J Physiol (2003), **551.3** *pp.* 765–776

DOI: 10.1113/jphysiol.2003.041707
www.jphysiol.org

Journal of Physiology

The role of cGMP in the regulation of rabbit airway ciliary beat frequency

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The involvement of cyclic guanosine 3′,5′-monophosphate (cGMP) and cGMP-dependent protein kinase (PKG) and their interaction with the Ca^{2+}-dependent mechanisms in the regulation of ciliary activity are not well understood. To investigate how cGMP regulates ciliary activity, changes in ciliary beat frequency (CBF) and intracellular calcium concentration ($[Ca^{2+}]_i$) of rabbit tracheal ciliated cells in response to 8-bromo-cGMP (Br-cGMP) were simultaneously quantified using digital, high-speed phase-contrast and fluorescence imaging. Br-cGMP induced a response in ciliary activity that could be separated into two parts. Firstly, Br-cGMP induced a concentration-dependent increase in the basal CBF that occurred without increasing the $[Ca^{2+}]_i$. This response was not affected by excessively buffering the $[Ca^{2+}]_i$ with BAPTA but was abolished by KT5823, a PKG inhibitor. Secondly, Br-cGMP induced a series of transient increases in CBF that were superimposed on the sustained increases in CBF. These transient increases in CBF correlated with the stimulation of a series of transient increases in $[Ca^{2+}]_i$ and were abolished by BAPTA, but were unaffected by KT5823. The magnitude of the transient increases in CBF and $[Ca^{2+}]_i$ were not dependent on the concentration of Br-cGMP. The Ca^{2+}-dependent changes in CBF induced by ionomycin or ATP were not affected by KT5823. From these results, we propose that cGMP increases CBF in two ways: firstly through a Ca^{2+}-independent mechanism involving PKG, and secondly through a Ca^{2+}-dependent mechanism following the stimulation of changes in $[Ca^{2+}]_i$. In addition, we suggest that the Ca^{2+}-dependent stimulation of rabbit airway ciliary activity does not initially require PKG activation.

(Received 19 February 2003; accepted after revision 19 June 2003; first published online 20 June 2003)
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Changes in ciliary beat frequency (CBF) are believed to be a key factor in the regulation of mucociliary transport and the defence mechanisms of the respiratory tract (Satir & Sleigh, 1990; Wanner *et al.* 1996). For example, a relatively small increase in CBF (16 %) can result in a large increase (56 %) in surface liquid velocity (Seybold *et al.* 1990), a response that is likely to enhance mucus clearance. It has been well established that airway CBF is strongly regulated by second messengers, such as Ca^{2+} and cAMP, and substantial evidence now exists for a regulatory role of cyclic guanosine 3′,5′-monophosphate (cGMP) (Tamaoki *et al.* 1991; Jain *et al.* 1993; Geary *et al.* 1995; Yang *et al.* 1997; Wyatt *et al.* 1998; Runer & Lindberg, 1999; Uzlaner & Priel, 1999; Li *et al.* 2000; Shirakami *et al.* 2000; Braiman *et al.* 2001; Zagoory *et al.* 2002). However, the effects of cGMP on ciliary activity remain controversial.

In other cells, cGMP has been found to modulate many cellular functions including smooth muscle cell contraction, cardiac function and platelet aggregation (Lincoln & Cornwell, 1993; Hobbs & Ignarro, 1996; Murad, 1996; Vaandrager & de Jonge, 1996) and is formed by activation of either soluble or membrane-bound guanylate cyclase (GC). While the membrane-bound or receptor form of GC is stimulated by ligands such as atrial natriuretic peptide (ANP), soluble GC is stimulated by nitric oxide (NO) (Schmidt & Walter, 1994; McDonald & Murad, 1995; Vaandrager & de Jonge, 1996). Increases in cGMP generally lead to the activation of cGMP-dependent protein kinase (PKG) (McDonald & Murad, 1995) and phosphorylation of target proteins (Bonini & Nelson, 1990; Walczak & Nelson, 1994; Porter & Sale, 2000).

The involvement of cGMP–PKG-mediated phosphorylation in ciliary motility is suggested by the immunoreactivity of rat tracheal ciliated cells for PKG Iβ (Zhan *et al.* 1999), the presence of a PKG substrate in the cilia of *Paramecium* (Bonini & Nelson, 1990) and a cGMP-stimulated PKG activity in bovine airway epithelial cells (Wyatt *et al.* 1998). However, cGMP has been reported to either inhibit (Tamaoki *et al.* 1991) or have no effect on the CBF (Uzlaner & Priel, 1999; Braiman *et al.* 2001) of rabbit tracheal cells or to stimulate CBF in rat (Li *et al.* 2000), bovine (Wyatt *et al.* 1998) and human (Geary *et al.* 1995; Runer & Lindberg, 1999) airway cells.

One possibility that may contribute to these inconsistencies is the relationship between Ca^{2+} and cGMP–PKG regulation of ciliary activity. It has been postulated, for rabbit airway and frog palate cells, that Ca^{2+} is incapable of increasing CBF without the activation of PKG (Uzlaner & Priel, 1999; Braiman *et al.* 2001; Ma *et al.* 2002; Zagoory *et al.* 2002). One implication of this idea is that there should be a significant delay between the increase in $[Ca^{2+}]_i$ and the increase in CBF to accommodate the activation process of PKG and the phosphorylation of specific targets. However, by using high-speed phase-contrast (240 frames s^{-1}) and fast fluorescence imaging (30 frames s^{-1}), combined with a beat-by-beat analysis, we have found that, in response to mechanical stimulation or ATP, the changes in $[Ca^{2+}]_i$ and CBF during Ca^{2+} waves or oscillations were very tightly coupled in rabbit airway ciliated cells (Evans & Sanderson, 1999; Lansley & Sanderson, 1999; Zhang & Sanderson, 2003). While it is possible that PKG activation may precede the onset of ATP-induced Ca^{2+} oscillations, it does not seem likely that PKG activation occurs during the propagation of intercellular Ca^{2+} waves through unstimulated adjacent cells (Lansley & Sanderson, 1999). In these cells, the latency between increases in $[Ca^{2+}]_i$ and increases in CBF was very brief (~100 ms at 37 °C). A similar dependency of the CBF on the $[Ca^{2+}]_i$, which was not influenced by PKG inhibitors, was also found in ovine airway epithelium (Salathe & Bookman, 1999; Salathe *et al.* 2000).

In view of the uncertainties of cGMP in the control of CBF, the aim of this study was to use our high-speed digital recording techniques (Zhang & Sanderson, 2003) to resolve the changes in CBF and $[Ca^{2+}]_i$ during the activation of cGMP–PKG. From the data obtained, we suggest that cGMP regulates rabbit airway CBF in both a Ca^{2+}-dependent manner and a Ca^{2+}-independent manner and that the cGMP–PKG signalling pathway is not essential for Ca^{2+}-dependent increases in CBF.

METHODS

Materials

Hanks' balanced salt solution (HBSS) without Phenol Red and Dulbecco's modified Eagle's Medium (DMEM) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). HBSS was supplemented with 25 mM Hepes (sHBSS, pH 7.4). Fura-2 AM, Oregon Green 488 BAPTA-1 AM, calcium calibration buffers and fluorescent microspheres were obtained from Molecular Probes (Eugene, OR, USA). Br-cGMP (8-bromo-guanosine 3′,5′-cyclic monophosphate, sodium salt), KT5823, ionomycin, BAPTA AM and Pluronic F-127 were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA, USA). ATP, sulfobromophthalein, L-ascorbic acid and DMSO were obtained from Sigma-Aldrich Corporation (St Louis, MO, USA). Oregon Green 488 BAPTA-1 AM, BAPTA AM, fura-2 AM, KT5823 and ionomycin were dissolved in DMSO and diluted in sHBSS to the final working concentrations with DMSO concentrations of 0.8, 0.2, 0.1, 0.04 and 0.002 %, respectively.

Cell culture

Primary cultures of rabbit tracheal epithelial cells were prepared as previously described (Dirksen *et al.* 1995; Evans & Sanderson, 1999). Adult New Zealand White rabbits (~1.5 kg) were initially sedated with intramuscular xylazine (0.375 ml of 20 mg ml^{-1}; 5 mg kg^{-1}) and ketamine HCl (0.7 ml of 100 mg ml^{-1}; 35 mg kg^{-1}) and killed with intravenous pentobarbital sodium (3 ml of 50 mg ml^{-1}, 100 mg kg^{-1}) according to the protocol approved by Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. After removal by dissection, the tracheal mucosa was cut into ~0.5 mm squares, plated on collagen-coated glass coverslips, and cultured in DMEM supplemented with 10 % fetal bovine serum and penicillin–streptomycin at 37 °C in 10 % CO_2 for 7–11 days.

