc. Pharmacol. Ther.,* **13,** 1–11.

MITCHELL, C.H., CARRE, D.A., MCGLINN, A.M. & STONE, R.A. (1998). A mechanism for stored ATP in ocular ciliary epithelial cells. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 7174–7178.

MITTAG, T.W. (1989). Adrenergic and dopaminergic drugs in glaucoma. In *The Glaucomas* eds. Ritch, R., Shields, M.B & Krupin, T. pp. 523–527. St. Louis, MO: CV Mosby.

MITTAG, T.W., TORMAY, A., ORTEGA, M. & SEVERIN, C. (1987). Atrial natriuretic peptide (ANP), guanylyl cyclase and intraocular pressure in the rabbit eye. *Curr. Eye Res.,* **6,** 1189–1196.

NATHANSON, J.A. (1980). Adrenergic regulation of intraocular pressure: identification of β_2-adrenergic-stimulated adenylate cyclase in ciliary process epithelium. *Proc. Natl. Acad. Sci. U.S.A.,* **77,** 7420–7424.

NATHANSON, J.A. (1987). Atriopeptin-activated guanylyl cyclase in the anterior segment. *Invest. Ophthalmol. Vis. Sci.,* **28,** 1357–1364.

NEUFELD, A.H., JAMPOL, L.M. & SEARS, M.L. (1972). Cyclic AMP in the aqueous humour: the effects of adrenergic agents. *Exp. Eye Res.,* **14,** 242–250.

POZZAN, T., RIZZUTO, R., VOLPE, P. & MELDOLESI, J. (1994). Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.,* **74,** 595–636.

RAEYMAKERS, L., HOFMANN, F. & CASTEELS, R. (1988). Cyclic GMP-dependent protein kinase phosphorylates phospholamban in isolated sarcoplasmic reticulum from cardiac and smooth muscle. *Biochem. J.,* **252,** 269–273.

ROONEY, T.A., JOSEPH, S.K., QUEEN, C. & THOMAS, A.P. (1996). Cyclic GMP induces oscillatory calcium signals in rat hepatocytes. *J. Biol. Chem.,* **271,** 19817–19825.

SCHÜTTE, M., DIADORI, A., WANG, C. & WOLOSIN, J.M. (1996). Comparative adrenocholinergic control of intracellular Ca^{2+} in the layers of the ciliary body epithelium. *Invest. Ophthalmol. Vis. Sci.,* **37,** 212–220.

SHAHIDULLAH, M. & WILSON, W.S. (1997). Mobilization of intracellular calcium by $P2Y_2$ receptors in cultured, non-transformed bovine ciliary epithelial cells. *Curr. Eye Res.,* **16,** 1006–1016.

SHAHIDULLAH, M., WILSON, W.S. & MILLAR, J.C. (1995). Effects of timolol, terbutaline and forskolin on IOP, aqueous humour formation and ciliary cyclic AMP levels in the bovine eye. *Curr. Eye Res.,* **14,** 519–528.

STEARDO, L. & NATHANSON, J. (1987). Brain barrier tissues: end organ for atriopeptins. *Science,* **235,** 470–472.

SUGRUE, M.F. & VIADER, M.P. (1986). Synthetic atrial natriuretic factor lowers rabbit intraocular pressure. *Eur. J. Pharmacol.,* **130,** 349–350.

SUZUKI, Y., NAKANO, T. & SEARS, M. (1997). Calcium signals from intact rabbit ciliary epithelium observed with confocal microscopy. *Curr. Eye Res.,* **16,** 166–175.

TSUNODA, Y. (1993). Receptor-operated Ca^{2+} signalling and crosstalk in stimulus-secretion coupling. *Biochim. Biophys. Acta,* **1154,** 105–156.

VROLIX, M., RAEMAEKERS, L., WUYTACK, F., HOFMANN, F. & CASTEELS, R. (1988). Cyclic GMP-dependent protein kinase stimulates the plasmalemmal Ca^{2+} pump of smooth muscle via phosphorylation of phosphatydilinositol. *Biochem. J.,* **255,** 855–863.

WILSON, W.S., SHAHIDULLAH, M. & MILLAR, J.C. (1993). The bovine arterially-perfused eye: an *in vitro* method for the study of drug mechanisms on IOP, aqueous humour formation and uveal vasculature. *Curr. Eye Res.,* **12,** 609–620.

YOSHIMURA, N., TANABE-OHUCHI, T., TAKAKI, H. & HONDA, Y. (1995). Drug-dependent Ca^{2+} mobilization in organ-cultured ciliary processes. *Curr. Eye Res.,* **14,** 629–635.

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