PLOS ONE

Terbutaline, forskolin and cAMP reduce secretion of aqueous humour in the isolated bovine eye

Mohammad Shahidullah[1]*, **William Stuart Wilson**[2], **Kazi Rafiq**[3], **Mahmudul Hasan Sikder**[3], **Jannatul Ferdous**[3], **Nicholas Anthony Delamere**[1]

1 Department of Physiology and the Department of Ophthalmology and Vision Science, University of Arizona, Tucson, AZ, United States of America, **2** College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom, **3** Department of Pharmacology, Bangladesh Agricultural University, Mymensing, Bangladesh

* shahidua@arizona.edu

Abstract

In order to elucidate involvement of cyclic AMP and intracellular Ca^{2+}, $[Ca^{2+}]_i$, in the modulation of aqueous humour formation (AHF), we studied the effects of terbutaline, forskolin and 8-Br-cAMP in the isolated bovine eye. We also studied the interaction of cAMP on calcium signaling in cultured ciliary epithelial (CE) cells. Drug effects on AHF were measured by fluorescein dilution. Drug effects on $[Ca^{2+}]_i$ were studied by the fura-2 fluorescence ratio technique. Terbutaline (100 nmol-100 M), forskolin (30 nM-100 M) or 8-Br-cAMP (100 nM–10 μM), administered in the arterial perfusate produced significant reductions in AHF. The AH reducing effect of terbutaline was blocked by a selective inhibitor of protein kinase A (KT-5720). ATP (100 M) caused a rapid, transient (peak) increase in $[Ca^{2+}]_i$ followed by a sustained plateau phase lasting more than 5 minutes. Preincubation of the cells (6 min) with terbutaline, forskolin or 8-Br-cAMP significantly reduced the peak calcium response to ATP. The sustained plateau phase of the response, on the other hand, was augmented by each of the agents. KT-5720 partially reversed the inhibitory effect of terbutaline on the peak and totally inhibited its effect on the plateau phase. These data indicate: (a) that AHF in the bovine eye can be manipulated through cyclic AMP, operating via protein kinase A, (b) that protein kinase A can affect $[Ca^{2+}]_i$ homeostasis, (c) that calcium release from the intracellular store, not the entry, affects AHF, and (d) that interaction of $[Ca^{2+}]_i$ with cAMP plays a role in modulating AH secretion.

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 muscular ciliary body. Cyclic AMP (cAMP) has long been considered to be an important regulator of aqueous humour formation (AHF) and this area has been extensively studied [1–4]. However, the mechanisms involved are not clearly understood, not least, since reduction of intraocular pressure (IOP)

and AHF by both β-adrenergic agonists and antagonists occurs in some species [5–11]. In the bovine isolated perfused eye we have reported that AHF is suppressed by the β_2 agonist, terbutaline, but that there seems to be no correlation between drug effects on AHF and those on the cAMP content of the CE [12].

In many tissues, other important intracellular second messengers have become firmly established including Ca^{2+}. Ca^{2+} is well established as a regulator of a number of cellular functions including secretion [13,14]. Many workers have reported "transients" or sudden changes in intracellular calcium, $[Ca^{2+}]_i$, in cultured CE cells [15–20]. We have previously identified and characterised P2Y2 receptors in cultured bovine CE [18]. Stimulation of these receptors with ATP or UTP causes $[Ca^{2+}]_i$ release and a rise in intracellular free Ca^{2+} concentration [18]. Release of Ca^{2+} has also been reported in CE cells by stimulation of various other receptors including histamine H1 and vasopressin V1 receptors [21–23], 1 and 2-adrenergic, muscarinic and adenosine receptors [15–17,19].

We have previously published evidence of links between AH production and $[Ca^{2+}]_i$ by examining the effects of drugs acting through cyclic GMP on AHF in bovine perfused eye and on ATP-induced increase in $[Ca^{2+}]_i$ in bovine cultured CE [24]. Such data help to correlate physiological effects with biochemical phenomena. In the present investigation, we present data establishing similar links using drugs which act via cAMP and which affect both AHF and Ca^{2+} signaling in bovine CE cells.

Materials and methods

Drugs and chemicals

ATP, terbutaline, forskolin, 8-bromo cAMP, 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-5720 was obtained from Calbiochem. Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), new-born calf serum (NCS) and trypsin EDTA (1X) were obtained from Life Technologies and collagenase A from Boehringer Mannheim. MnCl2 was obtained from BDH and $NiCl_2$ from Hopkin and Williams. All other reagents were of analytical grade. Drugs were dissolved in appropriate concentrations either in distilled water or in dimethyl sulfoxide (DMSO) according to their solubility.

Bovine eye

Bovine eyes were used for whole eye perfusion study to estimate aqueous humor secretion and for obtaining primary culture of ciliary epithelium to measure intracellular calcium. Bobine eyes were obtained from a local abattoir. The use of animal tissue was approved by the University of Arizona, Institutional Animal Care and Use Committees (Protocol number: 12–395) and conformed to the ARVO Resolution for the Use of Animals in Ophthalmic and Vision Research.

Estimation of AHF

AHF rate was measured by the fluorescein dilution technique as described earlier [25,26]. In brief, bovine eyes were perfused through the ophthalmic artery at 37C with an oxygenated Krebs' solution comprising (mM): NaCl, 118; KCl, 4.7; CaCl2, 2.5; KH2PO4, 1.2; MgSO4, 1.2; NaHCO3, 25; glucose, 11.5; ascorbate, 0.05. Drugs were administered at fixed concentrations in the arterial perfusate. AHF rate was estimated for 30 min before addition of drug solution into the perfusate to obtain control values (this excluded individual variation among different

eyes). Estimation of AHF rate was then restarted 15–20 min after exposure to perfusate containing the drug and continued for a further 60 min.

Culture of CE cells and measurement of intracellular Ca^{2+}

The tips (1–2 mm) of ciliary processes were isolated from bovine eyes and CE cells were cultured as previously described [12]. Cells from the 3rd or 4th passage were placed on sterile glass coverslips and cultured overnight before being loaded with fura-2 for studies on $[Ca^{2+}]_i$. The cells were incubated for 30 min at 37C with fura-2 AM (2 M) in HEPES (25 mM)-buffered DMEM containing bovine serum albumin (1%) under constant gentle shaking. The coverslips containing cells loaded with fura-2 were transferred to HEPES (10 mM)-buffered Krebs' solution (pH 7.4) containing (mM): NaCl, 118; KCl, 4.8; MgSO4, 1.0; NaHCO3, 2.4; glucose, 11.0; HEPES 10.0 and CaCl2, 1.8 and left at room temperature for 20 min, to allow for hydrolysis of fura-2 AM to the Ca2+-sensitive acid form. Coverslips with fura-2 loaded cells were then mounted in a small bath attached to the stage of an inverted microscope, where the cells were continuously superperfused (approximately 2.5 ml/min, at 37°C) with oxygenated HEPES buffered Krebs' solution mentioned above. All experiment was carried out using this HEPES-buffered Krebs' solution, but in some, the $CaCl_2$ was omitted, as indicated in the result. In experiments involving $NiCl_2$ (4mM), the same solution was used, except that $MgSO_4$ was replaced with 1.0 mM $MgCl_2$ and $NaHCO_3$ was omitted. The drugs were added to the perfusate at appropriate concentration.

Measurement and estimation of $[Ca^{2+}]_i$ was done according to the dual excitation method of Grynkiewicz [27] and using Quanticell 500 imaging system (Applied Imaging). The Fura-2 fluorescence signal (Ex 340/380 nm; Em 515nm) were acquired with a Nikon Diaphot inverted microscope and a CCD camera. At the end of each experimental run, background auto fluorescence was obtained according to Hallam et al [28]. Ionomycin (2 μM) was added to permeabilize the cells to divalent cations and $MnCl_2$ (2mM) 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 computed using the Grynkiewicz equation,

$$[Ca^{2+}]_I = K_d \times \frac{(R - R_{min})S_{f2}}{(R_{max} - R)S_{b2}}$$

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 the 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 [27]. Values of Rmax, Rmin, Sf2 and Sb2 were experimentally determined in fura-2 loaded CE cell suspensions according to the method described earlier [18]. The following calibration values were obtained and used throughout to calculate $[Ca^{2+}]_i$: Rmax, 17.59 0.34 (n = 4); Rmin, 0.91 0.04 (n = 4); Sf2/Sb2, 6.89 0.02 (n = 13).

Statistical analysis

Data on AHF were subjected to one-way analysis of variance (ANOVA) and paired Student's t-test. Data on $[Ca^{2+}]_i$ were analysed using one-way ANOVA followed by Bonferoni's multiple comparison test. Graphs were drawn using the GraphPad Prism computer package.