Measurement of CBF with high-speed digital microscopy

Rabbit airway CBF was detected and quantified with a high-speed digital imaging system combined with a beat-by-beat analysis as previously described (Zhang & Sanderson, 2003). In general, phase-contrast images (648 pixels × 200 lines), formed with red light from a tungsten–halogen bulb, were collected at 240 frames s^{-1} with a high-speed CCD (charge-coupled device) camera (TM-6710, Pulnix America, Sunnyvale, CA, USA) in conjunction with a frame grabber ('Road Runner', BitFlow Inc., Woburn, MA, USA) and recording-software called 'Video Savant' (IO Industries, London, ON, Canada). CBF was determined from the variation in the light intensity of the phase-contrast image that resulted from the repetitive motion of cilia. The mean grey-intensity of a region of interest (ROI, area of 0.87 μm × 0.80 μm, 3 pixels × 3 pixels) positioned over the cilia of interest was calculated for each image and plotted with respect to time (i.e. frame number) to form a grey-intensity waveform. The frequency of each ciliary beat cycle was determined from the period of each cycle of the grey-intensity waveform by using a beat-by-beat analysis (Zhang & Sanderson, 2003).

Measurement of $[Ca^{2+}]_i$ with imaging of fura-2

The details of $[Ca^{2+}]_i$ measurement have been published elsewhere (Leybaert *et al.* 1998; Zhang & Sanderson, 2003). Briefly, cells were incubated in 1 μM fura-2 AM in sHBSS containing 100 μM sulfobromophthalein for 1 h at 37 °C, washed in sHBSS containing 100 μM sulfobromophthalein and allowed at least 30 min for de-esterification of the fura-2 AM. The coverslip bearing the cells was mounted on a Nikon Diaphot 300 inverted microscope equipped with a × 40, 1.3 NA, Ph 4, oil-immersion objective. The cells were equilibrated in sHBSS for at least 10 min to reach the warm working temperature of ~29.5 ± 1 °C. At higher temperatures, evaporation artifacts were difficult to control.

Fluorescence (at 510 nm), generated by exciting the fura-2-loaded cells with 340 or 380 nm light, was detected with a silicon-intensified target (SIT) camera (Cohu, San Diego, USA). An optical memory disc recorder (OMDR, Panasonic TQ3031F) was used to record the images in time-lapse (4 frames s^{-1}) without frame averaging. Calcium measurements were made by determining the normalized change in fluorescence (F_t/F_0 where F_t is the measured fluorescence and F_0 is the starting fluorescence) from a ROI (area of 1.8 μm × 1.6 μm, 6 pixels × 6 pixels) at the base of the cilia. The $[Ca^{2+}]_i$ was calculated from this value using the original $[Ca^{2+}]_i$, the starting fluorescence and reference $[Ca^{2+}]_i$ as described previously (Leybaert *et al.* 1998).

The temporal alignment of the phase-contrast and fluorescence images was achieved by simultaneously recording a time marker

consisting of a fluorescent glass micropipette with each camera. The spatial alignment of the two image sets was achieved by recording a phase-contrast sequence of ciliary movement with the SIT camera. As previously described, an *in vitro* calibration was performed using a thin glass chamber to determine the K_d (132 ± 14 nM, $n = 8$) of fura-2 and the fluorescence ratio in the absence of Ca^{2+} (R_{min}), the fluorescence ratio in the presence of saturating Ca^{2+} (R_{max}) and the fluorescence ratio at 380 nm (F_0/F_s) in the absence (F_0) and presence of saturating (F_s) Ca^{2+} for the dye (Lansley & Sanderson, 1999; Zhang & Sanderson, 2003). The R_{min} and R_{max} measured with our imaging system on separate occasions over 11 months were 0.20 ± 0.01 and 5.19 ± 0.14, respectively ($n = 8$).

Estimation of the $[Ca^{2+}]_i$ with imaging of Oregon Green

When cells were treated with KT5823, they became sensitive to UV light (see later) and, as a result, fura-2 could not be used for $[Ca^{2+}]_i$ measurement. As an alternative Oregon green, which is excited by longer wavelength light, was used to monitor changes in $[Ca^{2+}]_i$. Cells were loaded with 20 μM Oregon Green 488 BAPTA-1 AM in sHBSS containing 0.2 % Pluronic F-127 for 1 h at 37 °C followed by 30 min of de-esterification in sHBSS. The solutions contained 100 μM sulfobromophthalein to inhibit ion pump activity and 3 mg ml^{-1} ascorbic acid to reduce bleaching (Bergner & Sanderson, 2002). Fluorescence (at 523 nm), generated by exciting Oregon Green-loaded cells with 485 nm light, was detected with the SIT camera and recorded at 4 frames s^{-1}. The change in $[Ca^{2+}]_i$ was represented by the normalized change in fluorescence by dividing the measured fluorescence by starting fluorescence (F_t/F_0).

Simultaneous measurement of CBF and $[Ca^{2+}]_i$

Simultaneous imaging of CBF and $[Ca^{2+}]_i$ was achieved by directing the different wavelengths of light forming the phase-contrast and fluorescence images to the respective cameras (CCD, SIT) with a dichroic beam splitter (655 nm) (Sanderson, 2000). When fluorescence images were not required, the excitation light was turned off. To replace the solution in the experimental chamber (300 μl), 1 ml of experimental solution, warmed to the working temperature, was drawn through the chamber with suction.

Data analysis and statistics

The basal CBF and $[Ca^{2+}]_i$ were calculated from the first 10 s of data. The change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) (using fura-2) was calculated by subtracting the basal $[Ca^{2+}]_i$ from the observed $[Ca^{2+}]_i$. Normalized CBF was obtained by dividing the measured CBF by the starting basal CBF. Normalized CBF and $\Delta[Ca^{2+}]$ were used to facilitate the comparison of data from cells with slightly different starting conditions and to be compatible with other studies in the literature. All data were expressed as means ± S.E.M. Statistical analysis was performed with Student's paired or unpaired *t* test, or one-way analysis of variance (ANOVA) (Student-Newman-Keuls method for further multiple pair-wise comparisons). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Changes in CBF and $[Ca^{2+}]_i$ induced by Br-cGMP

In general, the changes in CBF induced by Br-cGMP could be divided into two responses. These consisted of (1) a moderate but sustained increase in CBF and (2) a series of transient increases in CBF that were superimposed on the sustained increase in CBF (Fig. 1, black traces). These transient increases in CBF were considerably larger than the sustained increases in CBF. By contrast, the changes in $[Ca^{2+}]_i$ induced by Br-cGMP consisted only of a series of transient increases arising from a steady basal level (Fig. 1, grey traces).

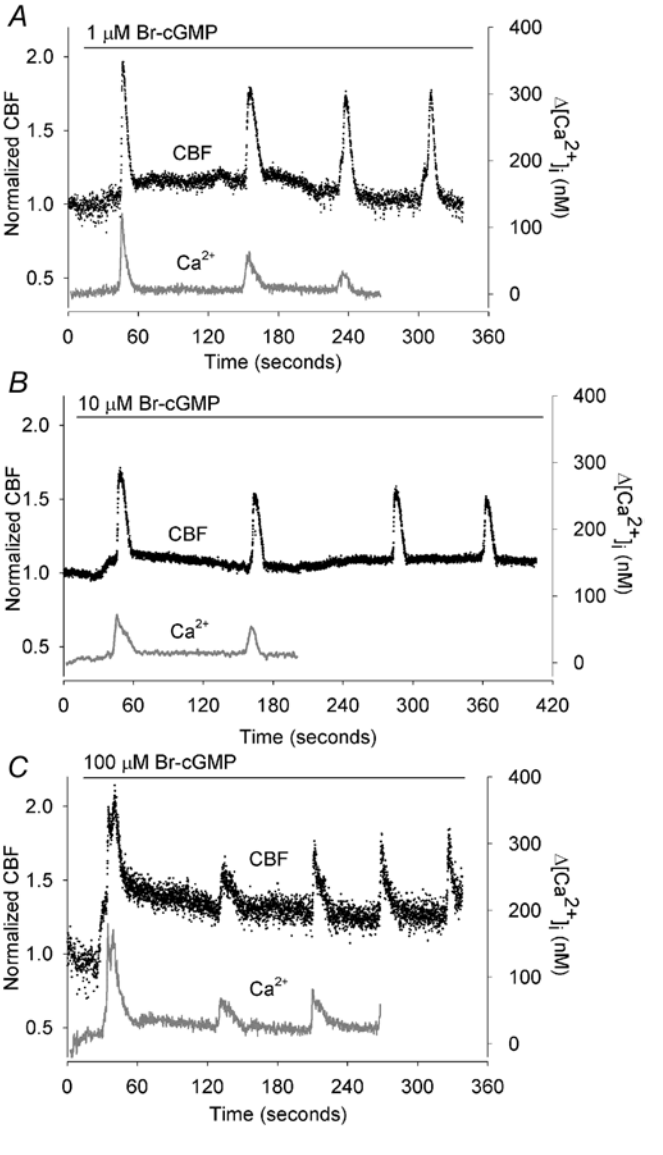

Figure 1. The effects of 8-Bromo-cGMP (Br-cGMP) on the ciliary beat frequency (CBF) and $[Ca^{2+}]_i$ of rabbit airway ciliated cells

