Purinergic stimulation of rabbit ciliated airway epithelia: control by multiple calcium sources

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1. Simultaneous measurements of average intracellular calcium concentration ($[Ca^{2+}]_i$) and ciliary beat frequency (CBF) were carried out on ciliated rabbit tracheal cells in order to determine quantitatively the role of calcium in the regulation of mucus-transporting cilia.
2. Extracellular ATP caused a rapid increase in both $[Ca^{2+}]_i$ and CBF in the 0·1–1000 μM concentration range. The rise in $[Ca^{2+}]_i$ levelled off to an elevated $[Ca^{2+}]_i$ plateau while the cilia remained in a high activation state. The magnitude of the rise in $[Ca^{2+}]_i$ and CBF as well as the value of the elevated $[Ca^{2+}]_i$ plateau and the value of the sustained CBF were dependent on the concentration of ATP in the solution.
3. No correlation was found between the mean values of $[Ca^{2+}]_i$ and CBF at rest but a sigmoidal relationship was found to exist between the maximal rises of these parameters following excitation with extracellular ATP. This sigmoidal correlation incorporated the experiments where $[Ca^{2+}]_i$ rise was induced by depletion of internal calcium stores with thapsigargin or by entry of calcium induced by ionomycin.
4. Extracellular ATP caused both the release of calcium from internal stores and calcium influx from the extracellular solution. The release of calcium was identified as originating from a thapsigargin-sensitive and a thapsigargin-insensitive calcium store. It is suggested that the release of calcium from these stores induces the initial rise in CBF.
5. The sustained activation of the cilia and elevated calcium plateau were found to be the result of the extracellular ATP-induced calcium influx. This calcium influx was insensitive to the voltage-gated calcium channel inhibitors verapamil and diltiazem, but was completely eliminated by lowering the extracellular calcium concentration to 0·1 μM.
6. We propose that the initial jump in the CBF is mediated by the calcium released from a thapsigargin-insensitive calcium store adjacent to the cilia, while the later, and longer, rise in CBF is the result of the calcium emanating from the thapsigargin-sensitive store which is positioned further away from the cilia within the cell cytoplasm. The calcium influx that follows is responsible for sustaining the cilia at a high level of excitation.

The mammalian airway tract contains an ingenious waste disposal system. This system uses mucus as a vehicle, driven by a 'conveyor belt' of co-operatively beating cilia, to transport unwanted particles (trapped in the mucus) away from the lungs (Sleigh, Blake & Liron, 1988). This assemblage can cope with the titanic task of mucus transport because of the high degree of co-ordination between the individual cilia and because of the ability of the cilia to respond, quickly and for prolonged periods of time, to various stimuli by increasing the ciliary beat frequency (CBF). Although much has been accomplished in the understanding of the underlying mechanisms of ciliary activation, the entire spectrum of mucociliary activation (i.e. initial stimulation, prolonged activation and return to resting activity) still remains to be investigated.

The cellular event most implicated in ciliary stimulation is change in intracellular calcium concentration ($[Ca^{2+}]_i$). In water-propelling cilia, calcium influx through voltage-gated calcium channels is responsible for the change in the organism's swimming behaviour (Naitoh & Kaneko, 1972; Eckert, 1972). In mucus-transporting cilia, $[Ca^{2+}]_i$ is essential for ciliary beating (Verdugo, 1980) and a rise in CBF correlates with a rise in $[Ca^{2+}]_i$ (Girard & Kennedy, 1986; Sanderson, Chow & Dirksen, 1988; Dirksen & Sanderson, 1989; Villalon, Hinds & Verdugo, 1989; Di Benedetto, Magnus, Gray & Mehta, 1991; Lansley, Sanderson &

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Dirksen, 1992; Hansen, Boitano, Dirksen & Sanderson, 1993, 1995; Korngreen & Priel, 1994; Salathe & Bookman, 1995; Tarasiuk, Bar-Shimon, Gheber, Korngreen, Grossman & Priel, 1995). Although the link between $[Ca^{2+}]_i$ and CBF has been established, many details of that relationship have yet to be understood: the role of the resting value of $[Ca^{2+}]_i$ in the control of CBF; the quantitative relationship between CBF and $[Ca^{2+}]_i$; and the interplay between the various calcium sources and the processes of ciliary activation. Is the activation of the cilia integrated by a single calcium source, such as intracellular calcium stores (Verdugo, 1980) or a voltage-gated calcium channel (Eckert, 1972), or by several co-ordinated cellular calcium sources? Finally, the mechanism by which a rise in the concentration of the calcium ions induces the cilia to beat faster is also unknown.