3. Results

Effects of terbutaline and forskolin and 8-Br-cAMP on AH formation

The specific β_2-adrenoceptor agonist, terbutaline, caused a highly significant reduction of AH formation rate (Fig 1) as did the direct stimulator of adenylate cyclase, forskolin, (Fig 2) and 8-Br-cAMP, the cell permeable analogue of endogenous cAMP (Fig 3).

Effects KT-5720 on the AH-reducing action terbutaline

KT-5720 (10nM), a specific inhibitor of cAMP-dependent protein kinase (PKA), was added to the perfusate 10 minutes prior to terbutaline and this abolished the AH-suppressing effect of terbutaline (100 M) (Fig 4).

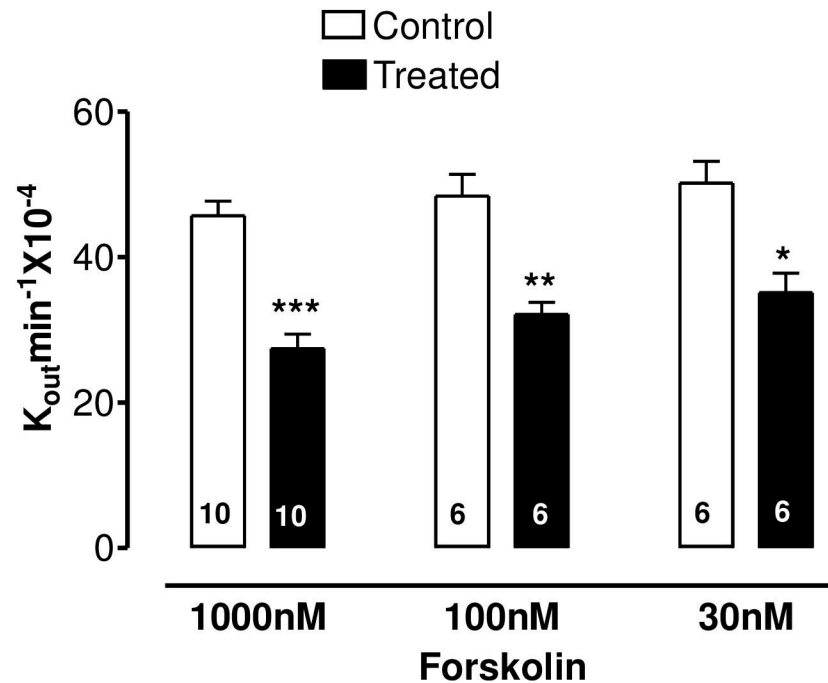### Effects of terbutaline, forskolin and 8-bromo cAMP on ATP-induced Ca^{2+} transients in CE

Addition of 100 M ATP to cultured CE, in the presence of extracellular calcium, caused a highly reproducible Ca2+ transient whose peak was followed by a slow decline and plateau (see typical trace in Fig 5A). The plateau phase was completely absent when cells were exposed to ATP in calcium free solution (Fig 5B), indicating that the plateau phase is due to entry of calcium from extracellular space. The peak of the Ca2+ transient was usually reached within 30s.

When the CE cells were incubated in a medium containing terbutaline, forskolin or 8-bromo cAMP, the magnitude of the peak value of the Ca2+ transient triggered by ATP was decreased, but the sustained plateau phase was increased (Figs 6, 7 and 8). In each case the effects of these three agents were concentration-dependent and the time course of the effects
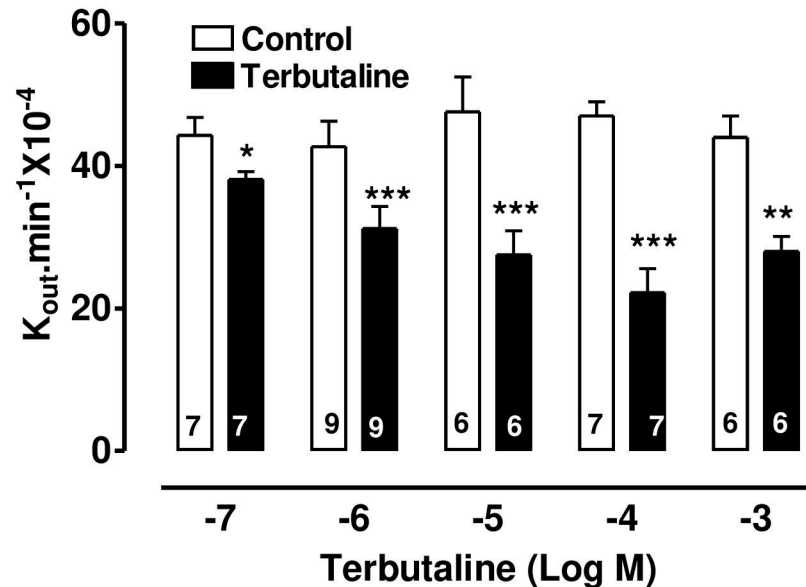
Fig 1. The effect of terbutaline on AH formation rate in the bovine isolated perfused eye measured by fluorescein dilution technique. AH formation rate was expressed as the rate constant ($K_{out}.min^{-1} \times 10^{-4}$), defined as the slope of the regression line drawn on LN (Log_e) of the changes of fluorescence with time (min). Each value is a mean s.e.mean of the number (n) of experiments shown at the base of each column. In order to eliminate individual variation, control and treated data were obtained from the same eye. AH formation rate measured during the first 30 min prior to drug application was taken as the control value. AH formation rate for the subsequent 40–60 min after establishment of drug effects was taken as the treated value. Note that after addition of drug, 20 min stabilization period was allowed to establish drug effect. Significance of differences from controls; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

https://doi.org/10.1371/journal.pone.0244253.g001

Fig 2. The effect of forskolin on AH formation rate in the bovine isolated perfused eye measured by fluorescein dilution technique. AH formation rate was expressed as the rate constant ($K_{out}.min^{-1} \times 10^{-4}$), defined as the slope of the regression line drawn on LN (Log_e) of the changes of fluorescence with time (min). Each value is a mean s.e.mean of the number (n) of experiments shown at the base of each column. In order to eliminate individual variation, control and treated data were obtained from the same eye. AH formation rate measured during the first 30 min prior to drug application was taken as the control value. AH formation rate for the subsequent 40–60 min after establishment of drug effects was taken as the treated value. Note that after addition of drug, 20 min stabilization period was allowed to establish drug effect. Significance of differences from controls; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

was very similar from one agent to the next. In the case of terbutaline (100 μM), the maximum degree of inhibition of peak height of the Ca^{2+} transient was 48.7 ± 4.5% (mean ± s.e.mean, n = 10) of control response while the degree of augmentation of the plateau phase (at 180 s) was 67.8 ± 7.3% (Fig 6). The lowest concentration of terbutaline (1 μM) which significantly depressed peak heights was also the lowest effective concentration in terms of augmenting the plateau phase (calcium entry).

In the case of forskolin (10μM), the maximum degree of inhibition of peak height of the Ca^{2+} transient was 50.1± 4.5% (mean ± s.e.mean, n = 8) of control response while the degree of augmentation of the plateau phase (at 180 s) was 63.7 ± 6.2% (Fig 7). In the case of 8-bromo cAMP (10 μM), the maximum degree of inhibition of peak height of the Ca^{2+} transient was 44.2± 5.5% (mean ± s.e.mean, n = 8) of control response while the degree of augmentation of the plateau phase (at 180 s) was 59.0 ± 10.5% (Fig 8). The lowest concentrations of forskolin and 8-bromo cAMP which significantly depressed peak heights were also the lowest effective concentrations in augmenting the plateau phase in each case.