Data for both CBF and $[Ca^{2+}]_i$ are normalized with the initial basal value and are representative of multiple cells. *A*, 1 μM Br-cGMP induced a two-part response in the CBF consisting of a small increase in the basal CBF (basal CBF = 10.4 Hz) that reached a maximum level within 60 s and declined thereafter, and larger transient increases in CBF that were superimposed on the basal CBF. By contrast, Br-cGMP only induced a series of transient increases in the $[Ca^{2+}]_i$ without affecting the basal $[Ca^{2+}]_i$ ($n = 10$). *B*, 10 μM Br-cGMP induced a higher sustained increase in the basal CBF (basal CBF = 15.9 Hz) but the superimposed transient increases in CBF, as well as the transient increases in $[Ca^{2+}]_i$, were unchanged ($n = 11$). *C*, 100 μM Br-cGMP only induced a further increase in the sustained CBF increase (basal CBF = 14.1 Hz) ($n = 11$). *A–C*, in all cases, the sustained increases in CBF occurred in the absence of changes in $[Ca^{2+}]_i$ while the transient changes in CBF and $[Ca^{2+}]_i$ were tightly coupled.

The correlation between the CBF and $[Ca^{2+}]_i$ changes induced by Br-cGMP

With the high-temporal resolution data provided by our recording technique, it is possible to accurately correlate the changes in CBF with the changes in $[Ca^{2+}]_i$ induced by Br-cGMP. In all cases, at either low or high concentrations of Br-cGMP, we found that the sustained increases in CBF (Fig. 2, blue dots) were initiated without a substantial change in $[Ca^{2+}]_i$. In addition, the sustained increases in CBF always occurred before the stimulation of the transient increases in CBF (Fig. 2, red dots). By contrast, these secondary transient increases in CBF were always tightly coupled with the transient increases in $[Ca^{2+}]_i$ (Fig. 2); the increase in $[Ca^{2+}]_i$ always preceded the increase in CBF. The change in CBF with respect to the $[Ca^{2+}]_i$ during each phase of the Br-cGMP-induced response is clearly indicated in Fig. 2*B* and *D*. During the initial stages, the CBF increases (blue dots, vertical arrow) without a change in $[Ca^{2+}]_i$ whereas as the subsequent increases in $[Ca^{2+}]_i$ are associated with increases in CBF (red dots, diagonal arrow). These results imply that the sustained and moderate increases in CBF were induced directly by Br-cGMP whereas the transient increases in CBF were induced indirectly via changes in $[Ca^{2+}]_i$ induced by Br-cGMP.

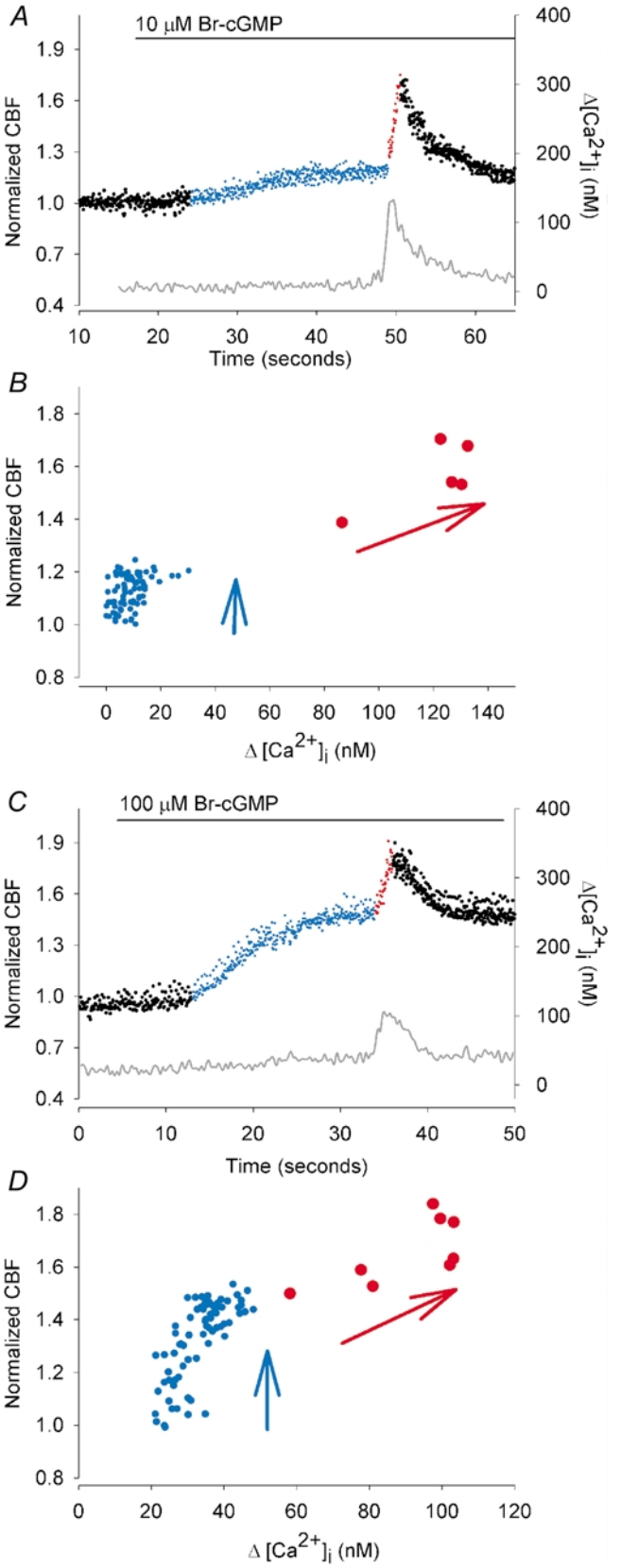

Figure 2. The correlation of the initial changes in CBF (coloured dots) with changes in $[Ca^{2+}]_i$ (grey line) induced by 10 (*A* and *B*) or 100 μM (*C* and *D*) Br-cGMP

A, after the addition of 10 μM Br-cGMP, the CBF (normalized with basal CBF) slowly increased (blue dots) from the basal CBF (17.9 Hz, left black dots) while the $[Ca^{2+}]_i$ remained unchanged. Approximately 30 s after the addition of Br-cGMP, the first transient increase in $[Ca^{2+}]_i$ occurred and this was strongly correlated with a transient increase in CBF (red dots). *B*, the normalized CBF plotted against the corresponding $[Ca^{2+}]_i$ for the time period illustrated in *A*. The vertical distribution of the blue dots, which represent the initial CBF increase induced by Br-cGMP, indicate that this phase of the response occurs independently of the $[Ca^{2+}]_i$. The inclined distribution of the red dots, which represent the CBF during the transient increase in $[Ca^{2+}]_i$, indicate that the CBF is dependent on the $[Ca^{2+}]_i$ during this phase of the response. *C* and *D*, similar temporal and correlative representations of the changes in CBF and $[Ca^{2+}]_i$ induced by 100 μM Br-cGMP. The initial increase in CBF (blue dots) is larger (basal CBF = 14.8 Hz) but still independent of the $[Ca^{2+}]_i$. The transient changes in CBF (red dots) are dependent on the $[Ca^{2+}]_i$.

Br-cGMP induces a concentration-dependent increase in CBF

The sustained increase in CBF induced directly by Br-cGMP was concentration dependent (Figs 1 and 3). For example, 1 μM Br-cGMP induced a small but significant increase in CBF (relative increase of 1.13 ± 0.02, $n = 10$, $P < 0.001$) that usually reached a maximum level within 60 s. This was followed by a gradual decline in CBF to the basal rate after about 240–420 s (1.02 ± 0.02, $n = 10$, $P > 0.05$) (Figs 1*A* and 3*A*). At higher concentrations, Br-cGMP induced significantly higher sustained increases in CBF that did not decline. For example, 10 μM Br-cGMP induced an initial relative increase in CBF of 1.23 ± 0.04 ($n = 11$, $P < 0.001$) that was sustained, after 240–420 s, at

1.17 ± 0.04 ($n = 11$, $P < 0.001$) (Figs 1*B* and 3*A*). Similarly, 100 μM Br-cGMP induced an initial relative increase of 1.28 ± 0.05, ($n = 11$, $P < 0.001$) and this was sustained, after 240–420 s, at 1.24 ± 0.04, ($n = 11$, $P < 0.001$) (Figs 1*C* and 3*A*). The initial increases in CBF induced by 100 μM Br-cGMP were also significantly greater than the increases in CBF induced by 1 μM Br-cGMP ($P < 0.05$). Similarly, the sustained increases in CBF induced by 10 and 100 μM Br-cGMP were significantly higher than the sustained increases in CBF induced by 1 μM Br-cGMP ($P < 0.01$ and $P < 0.001$, respectively). In control experiments, the application of sHBSS had no significant effect on either CBF or $[Ca^{2+}]_i$ ($n = 6$).