Effect of KT-5720 on terbutaline inhibition of the ATP-induced Ca^{2+} release

KT-5720 (10 nM) significantly ($P < 0.001$) reduced the inhibitory effect of terbutaline on the ATP-induced peak rise in [Ca2+]i from 48.7 ± 4.5% to 22.7 ± 6.2% (mean ± s.e.mean, n = 7).
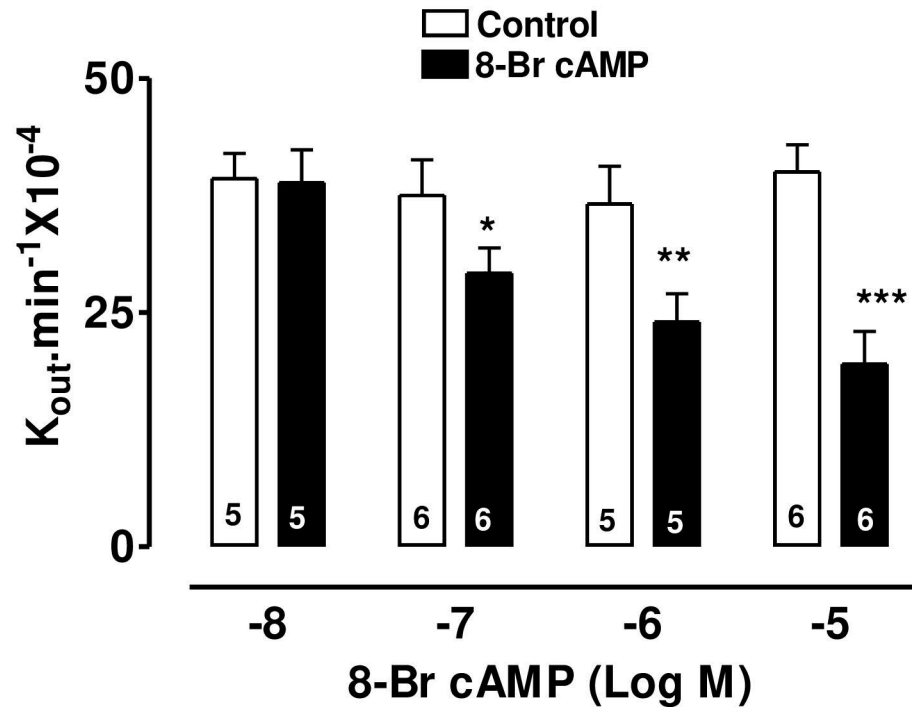
Fig 3. The effect of 8-Br-cAMP on AH formation rate in the bovine isolated perfused eye measured by fluorescein dilution technique. AH formation rate was expressed as the rate constant ($K_{out}.min^{-1} \times 10^{-4}$), defined as the slope of the regression line drawn on LN (Log_e) of the changes of fluorescence with time (min). Each value is a mean s.e.mean of the number (n) of experiments shown at the base of each column. In order to eliminate individual variation, control and treated data were obtained from the same eye. AH formation rate measured during the first 30 min prior to drug application was taken as the control value. AH formation rate for the subsequent 40–60 min after establishment of drug effects was taken as the treated value. Note that after addition of drug, 20 min stabilization period was allowed to establish drug effect. Significance of differences from controls; * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

https://doi.org/10.1371/journal.pone.0244253.g003

On the other hand, KT-5720 (10 nM) completely blocked the terbutaline's effect on the plateau phase of the Ca^{2+} response (Fig 9).

Effect of terbutaline on ATP-induced Ca_{2+} transients in the absence of extracellular Ca^{2+} or in presence of Ni^{2+}

When CE cells were incubated in a calcium-free medium containing EGTA (40 mM) (Fig 10), or a medium with calcium but containing $NiCl_2$ (4mM) (Fig 11), the peak phase of the ATP-induced $[Ca^{2+}]_i$ transient was unchanged, however, the plateau phase was virtually absent. Under these conditions, terbutaline still suppressed the peak phase, but the sustained plateau phase was no longer augmented (Figs 10 and 11). This result confirms that the plateau phase is due to entry of calcium from extracellular space.

Discussion and conclusion

The present study demonstrates that terbutaline, forskolin and 8-bromo cAMP, perfused arterially at appropriate concentrations, decrease AHF in the bovine isolated eye preparation. This confirms our previous report [12] that terbutaline lowers IOP and AHF, although our failure (in that earlier work) to demonstrate a similar effect of forskolin or 8-bromo cAMP may have been due to the nature of drug administration–by single bolus injection rather than continuous arterial perfusion. In the present work, all drugs were delivered continuously at a fixed
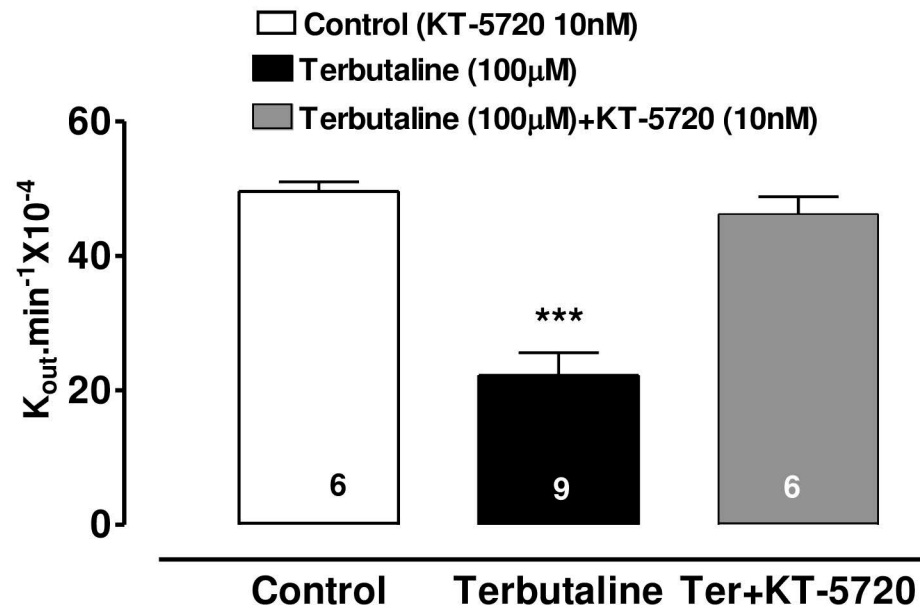
Fig 4. Effect of KT-5720 (10nM) on the inhibitory effect of terbutaline (100μM) on AH formation in the bovine perfused eye. Stock solution of the PKA inhibitor (KT-5720) in DMSO was added to the perfusate to obtain a final concentration of 10 nM. AH formation rate (shown as $K_{out} \cdot min^{-1} \ 10^{-4}$) was measured in separate groups of eyes for 90 min after injection of vehicle or drug. Each value is a mean s.e.mean of the number (n) of experiments shown at the base of each column. Control: injection of vehicle and PK inhibitor. Significance of difference from control; *** $p < 0.001$.

concentration in the perfusate. A further implication of this is that, in the present work, drug concentrations perfused through the vasculature of the whole eye should closely approximate actual drug concentrations in the ciliary body and can therefore be directly compared to those applied to the CE cells in culture in the study of calcium movements.

The involvement of cAMP in the mechanism of the β-agonist effect on intraocular pressure was proposed by Neufeld [4]. We have previously summarised [12] the controversy over adrenergic effects on AH dynamics. A bolus dose of terbutaline decreased AHF in the bovine perfused eye and that the same dose also increased cAMP in the ciliary processes, although this increase was only measurable when the phosphodiesterase inhibitor, IBMX, was also present [12]. Forskolin increases cAMP synthesis by a different mechanism from terbutaline, yet it too decreases AHF, encouraging the idea that this nucleotide may mediate the effects of both drugs on AHF. Our present evidence in the bovine eye that continuous perfusion of forskolin or 8-Br-cAMP decreases AHF and that KT-5720 (a selective PKA inhibitor) inhibits the terbutaline-induced suppression of AH secretion, throws the balance of evidence in favour of the hypothesis that cAMP is the most likely mediator of the terbutaline effect on AHF in the bovine eye. The lack of an effect of KT5720 on its own also indicates that endogenous levels of cAMP in the isolated eye may be too low to exert a tonic effect on AH secretion. The exact mechanism by which cAMP reduces AH secretion in the bovine eye is not known. However, cAMP has been shown, by dual patch clamp and lucifer yellow (LY) transfer studies, to inhibit gap junctional communication between PE and nonpigmented ciliary epithelim (NPE) of bovine CE [29]. Gap junctional communication between PE and NPE is fundamentally important function in AH secretion. This is because AH is formed by ion and fluid transfer from the ciliary stroma sequentially across the PE cells, gap junctions, NPE cells and then into the posterior aqueous humor chamber of the eye [30,31]. Cyclic AMP also activate chloride channels in