Br-cGMP-induced changes in Ca^{2+} signalling

As indicated previously, Br-cGMP also initiates a series of transient increases in $[Ca^{2+}]_i$. In response to 1 μM Br-cGMP, the Ca^{2+} transients had a mean magnitude of 83 ± 10 nM ($n = 10$) and occurred at a frequency of 0.67 ± 0.02 min^{-1} ($n = 5$) (Figs 1*A* and 3*B* and *C*). The frequency was calculated from the duration of at least three sequential oscillations. However, not all cells (5 of 10 cells) displayed three oscillations during the relatively long recording period (up to 420 s) indicating that these values may represent the strongest response. Importantly, in all cases, these Ca^{2+} transients were accompanied by large transient changes in CBF (relative change = 1.60 ± 0.08, $n = 10$, Fig. 3*A*).

In response to 10 μM Br-cGMP, the $[Ca^{2+}]_i$ transients had a mean magnitude of 94 ± 10 nM ($n = 9$) and an associated relative increase in CBF of 1.73 ± 0.08 ($n = 10$) at a frequency of 0.82 ± 0.13 min^{-1} ($n = 5$) (Figs 1*B* and 3). In response to 100 μM Br-cGMP, the $[Ca^{2+}]_i$ transients had a mean magnitude of 92 ± 10 nM ($n = 10$) and an associated relative increase in CBF of 1.77 ± 0.06 ($n = 11$) at a frequency of 1.07 ± 0.14 min^{-1} ($n = 4$) (Figs 1*C* and 3). Although both the magnitude and the frequency of the

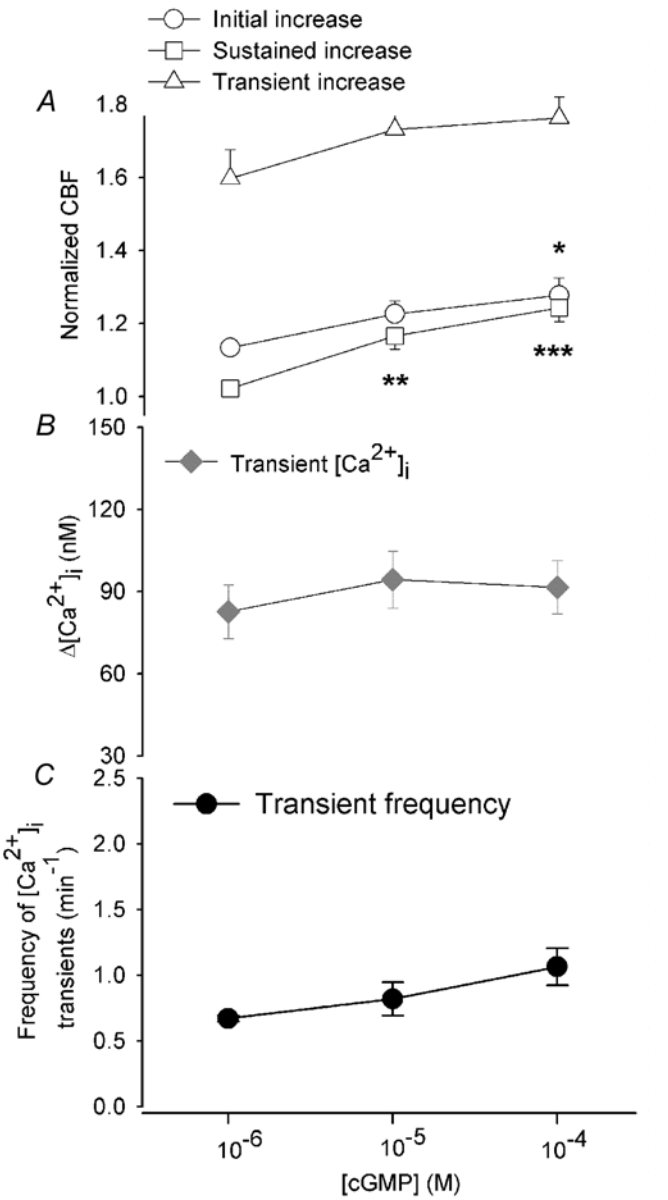

Figure 3. The concentration–response relationship of CBF and $[Ca^{2+}]_i$ in airway epithelial cells to Br-cGMP

A, the initial increase in CBF (○) in response to 1, 10 and 100 μM Br-cGMP was 1.13 ± 0.02 ($n = 10$), 1.23 ± 0.04 ($n = 11$) and 1.28 ± 0.05, ($n = 11$), respectively, and was significantly greater (all $P < 0.001$) with respect to the basal rate with the increasing Br-cGMP concentration. The CBF increase induced by 100 μM Br-cGMP was also significantly greater than that induced by 1 μM Br-cGMP (* $P < 0.05$). The sustained increase in CBF (□) in response to 1, 10 and 100 μM Br-cGMP was 1.02 ± 0.02 ($n = 10$), 1.17 ± 0.04 ($n = 11$) and 1.24 ± 0.04 ($n = 11$), respectively, and this was also significantly greater (all $P < 0.001$) with the increasing Br-cGMP concentration. The sustained increase in CBF induced by 10 and 100 μM Br-cGMP was also significantly greater (** $P < 0.01$, *** $P < 0.001$) than that induced by 1 μM Br-cGMP. The transient increases in CBF (△) in response to 1, 10 and 100 μM Br-cGMP were 1.60 ± 0.08 ($n = 10$), 1.73 ± 0.08 ($n = 10$) and 1.77 ± 0.06 ($n = 11$), respectively, but these were not significantly increased with increasing Br-cGMP concentration. The starting basal CBF (12.8 ± 0.9 Hz, $n = 10$; 11.9 ± 0.9 Hz, $n = 11$; 12.4 ± 0.6 Hz, $n = 11$) for each concentration of Br-cGMP was similar ($P > 0.05$). *B*, the concentration–response relationship of the transient change in $[Ca^{2+}]_i$ to Br-cGMP. The transient changes in $[Ca^{2+}]_i$ (◆) in response to 1, 10 and 100 μM Br-cGMP were 83 ± 10 nM, ($n = 10$), 94 ± 10 nM ($n = 9$) and 92 ± 10 nM ($n = 10$), respectively, but were not significantly different with increasing Br-cGMP concentration ($P > 0.05$). The basal $[Ca^{2+}]_i$ (30 ± 4 nM, $n = 10$; 36 ± 4 nM, $n = 11$; 32 ± 5 nM, $n = 11$) for each concentration was similar ($P > 0.05$). *C*, the concentration–response relationship of the frequency of the transient changes in $[Ca^{2+}]_i$ or CBF oscillations (●) to Br-cGMP. The frequency in response to 1, 10 and 100 μM Br-cGMP was 0.67 ± 0.02 min^{-1} ($n = 5$), 0.82 ± 0.13 min^{-1} ($n = 5$) and 1.07 ± 0.14 min^{-1} ($n = 4$), and not significantly different with the increasing Br-cGMP concentration ($P > 0.05$).

$[Ca^{2+}]_i$ and CBF transients had a tendency to increase with the increasing Br-cGMP concentrations, no statistical difference was found (Fig. 3).

The effect of buffering $[Ca^{2+}]_i$ on the response to Br-cGMP

To confirm that Br-cGMP was capable of increasing CBF independently of changes in $[Ca^{2+}]_i$, the $[Ca^{2+}]_i$ was buffered with the Ca^{2+} chelator BAPTA. After loading with fura-2, the cells were subsequently loaded with 20 μM BAPTA AM to establish a high intracellular concentration of BAPTA (Zhang & Sanderson, 2003). Upon treatment with 100 μM Br-cGMP, an increase in CBF was observed

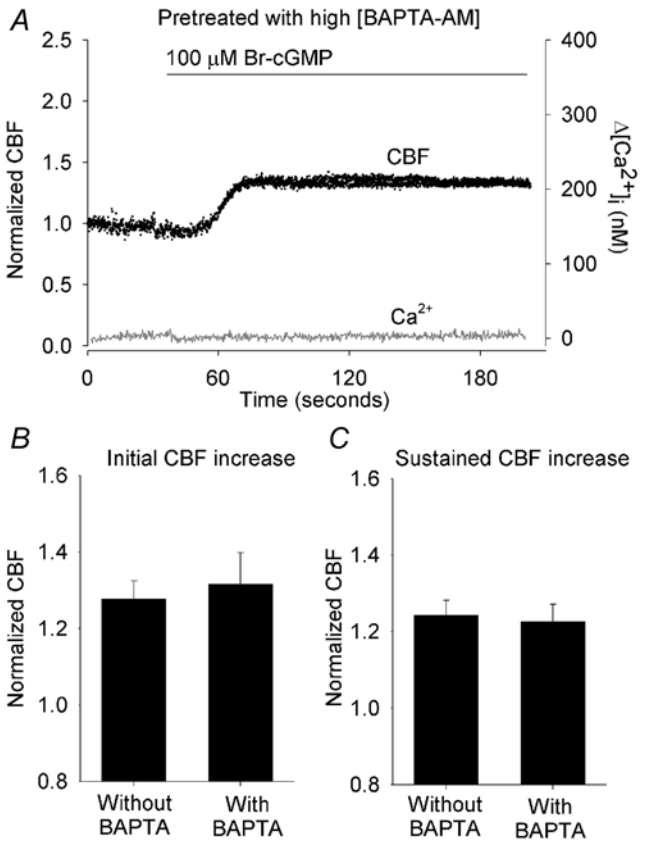

Figure 4. The effect of buffering the $[Ca^{2+}]_i$ with BAPTA in response to 100 μM Br-cGMP

A, a representative response of a ciliated cell pretreated with 20 μM BAPTA AM and exposed to Br-cGMP (bar). The BAPTA buffering prevented any changes in $[Ca^{2+}]_i$ (grey line). However, the CBF (black dots) still increased (to an initial normalized CBF = 1.36) and remained sustained (at a normalized CBF = 1.32). Transient increases in $[Ca^{2+}]_i$ or CBF did not occur. The basal CBF was 12.1 Hz. *B* and *C*, the effect of buffering the $[Ca^{2+}]_i$ with BAPTA on the initial (*B*) and sustained (*C*) changes in CBF induced by Br-cGMP. In control experiments, without BAPTA treatment, the initial and sustained increases in CBF induced by Br-cGMP were 1.28 ± 0.05 (n = 11) and 1.24 ± 0.04 (n = 11) respectively. These changes were indistinguishable ($P > 0.05$) from the initial (1.32 ± 0.08, n = 6) and sustained (1.23 ± 0.05, n = 6) increases in CBF induced by Br-cGMP in the presence of BAPTA. The basal CBFs for cells with (12.4 ± 1.0, n = 6) or without BAPTA treatment (12.4 ± 0.6, n = 11) were similar ($P > 0.05$).

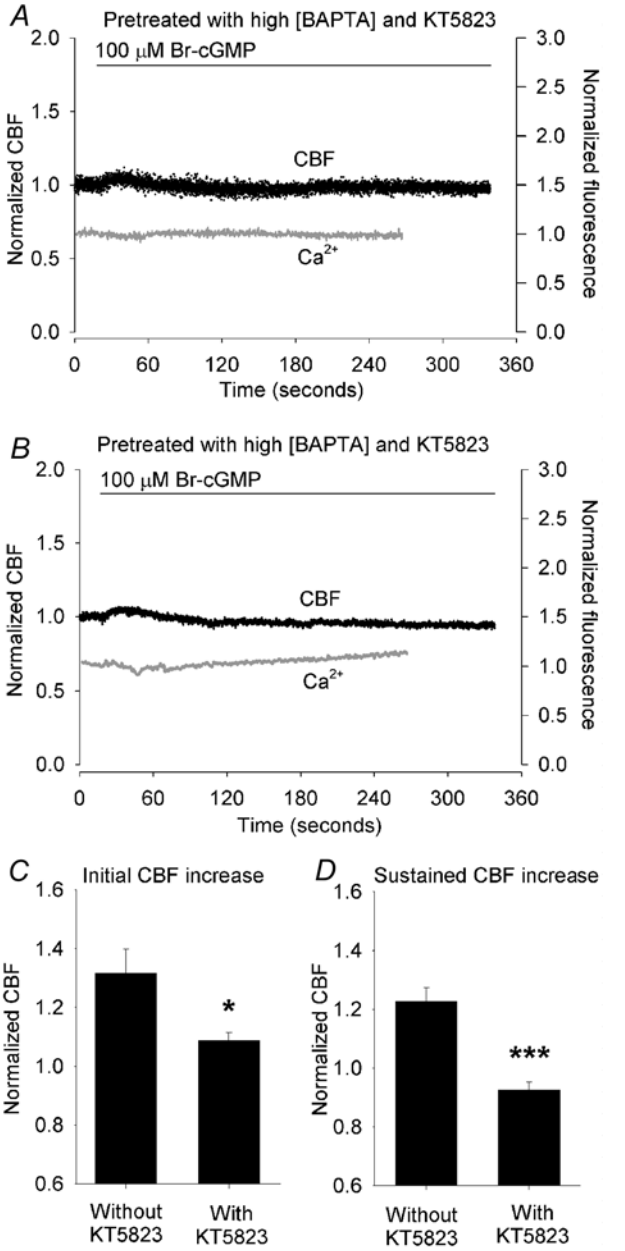

Figure 5. The combined effect of buffering $[Ca^{2+}]_i$ with BAPTA and inhibiting PKG activity with KT5823 on the response to Br-cGMP

A and *B*, two representative cells illustrate the combined effect of the pretreatment of cells with KT5823 and BAPTA AM on the changes in CBF (black dots) and $[Ca^{2+}]_i$ (grey lines) induced by 100 μM Br-cGMP (bar). KT5823 and BAPTA AM treatment prevented any significant increases in the $[Ca^{2+}]_i$ and CBF in response to Br-cGMP. The basal CBF of the cells was 17.7 Hz (*A*) and 12.5 Hz (*B*). *C* and *D*, summaries of the combined effects of KT5823 and BAPTA AM on the initial (*C*) and sustained (*D*) increases in CBF induced by 100 μM Br-cGMP. The increase in the initial CBF (1.09 ± 0.03, n = 7) induced by Br-cGMP in the presence of KT5823 and BAPTA AM was significantly lower than the increase induced in cells without treatment (1.32 ± 0.08, n = 6, * $P < 0.05$). Similarly, the sustained increase in CBF (0.93 ± 0.03, n = 7) was significantly lower compared to cells without treatment (1.23 ± 0.05, n = 6, *** $P < 0.001$). The basal CBFs from each group were similar (12.4 ± 1.0 Hz, n = 6 and 15.3 ± 1.2 Hz, n = 7, $P > 0.05$).

(Fig. 4) that consisted of an initial increase of 1.32 ± 0.08 ($n = 6$) that was followed by a sustained increase in CBF of 1.23 ± 0.05 ($n = 6$). These changes in CBF were indistinguishable from sustained CBF changes induced by 100 μM Br-cGMP in the absence of $[Ca^{2+}]_i$ buffering ($P > 0.05$, respectively) (Fig. 4*B* and *C*). However, as expected in the presence of intracellular BAPTA, 100 μM Br-cGMP was unable to induce observable changes in the $[Ca^{2+}]_i$. These results further support the idea that the sustained increases in CBF are mediated by Br-cGMP whereas the transient increases in CBF are dependent on transient changes in $[Ca^{2+}]_i$.

The effect of KT5823 on the response to Br-cGMP with buffering $[Ca^{2+}]_i$

Since it is widely accepted that cGMP activates PKG, we tested the hypothesis that Br-cGMP-induced changes in CBF are induced by PKG activity by examining the effects of the PKG inhibitor KT5823 on the Br-cGMP-induced increases in CBF. Unfortunately, we initially found that, following treatment with KT5832 (< 0.5 μM) and exposure to 380 nm excitation light for simultaneous $[Ca^{2+}]_i$ measurements, the CBF would gradually slow to a stop (after about 2 min). A similar phenomenon has been reported when the calmodulin inhibitors trifluoperazine and calmidazolium were applied to ovine airway ciliated cells (Salathe & Bookman, 1999). These results imply that the combination of exposure to 380 nm light and KT5823 is toxic to airway ciliated cells. To circumvent this problem, we used the Ca^{2+} indicator Oregon Green 488 BAPTA-1, which requires excitation light of 485 nm, to perform experiments with KT5832. A disadvantage of this approach is that the absolute value of the $[Ca^{2+}]_i$ cannot be obtained with this dye. However, the normalized changes in fluorescence of the dye have been well accepted to represent the changes in $[Ca^{2+}]_i$.