A

B
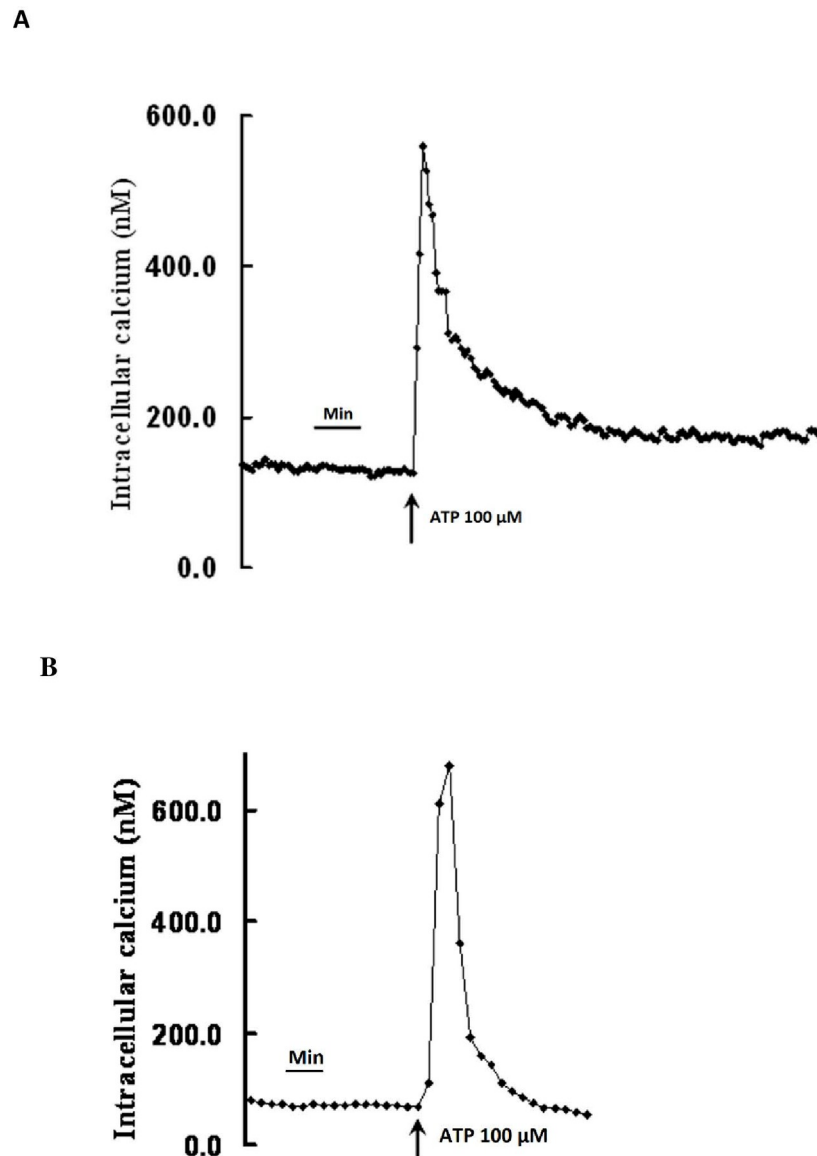
Fig 5. Increase in intracellular Ca^{2+} in bovine cultured ciliary epithelial cells in response to ATP (100 μM). (A) Typical trace, obtained in the presence of 1.8mM extracellular calcium, shows a rapid increase in [Ca^{2+}]$_i$ which is followed by a rapid decline and then a prolonged plateau above baseline. (B) Typical trace obtained in the absence extracellular calcium shows a rapid increase in [Ca^{2+}]$_i$ which is followed by a rapid decline to baseline without forming the plateau.

https://doi.org/10.1371/journal.pone.0244253.g005

the PE of bovine eye [1,32]. Activation of chloride channels in the PE would favour reabsorption of chloride ions into the stroma and hence potential reduction in AH secretion. Thus, present data also supports the hypothesis that chloride ion transport across the CE is an important contributor in AH secretion [33], particularly in the bovine eye [25].

The present study demonstrates that terbutaline, forskolin and cAMP are all potent inhibitors of cytoplasmic Ca^{2+} release induced by exogenous ATP in bovine CE cells. In an earlier study we have shown that in these cells ATP mobilises [Ca^{2+}]i mainly from internal stores [18]. Extracellular ATP acts on G-protein coupled cell-surface P2Y2 receptors to stimulate the membrane-bound enzyme phospholipase C, causing production of IP3, which then binds to
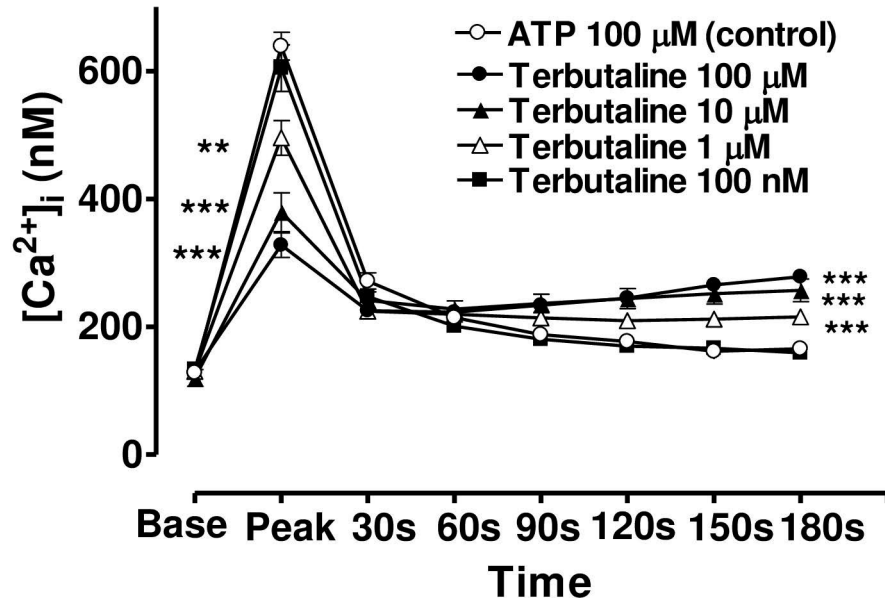
Fig 6. Effect of increasing concentration of terbutaline on the Ca²⁺-mobilizing response of ATP (100μM) in bovine cultured CE, both at the peak of the Ca²⁺ transient and at selected time intervals thereafter. Cultured cells were incubated for 6 min with appropriate concentration of terbutaline in HEPES bufererd Krebs' solution containing 1.8mM calcium. The cells were then exposed to 100μM of ATP. Each point represents a mean±s.e.mean of 8–10 experiments. Significance of differences between incubations with or without terbutaline; ** $p < 0.01$ and *** $p < 0.001$, Bonferroni's multiple comparison test. Base value indicates the $[Ca^{2+}]_i$ prior to addition of ATP.

https://doi.org/10.1371/journal.pone.0244253.g006
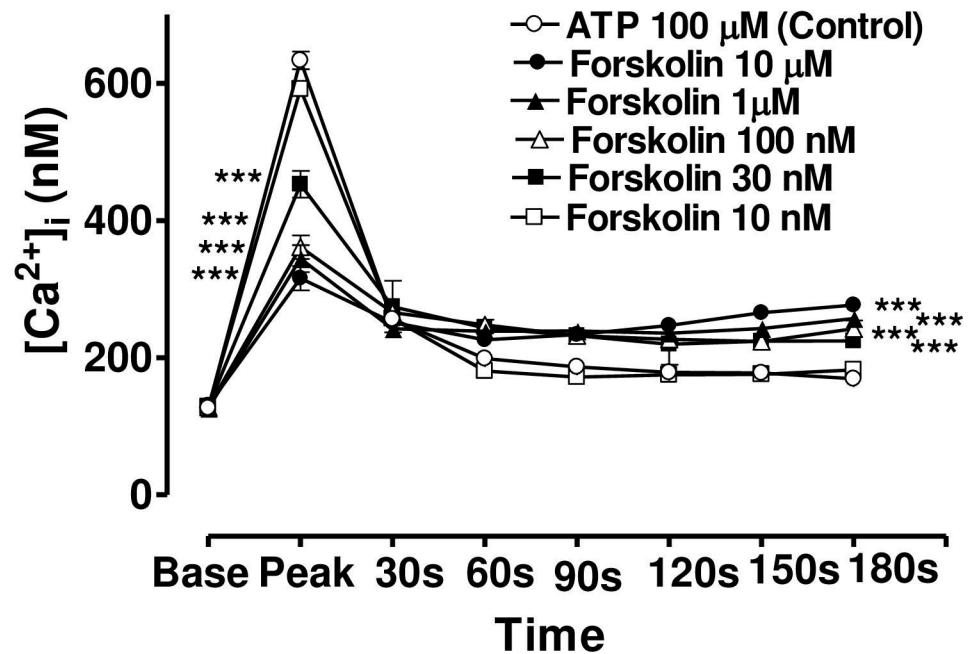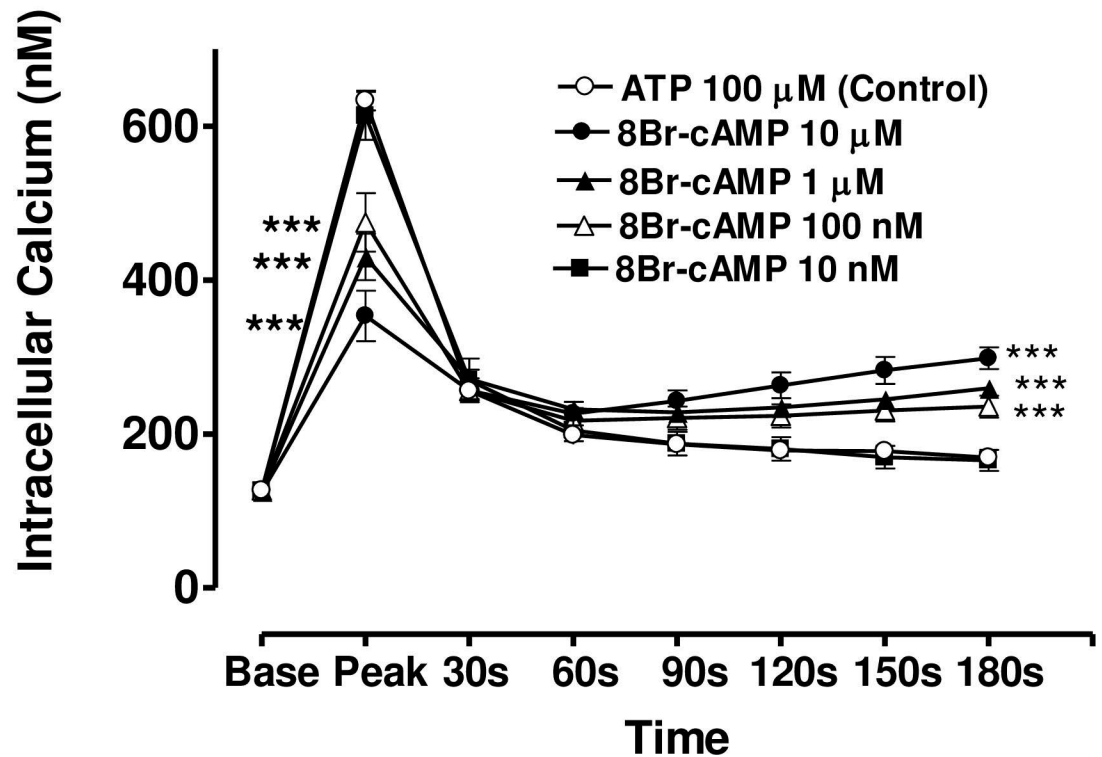