After the cells were loaded with Oregon Green 488 BAPTA-1 AM to monitor $[Ca^{2+}]_i$, the cells were also loaded with the Ca^{2+} buffer BAPTA AM to prevent changes in $[Ca^{2+}]_i$. The cells were then pretreated with 2 μM KT5823 before exposure to 100 μM Br-cGMP. Under these conditions, Br-cGMP did not induce an increase in either CBF or $[Ca^{2+}]_i$ (Fig. 5*A* and *B*, $n = 7$). A comparison of the Br-cGMP-induced initial or sustained changes in CBF in the presence or absence of KT5823 confirmed that the KT5823 abolished the effects of Br-cGMP (Fig. 5*C* and *D*). These results imply that Br-cGMP can induce changes in CBF via PKG.

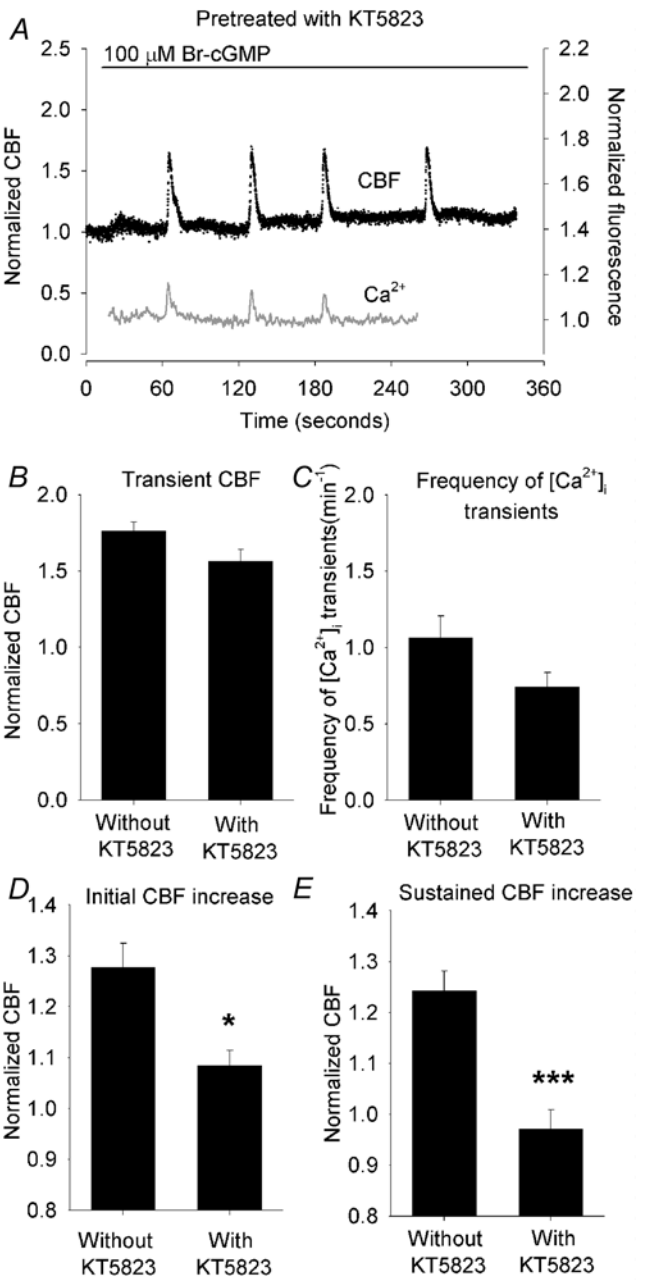

Figure 6. The effect of KT5823 treatment on changes in CBF and $[Ca^{2+}]_i$ induced by 100 μM Br-cGMP

A, Br-cGMP (bar) induced a very small initial increase in CBF (black dots) that rapidly returned to the basal rate without inducing significant increases in $[Ca^{2+}]_i$ (grey line). However, Br-cGMP still induced a series of transients in $[Ca^{2+}]_i$ accompanied with transient increases in CBF. The basal CBF was 15.2 Hz. *B* and *C*, summaries of the effects of KT5823 treatment on Br-cGMP-induced transient changes in CBF (*B*) and the frequency of $[Ca^{2+}]_i$ transients (*C*). KT5823 had no significant effect on the transient CBF (1.56 ± 0.08, $n = 6$ *versus* 1.77 ± 0.06, $n = 11$, $P > 0.05$) or the frequency of $[Ca^{2+}]_i$ transients (0.74 ± 0.09, $n = 4$ *versus* 1.07 ± 0.14, $n = 4$, $P > 0.05$) as compared to controls. The basal CBFs from each group were similar (14.1 ± 0.9 Hz, $n = 6$ and 12.4 ± 0.6 Hz, $n = 11$, $P > 0.05$). *D* and *E*, summaries of the effects of KT5823 on initial (*D*) and sustained (*E*) CBF induced by Br-cGMP. Br-cGMP at 100 μM induced a significantly lower initial increase in CBF (1.08 ± 0.03, $n = 6$) compared to cells without KT5823 treatment (1.28 ± 0.05, $n = 11$, $P < 0.05$) and a significantly lower sustained CBF increase (0.97 ± 0.04, $n = 6$) compared to cells without KT5823 treatment (1.24 ± 0.04, $n = 11$, $P < 0.001$). * $P < 0.05$, *** $P < 0.001$.

Table 1. The effects of pre-treatment of cells with KT5823 on the changes in CBF and $[Ca^{2+}]_i$ induced by 1 μM ionomycin

Pre-treatment KT5823 (n)	Basal CBF (Hz)	Normalized CBF (CBF_t/CBF_0)		Normalized maximum fluorescence (F_t/F_0)
		Maximum CBF	Sustained CBF	
Without (5)	[a]10.1 ± 1.2	[b]2.43 ± 0.16	[c]1.50 ± 0.08	[d]1.18 ± 0.05
With (5)	[a]12.2 ± 2.2	[b]2.28 ± 0.27	[c]1.74 ± 0.21	[d]1.13 ± 0.03

CBF, ciliary beat frequency. [a, b, c, d]No significant difference between groups ($P > 0.05$). Data values are represented as means ± S.E.M.

The effects of KT5823 on the $[Ca^{2+}]_i$ responses to cGMP

Because Br-cGMP induced both changes in CBF and $[Ca^{2+}]_i$, KT5823 was also used to assess whether PKG was involved in the stimulation of the Ca^{2+} transients. After cells were pretreated with KT5823, we found that exposure to Br-cGMP failed to increase the basal CBF, but a series of $[Ca^{2+}]_i$ transients, with associated increases in CBF, still occurred (Fig. 6*A*). Neither the frequency of the $[Ca^{2+}]_i$ transients (0.74 ± 0.09 min^{-1}, $n = 4$) nor the mean peak CBF associated with the transients (1.56 ± 0.08, $n = 6$) were statistically different from those of cells without KT5823 treatment ($[Ca^{2+}]_i$ transient frequency: 1.07 ± 0.14 min^{-1}, $n = 4$; peak CBF: 1.77 ± 0.06, $n = 11$) (Fig. 6*B* and *C*). In accordance with our previous experiments with KT5823, the ability of Br-cAMP to induce initial and sustained changes in CBF (not associated with $[Ca^{2+}]_i$ transients) was suppressed in comparison to those of cells not treated with KT5823 (Fig. 6*D* and *E*). These results suggest that Br-cGMP directly invokes changes in Ca^{2+} signalling and that increases in CBF can be mediated by increases in $[Ca^{2+}]_i$ independently of PKG activity.