Fig 7. Effect of increasing concentration of forskolin on the Ca²⁺-mobilizing response of ATP (100μM) in bovine cultured CE, both at the peak of the Ca²⁺ transient and at selected time intervals thereafter. Cultured cells were incubated for 6 min with appropriate concentration of forskolin in HEPES buffered Krebs' solution containing 1.8mM calcium. The cells were then exposed to 100μM of ATP. Each point represents a mean±s.e.mean of 8–15 experiments. Significance of differences between incubations with or without forskolin; ** $p < 0.01$ and *** $p < 0.001$, Bonferroni's multiple comparison test. Base value indicates the $[Ca^{2+}]_i$ prior to addition of ATP.

https://doi.org/10.1371/journal.pone.0244253.g007

Fig 8. Effect of increasing concentration of 8-Br-cAMP on the Ca^{2+}-mobilizing response of ATP (100μM) in bovine cultured CE, both at the peak of the Ca^{2+} transient and at selected time intervals thereafter. Cultured cells were incubated for 6 min with appropriate concentration of 8-Br-cAMP in HEPES bufererd Krebs' solution containing 1.8mM calcium. The cells were then exposed to 100μM of ATP. Each point represents a mean±s.e.mean of 8–15 experiments. Significance of differences between incubations with or without 8-BR-cAMP; ** p < 0.01 and *** p < 0.001, Bonferroni's multiple comparison test. Base value indicates the [Ca^{2+}]$_i$ prior to addition of ATP.

its receptor on the endoplasmic reticulum, thus mobilising stored Ca^{2+}. All three drugs raise cellular levels of cAMP and all produce a substantial and concentration-dependent inhibition of ATP-induced [Ca^{2+}]i release.

In our system, the [Ca^{2+}]$_i$ response of CE to ATP occurs in three phases. There is evidence to suggest the rapid initial rise is due to release of intracellular stored Ca$_{2+}$ [18] and this also occurs in other secretory cells [34]. The rapid decline which follows the peak is probably due to plasma membrane Ca-ATPase activity and sodium-calcium exchange expelling Ca$_{2+}$ through the plasma membrane [35] and/or to reuptake of Ca$_{2+}$ by the intracellular stores. There follows the third (plateau) phase, a much slower decline in Ca$_{2+}$ which, in bovine CE cells, we previously reported and re-confirmed in the present study, was completely abolished in the absence of extracellular Ca$_{2+}$ or when Ca$_{2+}$ entry was prevented with NiCl2 [18]. Some workers describe this phase as capacitative calcium entry, since it appears to be a means of refilling the cell's calcium stores from the extracellular pool.

In the present work, terbutaline, forskolin and cAMP all suppressed the release of stored calcium in a concentration-dependent manner. Similar inhibition of Ca^{2+} release by cAMP has been observed in many other tissues and cell types including platelets [36,37], aortic smooth muscle [38] and foetal lung epithelium [39]. It is believed that cAMP causes phosphorylation of the IP$_3$ receptor [40], thereby inhibiting its ability to release Ca$_{2+}$ as first suggested by Supattapone [41].
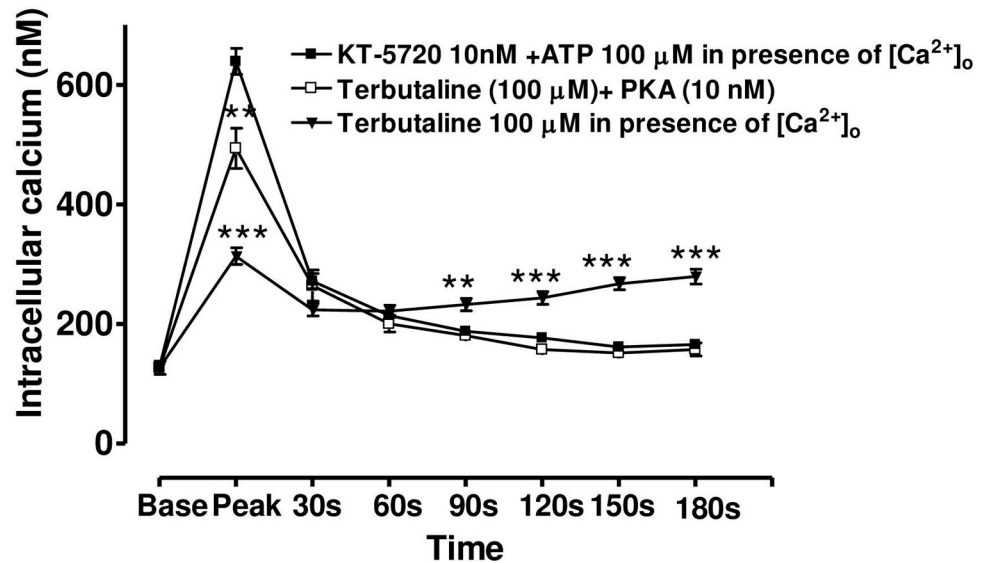
Fig 9. The effect of the PKA inhibitor, KT-5720 (10nM), on the terbutaline-induced inhibition of $[Ca^{2+}]_i$ increase by ATP. Cells were incubated with either KT-5720 (10nM), or terbutaline (100μM) alone or with KT-5720 (10nM) and terbutaline (100μM) together for 6 min before stimulating by ATP (100μM). KT-5720 largely reversed terbutaline-induced inhibition of the rapid phase of Ca2+ release by ATP. On the other hand, it completely abolished terbutaline-induced augmentation of the plateau phase of the ATP response. Each point is a mean±s.e.mean of 7–10 experiments. Significance of differences from 100μM terbutaline treated response; ** $p < 0.01$ and *** $p < 0.001$, Bonferroni's multiple comparison test. Base value indicates the $[Ca^{2+}]_i$ prior to addition of ATP.

https://doi.org/10.1371/journal.pone.0244253.g009

Terbutaline, forskolin and cAMP also had concentration-dependent effects on the plateau phase of the $[Ca^{2+}]_i$ record, significantly enhancing the entry of Ca_{2+}. This effect confirms the hypothesis that the plateau phase is due to calcium entry since it is absent in Ca_{2+}–free solutions or in the presence of $NiCl_2$. Such enhancement of calcium entry by cAMP has also been reported in other tissues and cell types, including foetal lung epithelium [39], rat salivary gland [42] and HL60 promyelocytes [43]. In the present work, KT-5720 entirely abolished the enhancement of Ca_{2+} entry by terbutaline (100μM), confirming the published report in promyelocyte [43] that this effect is PKA-dependent.