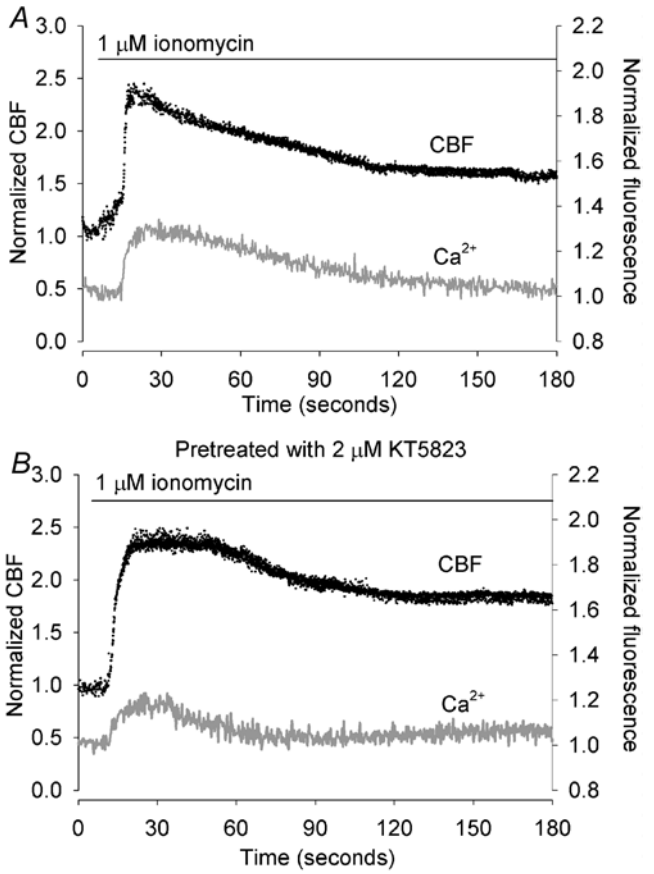

Figure 7. The effect of KT5823 treatment on ionomycin-induced increases in CBF and $[Ca^{2+}]_i$

A, a representative trace ($n = 5$) of the simultaneous changes in CBF (black dots) and $[Ca^{2+}]_i$ (grey line) in a ciliated epithelial cell in response to 1 μM ionomycin (bar). Ionomycin induced a rapid increase in CBF and $[Ca^{2+}]_i$. While the $[Ca^{2+}]_i$ gradually returned to the basal level, the CBF remained sustained at an elevated level. *B*, following the pretreatment of cells with 2 μM KT5823, the response induced by 1 μM ionomycin was indistinguishable from the control response ($n = 5$).

Response of CBF and $[Ca^{2+}]_i$ to ionomycin in the presence of KT5823

To confirm that CBF could be increased by increases in $[Ca^{2+}]_i$ when the cGMP–PKG signalling pathway was inhibited, cells were pretreated with 2 μM KT5823 and then stimulated with ionomycin to increase the $[Ca^{2+}]_i$. In control experiments without KT5823, 1 μM ionomycin induced a fast increase in $[Ca^{2+}]_i$ (normalized value of 1.18 ± 0.05, $n = 5$) and CBF (maximal rate: 2.43 ± 0.16, $n = 5$) (Fig. 7*A*). Subsequently, the $[Ca^{2+}]_i$ returned to the basal level while the CBF decreased to a lower but sustained level of 1.50 ± 0.08 ($n = 5$). However, the changes in the $[Ca^{2+}]_i$ and the initial maximum and sustained CBFs induced by ionomycin were unaffected by the pretreatment of the cells with KT5823 (Table 1, Fig. 7*B*). These results also suggest that the activation of PKG pathway was not necessary for the changes in CBF induced by increases in $[Ca^{2+}]_i$.

Response of CBF and $[Ca^{2+}]_i$ to ATP in the presence of KT5823

To further explore whether PKG signalling is a prerequisite for the Ca^{2+}-dependent increases in CBF induced by other agonists, we examined the effects of KT5823 on the oscillations in both CBF and $[Ca^{2+}]_i$ induced by ATP (Zhang & Sanderson, 2003). In brief, we found that all aspects of the oscillatory changes in CBF and $[Ca^{2+}]_i$ induced by ATP were unaffected by the pretreatment of cells with KT5823 (Table 2 and Fig. 8*A* and *B*). A similar initial increase in both CBF and $[Ca^{2+}]_i$

Table 2. The effects of pre-treatment of cells with KT5823 on the changes in CBF and $[Ca^{2+}]_i$ induced by 2 μM ATP

Pre-treatment KT5823 (*n*)	Basal CBF (Hz)	Oscillations in CBF (min^{-1})	Normalized CBF (CBF_t/CBF_0)		Normalized maximum fluorescence (F_t/F_0)
			Min CBF	Max CBF	
Without (5)	[a]12.8 ± 0.9	[b]1.40.2	[c]1.24 ± 0.05	[d]1.96 ± 0.20	[e]1.16 ± 0.03
With (5)	[a]11.8 ± 1.9	[b]1.4 ± 0.5	[c]1.13 ± 0.03	[d]1.91 ± 0.12	[e]1.18 ± 0.03

F_t, measured fluorescence; F_0, starting fluorescence. [a,b,c,d,e]No significant difference between groups ($P > 0.05$). Data values are represented as means ± S.E.M.

and stimulation of sustained oscillations in CBF and $[Ca^{2+}]_i$, was observed in cells with (Fig. 8*B*) and without (Fig. 8*A*) KT5823 treatment. As previously described (Zhang & Sanderson, 2003), the CBF oscillations could be characterized with the following parameters: (i) the mean maximum (peak) CBF of the CBF oscillations, (ii) the mean minimum CBF of the CBF oscillations and (iii) the frequency of the oscillations in CBF. None of these parameters were significantly altered by pretreatment with KT5823. These results indicate that the PKG signalling pathway has no role in Ca^{2+}-mediated changes in CBF or the mechanisms generating Ca^{2+} oscillations.

DISCUSSION

In previous studies, cGMP (Br-cGMP or dibutyryl cGMP (db-cGMP)) has been reported to increase CBF in human (Geary *et al.* 1995; Runer & Lindberg, 1999), rat (Li *et al.* 2000) and bovine (Wyatt *et al.* 1998) airway ciliated cells. However, Tamaoki *et al.* (1991) reported that cGMP inhibited CBF in rabbit cells. By using high-speed digital recording to continuously sample CBF at 240 Hz and a beat-by-beat analysis to determine the duration or frequency of each ciliary beat cycle (Zhang & Sanderson, 2003), we have found, in keeping with the other studies, which cGMP increased CBF (Geary *et al.* 1995; Wyatt *et al.* 1998; Runer & Lindberg, 1999; Li *et al.* 2000) (Figs 1 and 3*A*). But, unlike other studies, we have also found, by simultaneously measuring $[Ca^{2+}]_i$ with CBF, that the CBF responses induced by cGMP consisted of a Ca^{2+}-dependent response and a Ca^{2+}-independent response.

Since a variety of factors may potentially influence the magnitude of the CBF, we chose to use Br-cGMP as the PKG activator because it is a potent, membrane-permeant activator that does not affect cGMP-stimulated or cGMP-inhibited phosphodiesterases (cGS-PDEs and cGI-PDEs) and is relatively resistant to hydrolysis by phosphodiesterases (Smolenski *et al.* 1998). Temperature also affects the response; Wyatt *et al.* (1998) and Li *et al.* (2000) reported a CBF increase of ~10 % at room temperature, while Geary *et al.* (1995) and Runer *et al.* (1999) reported higher increases in CBF at warmer temperatures (~25 % at 33 °C; ~50 % at 36–37 °C). Our studies were conducted at ~30 °C. Higher temperatures led to difficulties with evaporation artifacts. We only investigated the effects of 1–100 μM Br-cGMP on CBF and $[Ca^{2+}]_i$ since higher concentrations did not further increase CBF (Wyatt *et al.* 1998; Li *et al.* 2000) and had the potential to activate PKA (Forte *et al.* 1992; Lincoln *et al.* 1996; Carvajal *et al.* 2000).

The Ca^{2+}-independent increase in CBF induced by Br-cGMP was relatively small but was sustained at a constant

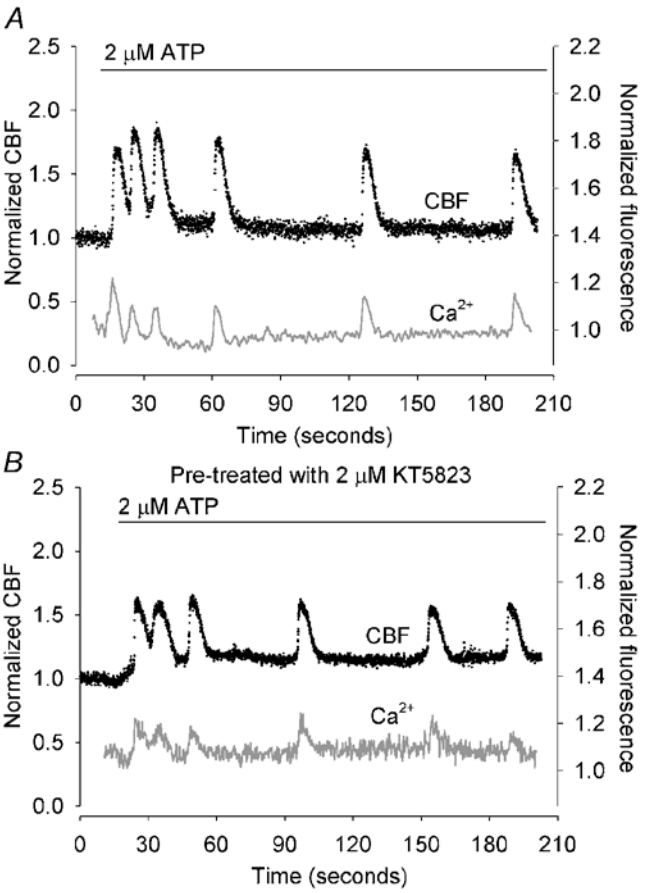

Figure 8. The effect of KT5823 treatment on ATP-induced changes in CBF and $[Ca^{2+}]_i$