It is interesting to note that as the release of stored Ca^{2+} is inhibited incrementally by increasing concentrations of terbutaline, forskolin or 8-Br-cAMP, so the stimulation of entry of extracellular Ca^{2+} increased. Hence this enhancement of calcium entry in bovine CE cells is not driven by the degree of store emptying, as has sometimes been observed [44,45] and could not be interpreted as stimulation of capacitative calcium entry. Rather it is mediated directly by cAMP itself on some other calcium entry pathways, since KT-5720 entirely blocked the terbutaline's ability to stimulate Ca^{2+} entry. Several pathways of calcium influx, through the plasma membrane, had been proposed in response to receptor stimulation by agonists. These include receptor operated channels (ROC), G-protein operated channels (GOC), second-messenger operated channels (SMOC), or by calcium-release activated channels (CRAC). The last pathway (CRAC) has been proposed to be due to the release of calcium influx factor (CIF) in response to emptying of store, and has been defined as capacitative calcium entry [46,47]. Which of these mechanisms is affected by cAMP to enhance calcium entry in bovine CE remains to be elucidated.

The apparent enhancement of Ca^{2+} entry by cAMP-PKA signaling is entirely different from the effect of cGMP on Ca^{2+} movements in CE cells, as we observed earlier [24]. Although both nucleotides suppressed ATP-induced release of stored Ca^{2+}, atriopeptin or
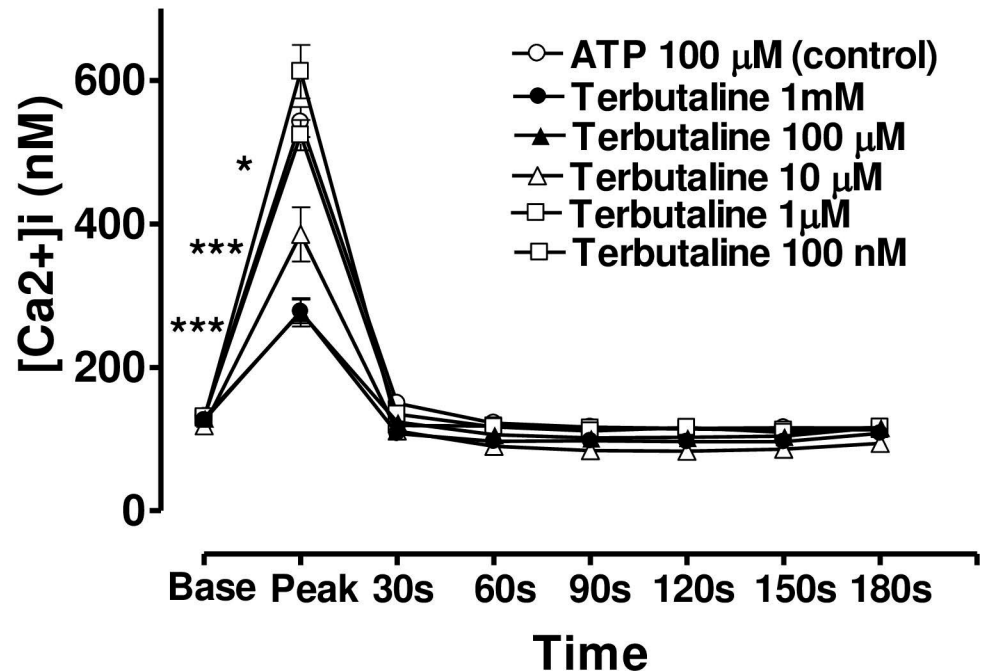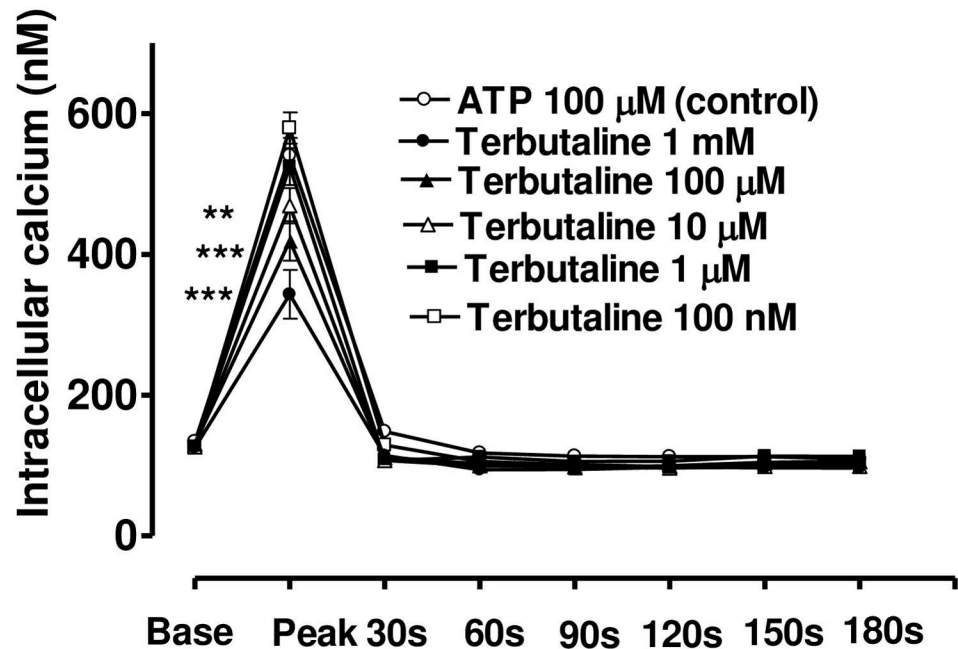
Fig 10. Effect of increasing concentration of terbutaline on the Ca^{2+}-mobilizing response of ATP (100μM) in bovine cultured CE, in the absence of extracellular calcium. Terbutaline's effect on both the peak Ca^{2+} transient and at selected time intervals thereafter had been shown. Cultured cells were incubated for 6 min with appropriate concentration of terbutaline in HEPES buffered Krebs' solution containing 40mM EGTA and no calcium. The cells were then exposed to 100μM of ATP. Each point represents a mean±s.e.mean of 6–11 experiments. Significance of differences between incubations with or without terbutaline; ** $p < 0.01$ and *** $p < 0.001$, Bonferroni's multiple comparison test. Base value indicates the [Ca^{2+}]$_i$ prior to addition of ATP.

https://doi.org/10.1371/journal.pone.0244253.g010

8-Br-cGMP suppressed Ca^{2+} entry while terbutaline, forskolin or 8-Br-cAMP enhanced the Ca^{2+} entry during the plateau phase of the response. Suppression of calcium entry by cGMP occured at a very low concentrations (lower threshold 100pM– 1.0 nM), while the enhancement of calcium entry by cAMP observed in the present study had a lower threshold concentration of 1.0μM. Furthermore, the effect of cGMP on calcium entry appeared not to be mediated by PKG [24].

How do we correlate the the biochemical response of cAMP on [Ca^{2+}]$_i$ with the cellular function of AH secretion by the CE? Terbutaline, forskolin and cAMP suppress the peak calcium response to a similar maximum extent as atriopeptin and cGMP [24] and also suppress AHF to a similar degree. Blockade of either protein kinase A and protein kinase G similarly leads to a partial inhibition of both the functional effect (on AH formation) and on [Ca^{2+}]i release. In contrast, cGMP and cAMP have entirely different effects on calcium entry, i.e., cGMP abolishes whereas cAMP augments the Ca^{2+} entry phase (plateau). However, both cGMP and cAMP reduce AHF in the bovine eye. Hence we may conclude that the store release phase of the Ca^{2+} response is more relevant in terms of cyclic nucleotide modulation of secretory function in the CE.

In summary, our previous evidence [24] showed that atriopeptin and azide decreased AHF through generating cGMP and also interfered with ATP-induced Ca^{2+} release by a cGMP-dependent mechanism. The present data show that drugs which increase cellular cAMP levels are similarly able to suppress AH formation and interfere with ATP-induced Ca^{2+} release. This lends substantial support to the hypothesis that Ca^{2+} could be involved in the mechanism by which many drugs affect secretory function in the CE.

Fig 11. Effect of increasing concentration of terbutaline on the Ca^{2+}-mobilizing response of ATP (100μM) in bovine cultured CE, in the present of extracellular calcium and NiCl$_2$ (4mM). Terbutaline's effect on both the peak Ca^{2+} transient and at selected time intervals thereafter had been shown. Cultured cells were incubated for 6 min with appropriate concentration of terbutaline in HEPES buffered Krebs' solution containing 4mM NiCl$_2$ and1.8mM calcium. The cells were then exposed to 100μM of ATP. Each point represents a mean±s.e.mean of 8–12 experiments. Significance of differences between incubations with or without terbutaline; ** $p < 0.01$ and *** $p < 0.001$, Bonferroni's multiple comparison test. Base value indicates the [Ca^{2+}]$_i$ prior to addition of ATP.

https://doi.org/10.1371/journal.pone.0244253.g011

Supporting information

S1 Table. Effect of terbutaline on aqueous humor formation.
(XLSX)

S2 Table. Effect of Forskolin on aqueous humor formation.
(XLSX)

S3 Table. Effect of 8-Br cAMP on aqueous humor formation.
(XLSX)

S4 Table. Effect of KT-5720 on terbutaline-induced reduction of aqueous humor formation.
(XLSX)

S5 Table.
(XLS)

S6 Table. Effect of Terbutaline on ATP-induced ntracellular calcium mobilization.
(XLSX)

S7 Table. Effect of Forskolin on ATP-induced intracellular calcium mobilization.
(XLSX)

S8 Table. Effect of 8-Br cAMP on ATP-induced intracellular calcium mobilization.
(XLSX)

S9 Table. Effect of PKA inhibitor (KT-5720) on the terbutaline-induced inhibition of intracellular calciun increase by ATP.
(XLSX)

S10 Table. Effect of terbutaline on ATP-induced intracellular calcium mobilization in the absence of extracellular calcium.
(XLSX)

S11 Table. Effect of terbutaline on ATP-induced intracellular calcium mobilization in presence of extracellular Nickel chloride.
(XLSX)

Acknowledgments

Author Contributions

Conceptualization: Mohammad Shahidullah.

Formal analysis: Mohammad Shahidullah.

Funding acquisition: Mohammad Shahidullah, William Stuart Wilson, Nicholas Anthony Delamere.

Investigation: Mohammad Shahidullah.

Methodology: Mohammad Shahidullah.

Project administration: Mohammad Shahidullah.

Resources: Mohammad Shahidullah.

Validation: Nicholas Anthony Delamere.

Writing – original draft: Mohammad Shahidullah, William Stuart Wilson, Kazi Rafiq, Mahmudul Hasan Sikder, Jannatul Ferdous, Nicholas Anthony Delamere.

Writing – review & editing: Mohammad Shahidullah, William Stuart Wilson, Kazi Rafiq, Mahmudul Hasan Sikder, Jannatul Ferdous, Nicholas Anthony Delamere.

References

1. Do C-W, Peterson-Yantorno K, Mitchell CH, Civan MM. cAMP-activated maxi-Cl⁻ channels in native bovine pigmented ciliary epithelial cells. American Journal of Physiology-Cell Physiology. 2004; 287(4): C1003–C11. https://doi.org/10.1152/ajpcell.00175.2004 PMID: 15189811

2. Mittag TW, Guo WB, Kobayashi K. Bicarbonate-activated adenylyl cyclase in fluid-transporting tissues. American Journal of Physiology-Renal Physiology. 1993; 264(6):F1060–F4. https://doi.org/10.1152/ajprenal.1993.264.6.F1060 PMID: 8322892

3. Nathanson JA. Adrenergic regulation of intraocular pressure: identification of beta 2-adrenergic-stimulated adenylate cyclase in ciliary process epithelium. Proc Natl Acad Sci U S A. 1980; 77(12):7420–4. https://doi.org/10.1073/pnas.77.12.7420 PMID: 6261257.

4. Neufeld AH, Jampol LM, Sears ML. Cyclic-AMP in the aqueous humor: The effects of adrenergic agents. Experimental Eye Research. 1972; 14(3):242–50. https://doi.org/10.1016/0014-4835(72)90009-7 PMID: 4344979

5. Brubaker RF, Gaasterland D. The effect of isoproterenol on aqueous humor formation in humans. Investigative Ophthalmology & Visual Science. 1984; 25(3):357–9. PMID: 6698753

6. Coakes RL, Brubaker RF. The Mechanism of Timolol in Lowering Intraocular Pressure: In the Normal Eye. Archives of Ophthalmology. 1978; 96(11):2045–8. https://doi.org/10.1001/archopht.1978.03910060433007 PMID: 363105

7. Dailey RA, Brubaker RF, Bourne WM. The Effects of Timolol Maleate and Acetazolamide on the Rate of Aqueous Formation in Normal Human Subjects. American Journal of Ophthalmology. 1982; 93 (2):232–7. https://doi.org/10.1016/0002-9394(82)90419-6 PMID: 7039328

8. Paterson GD, Paterson G. Drug therapy of glaucoma. Br J Ophthalmol. 1972; 56(3):288–94. https://doi.org/10.1136/bjo.56.3.288 PMID: 4260701.

9. Wettrell K. beta-adrenoceptor antagonism and intraocular pressure. A clinical study on propranolol, practolol and atenolol. Acta ophthalmologica Supplementum. 1977;(134):1–54. Epub 1977/01/01. PMID: 22980.

10. Wettrell K, Wilke K, Pandolfi M. Effect of beta-adrenergic agonists and antagonists on repeated tonometry and episcleral venous pressure. Experimental Eye Research. 1977; 24(6):613–9. https://doi.org/10.1016/0014-4835(77)90119-1 PMID: 17542

11. Yablonski ME, Zimmerman TJ, Waltman SR, Becker B. A fluorophotometric study of the effect of topical timolol on aqueous humor dynamics. Exp Eye Res. 1978; 27(2):135–42. Epub 1978/08/01. https://doi.org/10.1016/0014-4835(78)90083-0 PMID: 680032.

12. Shahidullah M, Wilson WS, Millar C. Effects of timolol, terbutaline and forskolin on IOP, aqueous humour formation and ciliary cyclic AMP levels in the bovine eye. Curr Eye Res. 1995; 14(7):519–28. Epub 1995/07/01. https://doi.org/10.3109/02713689508998398 PMID: 7587297.

13. Berridge MJ. Inositol trisphosphate and calcium signalling. Nature. 1993; 361(6410):315–25. Epub 1993/01/28. https://doi.org/10.1038/361315a0 PMID: 8381210.

14. Tsunoda Y. Receptor-operated Ca2+ signaling and crosstalk in stimulus secretion coupling. Biochimica et Biophysica Acta (BBA)—Reviews on Biomembranes. 1993; 1154(2):105–56. https://doi.org/10.1016/0304-4157(93)90008-C PMID: 8218335

15. Farahbakhsh NA, Cilluffo MC. Synergistic Increase in Ca2+Produced by A1Adenosine and Muscarinic Receptor Activation via a Pertussis-Toxin-Sensitive Pathway in Epithelial Cells of the Rabbit Ciliary Body. Experimental Eye Research. 1997; 64(2):173–9. https://doi.org/10.1006/exer.1996.0194 PMID: 9176050

16. Giovanelli A, Fucile S, Mead A, Mattei E, Eusebi F, Sears M. Spontaneous and Evoked Oscillations of Cytosolic Calcium in the Freshly Prepared Ciliary Epithelial Bilayer of the Rabbit Eye. Biochemical and Biophysical Research Communications. 1996; 220(2):472–7. https://doi.org/10.1006/bbrc.1996.0423 PMID: 8645329

17. Schütte M, Wolosin JM. Ca2+ mobilization and interlayer signal transfer in the heterocellular bilayered epithelium of the rabbit ciliary body. J Physiol. 1996; 496 (Pt 1)(Pt 1):25–37. https://doi.org/10.1113/jphysiol.1996.sp021662 PMID: 8910193.

18. Shahidullah M, Wilson WS. Mobilisation of intracellular calcium by P2Y2 receptors in cultured, non-transformed bovine ciliary epithelial cells. Curr Eye Res. 1997; 16(10):1006–16. Epub 1997/10/23. https://doi.org/10.1076/ceyr.16.10.1006.9018 PMID: 9330852.

19. Suzuki Y, Nakano T, Sears M. Calcium signals from intact rabbit ciliary epithelium observed with confocal microscopy. Curr Eye Res. 1997; 16(2):166–75. Epub 1997/02/01. https://doi.org/10.1076/ceyr.16.2.166.5095 PMID: 9068948.

20. Yoshimura N, Tanabe-Ohuchi T, Takagi H, Honda Y. Drug-dependent Ca^{2+} mobilization in organ-cultured rabbit ciliary processes. Curr Eye Res. 1995; 14(8):629–35. Epub 1995/08/01. https://doi.org/10.3109/02713689508998489 PMID: 8529397.