A, a representative trace of the simultaneous changes in CBF (black dots) and $[Ca^{2+}]_i$ (grey line) in ciliated epithelial cells in response to 2 μM ATP (bar). ATP induced a rapid increases in CBF and $[Ca^{2+}]_i$ which was followed by oscillations in both CBF and $[Ca^{2+}]_i$. The CBF oscillations occurred from an elevated minimum CBF while the $[Ca^{2+}]_i$ oscillations occurred from a baseline that declined to the basal level. *B*, ATP induced a similar response in CBF and $[Ca^{2+}]_i$ in cells that were pretreated with 2 μM KT5823 ($n = 5$).

rate and was dependent on the concentration of Br-cGMP (Fig. 3*A*). The sustained increase in CBF appeared to be mediated by the cGMP–PKG signalling pathway because the inhibition of PKG with KT5823 (in the presence or absence of the Ca^{2+} buffer, BAPTA) virtually abolished the Ca^{2+}-independent changes in CBF (Figs 5 and 6). Furthermore, when the Ca^{2+} buffering of the cell was increased by BAPTA, the sustained increases in CBF were still induced by Br-cGMP (Fig. 4).

The second major CBF response to cGMP was the large transient increases in CBF that were superimposed on the sustained elevation of CBF (Fig. 3). In all cases, we found that these transient increases in CBF were tightly-coupled with transient increases $[Ca^{2+}]_i$ (Figs 1 and 2) and that both the transient CBF and $[Ca^{2+}]_i$ changes were abolished by increased $[Ca^{2+}]_i$ buffering with BAPTA. In addition, the increases in $[Ca^{2+}]_i$ always occurred prior to the increases in CBF as has been previously described (Evans & Sanderson, 1999; Lansley & Sanderson, 1999). From these results, we conclude that the transient increases in CBF are dependent on the transient increases in $[Ca^{2+}]_i$ that are induced by Br-cGMP.

The mechanism by which $[Ca^{2+}]_i$ transients are induced by Br-cGMP is not fully understood. However, the transients in $[Ca^{2+}]_i$ still occurred in the presence of the PKG inhibitor KT5823 and this supports the possibility that cGMP acts directly, but independently of PKG, on the Ca^{2+} signalling machinery of the cell. We also found in this study that the frequency of Ca^{2+} transients induced by cGMP showed little or no change over the range of cGMP concentrations tested and this suggests that the sensitization of the Ca^{2+} signalling system may be readily saturated by small cGMP concentrations. The stimulation of the Ca^{2+} transients may result from increasing the sensitivity of the inositol 1,4,5-trisphosphate (IP_3) receptor or ryanodine receptor (RyR) to endogenous second messengers such as IP_3 or cyclic-adenosine diphosphate ribose (Berridge *et al.* 2000). However, contrary to these suggestions are other studies that have reported that cGMP can inhibit $[Ca^{2+}]_i$ transients (Ruth *et al.* 1993) and oscillations (Pauvert *et al.* 2000; Kwan *et al.* 2001) by targeting the IP_3 receptor (Ruth *et al.* 1993; Kwan *et al.* 2001) in epithelial cells and smooth muscle cells. The independence of the magnitude of the $[Ca^{2+}]_i$ transients (and therefore the CBF transients) from the cGMP concentration is readily attributable to the Ca^{2+} release properties of the IP_3 receptor that are inhibited by increasing $[Ca^{2+}]_i$ (Berridge *et al.* 2000). As a result, the release of Ca^{2+} by the cell is a self-limiting response. An alternative mechanism through which cGMP may act is the stimulation of cGS-PDEs, cGI-PDEs or cGMP-gated ion channels (McDonald & Murad, 1995; Leinders-Zufall *et al.* 1997; Lohmann *et al.* 1997; Carvajal *et al.* 2000) and, under certain circumstances, PKA to phosphorylate other target proteins (McCann *et al.* 1989; Forte *et al.* 1992; Lincoln *et al.* 1996; Braiman *et al.* 1998; Sisson *et al.* 1999; Carvajal *et al.* 2000). The fact that the Ca^{2+} transients were always induced later than the sustained increases in CBF by cGMP suggests the action of cGMP may require additional intermediate steps. However, the relevance of these mechanisms to the Ca^{2+} oscillations observed in this report requires further investigation.

Although the regulation of CBF appears to involve both cGMP–PKG and Ca^{2+} signalling, the interaction of these two signalling pathways is not well understood. A number of studies have suggested that Ca^{2+} could not alter CBF by itself, but required the prerequisite activation of PKG (Uzlaner & Priel, 1999; Braiman *et al.* 2000, 2001; Ma *et al.* 2002; Zagoory *et al.* 2002). For example, the inhibition of PKG with KT5823 was reported to abolish agonist-induced increase in CBF (e.g. acetylcholine (Zagoory *et al.* 2002) or ATP (Uzlaner & Priel, 1999)) even though the $[Ca^{2+}]_i$ responses were not significantly influenced. The proposed mechanism underlying this response was that PKG activation was stimulated by a cascade of signalling events associated with NO production. These included the Ca^{2+} activation of NO synthase to produce NO, which in turn activates GC to produce an elevation of cGMP. The subsequent increase in cGMP-dependent phosphorylation is proposed, together with the increased Ca^{2+}, to stimulate a large increase in CBF (Uzlaner & Priel, 1999; Braiman *et al.* 2000, 2001; Ma *et al.* 2002; Zagoory *et al.* 2002). Unfortunately, in these previous studies, no significant increases in either CBF or Ca^{2+} were induced by exposure to db-cGMP (100 μM) (Uzlaner & Priel, 1999; Braiman *et al.* 2001). As a result, it was not clear that the PKG activation could induce an increase in CBF. Similarly, it was not possible to distinguish between the increases in both CBF and $[Ca^{2+}]_i$ induced by ATP in the presence or absence db-cGMP (Braiman *et al.* 2001). Therefore, it was also unclear whether CBF increases induced by ATP were due to the direct effects of Ca^{2+} or the Ca^{2+}-activation of PKG. However, we show here that the Ca^{2+} and CBF responses induced by Ca^{2+} ionophore, ionomycin or by ATP were unaffected by the PKG inhibitor. This result also underscores the idea that stimulation by ATP, which is believed to act via the activation of PLC, does not rely on PKG activity. Similarly, the non-specific entrance of Ca^{2+} into the cell also does not need to activate PKG before it can elevate the CBF.

Support for the direct action of Ca^{2+} on the CBF has also been provided by Salathe & Bookman (1999) and Salathe *et al.* (2000) who found that tightly coupled changes in CBF and $[Ca^{2+}]_i$ were not influenced by inhibitors of NO synthase in ovine airway epithelium. Similarly, we have previously reported that Ca^{2+} waves are able to rapidly increase CBF in the absence of any prior stimulation (Lansley & Sanderson, 1992). The idea that Ca^{2+} and

cGMP regulate ciliary activity independently is also supported by studies at the axonemal level in which Ca^{2+}-dependent (Gundersen & Nelson, 1987; Son *et al.* 1993) and cyclic nucleotide-dependent (Miglietta & Nelson, 1988; Hochstrasser & Nelson, 1989) protein kinases were found to phosphorylate different axonemal proteins (Travis & Nelson, 1988; Bonini & Nelson, 1988, 1990; Hamasaki *et al.* 1989, 1991; Salathe *et al.* 1993; Walczak & Nelson, 1993, 1994; Satir *et al.* 1995; Porter & Sale, 2000).

In summary, we have found that Br-cGMP stimulates sustained increases in rabbit airway CBF in a PKG-dependent but Ca^{2+}-independent manner. By contrast, Br-cGMP also initiates transient increases in $[Ca^{2+}]_i$ that, in turn, lead to transient increases in CBF, which are independent of PKG. The inhibition of PKG had no significant influence on either CBF and $[Ca^{2+}]_i$ transients induced by ATP, or the Ca^{2+}-coupled CBF increase induced by ionomycin. As a result, we propose that cGMP can induce increases in CBF in a Ca^{2+}-independent and Ca^{2+}-dependent manner and that the stimulation of CBF by Ca^{2+} does not require the activation of PKG.

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Acknowledgements

This work was supported by NIH grants HL49288 and HL70882 to M.J.S.

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