21. Crook RB, Bazan NG, Polansky JR. Histamine H1 receptor occupancy triggers inositol phosphates and intracellular calcium mobilization in human non-pigmented ciliary epithelial cells. Curr Eye Res. 1991; 10(7):593–600. Epub 1991/07/01. https://doi.org/10.3109/02713689109013851 PMID: 1680603.

22. Crook RB, Lui GM, Polansky JR. Thrombin stimulates inositol phosphate formation, intracellular calcium fluxes and DNA synthesis in cultured fetal human non-pigmented ciliary epithelial cells. Exp Eye Res. 1992; 55(6):785–95. Epub 1992/12/01. https://doi.org/10.1016/0014-4835(92)90005-d PMID: 1486937.

23. Strauss O, Stahl F, Wiederholt M. Elevation of cytosolic free calcium in cultured ciliary epithelial cells by histamine: effects of verapamil and staurosporine. Journal of ocular pharmacology. 1992; 8(4):359–66. Epub 1992/01/01. https://doi.org/10.1089/jop.1992.8.359 PMID: 1336538.

24. Shahidullah M, Wilson WS. Atriopeptin, sodium azide and cyclic GMP reduce secretion of aqueous humour and inhibit intracellular calcium release in bovine cultured ciliary epithelium. Br J Pharmacol. 1999; 127(6):1438–46. https://doi.org/10.1038/sj.bjp.0702681 PMID: 10455294.

25. Shahidullah M, Wilson WS, Yap M, To CH. Effects of ion transport and channel-blocking drugs on aqueous humor formation in isolated bovine eye. Investigative Ophthalmology & Visual Science. 2003; 44 (3):1185–91. Epub 2003. https://doi.org/10.1002/jez.a.10303 PMID: 14598380.

26. Wilson WS, Shahidullah M, Millar C. 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. 1993; 12(7):609–20. https://doi.org/10.3109/02713689309001840 PMID: 7693396

27. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. The Journal of biological chemistry. 1985; 260(6):3440–50. Epub 1985/03/25. PMID: 3838314.

28. Hallam TJ, Pearson JD, Needham LA. Thrombin-stimulated elevation of human endothelial-cell cytoplasmic free calcium concentration causes prostacyclin production. Biochem J. 1988; 251(1):243–9. https://doi.org/10.1042/bj2510243 PMID: 3291862

29. Wang Z, Do CW, Valiunas V, Leung CT, Cheng AK, Clark AF, et al. Regulation of gap junction coupling in bovine ciliary epithelium. American journal of physiology Cell physiology. 2010; 298(4):C798–806. Epub 2010/01/22. https://doi.org/10.1152/ajpcell.00406.2009 PMID: 20089928; PubMed Central PMCID: PMC2853215.

30. Civan MM. Formation of the aqueous humor: transport components and their integration. In: Civan MM, editor. The Eye's Aqueous Humor San Diego, CA: Elsevier; 2008. p. 2–45.

31. Shahidullah M, Al-Malki WH, Delamere NA. Mechanism of Aqueous Humor Secretion, Its Regulation and Relevance to Glaucoma, Glaucoma—Basic and Clinical Concepts. In: Rumelt S, editor.: InTech; Available from: http://www.intechopen.com/articles/show/title/mechanism-of-aqueous-humor-secretion-its-regulation-and-relevance-to-glaucoma; 2011. p. 1–30.

32. Fleischhauer JC, Mitchell CH, Peterson-Yantorno K, Coca-Prados M, Civan MM. PGE2, Ca2+, and cAMP mediate ATP activation of Cl− channels in pigmented ciliary epithelial cells. American Journal of Physiology-Cell Physiology. 2001; 281(5):C1614–C23. https://doi.org/10.1152/ajpcell.2001.281.5.C1614 PMID: 11600425

33. Civan MM. The fall and rise of active chloride transport: implications for regulation of intraocular pressure. Journal of experimental zoology Part A, Comparative experimental biology. 2003; 300(1):5–13. Epub 2003/11/05. https://doi.org/10.1002/jez.a.10303 PMID: 14598380.

34. Leipziger J, Kerstan D, Nitschke R, Greger R. ATP increases [Ca2+]i and ion secretion via a basolateral P2Y-receptor in rat distal colonic mucosa. Pflugers Archiv: European journal of physiology. 1997; 434 (1):77–83. Epub 1997/05/01. https://doi.org/10.1007/pl00008079 PMID: 9094258.

35. Lincoln TM, Cornwell TL, Taylor AE. cGMP-dependent protein kinase mediates the reduction of Ca2+ by cAMP in vascular smooth muscle cells. The American journal of physiology. 1990; 258(3 Pt 1):C399–407. Epub 1990/03/01. https://doi.org/10.1152/ajpcell.1990.258.3.C399 PMID: 2156436.

36. Brüne B, Ullrich V. Cyclic nucleotides and intracellular-calcium homeostasis in human platelets. European journal of biochemistry. 1992; 207(2):607–13. Epub 1992/07/15. https://doi.org/10.1111/j.1432-1033.1992.tb17087.x PMID: 1321718.

37. Kageyama K, Mizobe T, Nozuchi S, Hiramatsu N, Nakajima Y, Aoki H. Toborinone and olprinone, phosphodiesterase III inhibitors, inhibit human platelet aggregation due to the inhibition of both calcium release from intracellular stores and calcium entry. Journal of anesthesia. 2004; 18(2):107–12. Epub 2004/05/06. https://doi.org/10.1007/s00540-004-0228-6 PMID: 15127258.

38. Jackson TS, Lerner E, Weisbrod RM, Tajima M, Loscalzo J, Keaney JF Jr. Vasodilatory properties of recombinant maxadilan. The American journal of physiology. 1996; 271(3 Pt 2):H924–30. Epub 1996/09/11. https://doi.org/10.1152/ajpheart.1996.271.3.H924 PMID: 8853326.

39. Niisato N, Nakahari T, Tanswell AK, Marunaka Y. Beta 2-agonist regulation of cell volume in fetal distal lung epithelium by cAMP-independent Ca2+ release from intracellular stores. Canadian journal of physiology and pharmacology. 1997; 75(8):1030–3. Epub 1997/08/01. PMID: 9360019.

40. Yang C, Tsao HL, Chiu CT, Fan LW, Yu SM. Regulation of 5-hydroxytryptamine-induced calcium mobilization by cAMP-elevating agents in cultured canine tracheal smooth muscle cells. Pflugers Archiv: European journal of physiology. 1996; 432(4):708–16. Epub 1996/08/01. https://doi.org/10.1007/s004240050189 PMID: 8764973.

41. Supattapone S, Danoff SK, Theibert A, Joseph SK, Steiner J, Snyder SH. Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium. Proc Natl Acad Sci U S A. 1988; 85(22):8747–50. https://doi.org/10.1073/pnas.85.22.8747 PMID: 2847175.

42. Abdollahi M, Isazadeh Z. Inhibition of rat parotid and submandibular gland functions by ofloxacin, a fluoroquinolone antibiotic. Fundamental & clinical pharmacology. 2001; 15(5):307–11. Epub 2002/03/21. https://doi.org/10.1046/j.1472-8206.2001.00041.x PMID: 11903499.

43. Song SK, Choi SY, Kim KT. Opposing effects of protein kinase A and C on capacitative calcium entry into HL-60 promyelocytes. Biochemical pharmacology. 1998; 56(5):561–7. Epub 1998/10/23. https://doi.org/10.1016/s0006-2952(97)00660-6 PMID: 9783724.

44. Gamberucci A, Innocenti B, Fulceri R, Bànhegyi G, Giunti R, Pozzan T, et al. Modulation of Ca2+ influx dependent on store depletion by intracellular adenine-guanine nucleotide levels. The Journal of biological chemistry. 1994; 269(38):23597–602. Epub 1994/09/23. PMID: 8089128.

45. Pozzan T, Rizzuto R, Volpe P, Meldolesi J. Molecular and cellular physiology of intracellular calcium stores. Physiological reviews. 1994; 74(3):595–636. Epub 1994/07/01. https://doi.org/10.1152/physrev.1994.74.3.595 PMID: 8036248.

46. Putney JW Jr. Capacitative calcium entry revisited. Cell calcium. 1990; 11(10):611–24. Epub 1990/11/11. https://doi.org/10.1016/0143-4160(90)90016-n PMID: 1965707.

47. Randriamampita C, Tsien RY. Emptying of intracellular Ca2+ stores releases a novel small messenger that stimulates Ca2+ influx. Nature. 1993; 364(6440):809–14. Epub 1993/08/26. https://doi.org/10.1038/364809a0 PMID: 8355806.