One reason these questions are still largely unanswered is the large fluctuations inherent in the values obtained for ciliary motility and $[Ca^{2+}]_i$. It is known that the CBF fluctuates over time and may vary between adjacent cells in the same culture. We have also observed that $[Ca^{2+}]_i$ levels and responses also vary greatly. Therefore, a statistically significant description of both $[Ca^{2+}]_i$ and CBF requires large numbers of experiments. However, in performing those, fine details of the experiment, which may provide important information regarding the dynamics of the system, are liable to be lost. To deal with this, we designed a tool that would allow the observation of *both* the cellular response and the signal transduction pathway in the *same* cell, thus rendering direct comparison between $[Ca^{2+}]_i$ and CBF possible. By combining fluorescence microscopy with bright-field observations we were able to monitor the changes in the cytoplasmic calcium concentration and CBF simultaneously from a restricted area of cultured rabbit airway epithelial cells (Korngreen & Priel, 1994). The direct application of calcium ions from the extracellular medium to the cilia, using the calcium ionophore ionomycin, markedly increased CBF even before $[Ca^{2+}]_i$ began to increase. This is most likely to have occurred from a rise in the calcium concentration near the membrane as the calcium moved into the cell triggering the ciliary activation. Only later did the calcium concentration in the cytoplasm reach an elevated level.

We have also used extracellular ATP to stimulate cilia. This agonist has been shown to influence various biological processes, including excitation, contraction and secretion, working through a diverse family of receptors called purinoceptors (Burnstock, 1976). Extracellular ATP has been shown to trigger myriad responses in many cell types by binding to these receptors (Dubyak & El-Moatassim, 1993), such as mobilization of calcium from internal stores, rise in $[Ca^{2+}]_i$ induced by calcium influx through voltage- and ligand-gated calcium channels in the plasma membrane (Bean, 1992), and increase in the permeability of transformed cells to calcium (Rozengurt, Heppel & Friedberg, 1977). This wide range of possible responses makes it essential to investigate fully the actions of extracellular ATP in every cell type studied. It has been found that extracellular ATP is one of the most potent activators of ciliary beating (Ovadyahu, Eshel & Priel, 1988; Villalon *et al.* 1989; Weiss, Gheber, Shoshan-Bramatz & Priel, 1992; Gheber & Priel, 1994; Hansen *et al.* 1995; Gheber, Priel, Aflalo & Shoshan-Bramatz, 1995; Tarasiuk *et al.* 1995).

Stimulation of rabbit airway epithelia with extracellular ATP caused a rise in $[Ca^{2+}]_i$ and a corresponding rise in CBF (Korngreen & Priel, 1994). Qualitatively this supports the hypothesis that the calcium ion plays an important role in the process of ciliary stimulation. Although direct measurement of the intraciliary calcium concentration would be preferable, the small dimensions (5–10 μm in length), rapid movement (5–35 Hz) and close packing (up to several hundred per cell) of the mucus-transporting cilia present great, and at present insurmountable, obstacles in the way of such measurements. Such direct measurements were recently accomplished on very large (77 μm in length) water-transporting cilia (Tamm & Terasaki, 1994). The results obtained by us (Korngreen & Priel, 1994) showed that viable information on calcium-induced ciliary stimulation could be obtained from simultaneous measurement of the average cytoplasmic calcium concentration and the CBF. The intricate pattern of $[Ca^{2+}]_i$ and ciliary stimulation induced by ionomycin and extracellular ATP in the rabbit airway epithelia have led us to hypothesize that this pattern is controlled by several calcium-activated processes.

In order to identify the various stages of this ciliary excitation process we have performed a quantitative investigation of the extracellular ATP-induced rise in $[Ca^{2+}]_i$ and CBF. In this paper we provide data on the correlation between $[Ca^{2+}]_i$ and CBF during rest and following stimulation. The results are discussed in the light of the physiological requirement that a mucus-transporting system should activate rapidly and remain in the activated state for the duration of the stimulus, and in consideration of the role and mode of action of extracellular ATP in cell physiology. Part of this work has appeared in abstract form (Korngreen & Priel, 1995).

METHODS

Chemicals and solutions

All chemicals, unless stated otherwise, were obtained from Merck (Darmstadt, Germany). All tissue culture reagents were from Biological Industries (Beit Haemek, Israel). The pentapotassium salt (K_5fura-2) and the acetoxymethyl ester (fura-2 AM) forms of the calcium indicator fura-2 were purchased from Molecular Probes (Eugene, OR, USA). The dye was stored as a solid at −20 °C and fresh solutions were made each day in dry dimethyl sulphoxide (DMSO). Ionomycin was obtained from Calbiochem (Lucerne, Switzerland). It was stored as a 5 mM stock solution in 100% ethanol at −20 °C, aliquots of which were diluted into the physiological solution as required. ATP sodium salt and thapsigargin were obtained from Sigma Chemical Co. ATP was dissolved in physiological solution prior to the experiments (the pH of the solution was corrected if necessary by addition of NaOH). Thapsigargin was stored as a 0·2 mM solution in dry DMSO at −20 °C. The physiological solution contained (mM): 140 NaCl, 1

Na_2HPO_4, 6 KCl, 1·5 $CaCl_2$, 1 $MgCl_2$, 5 D-glucose and 3 Hepes. The pH of the solution was adjusted to 7·4 with NaOH or HCl as required. Physiological solutions with low concentrations of Ca^{2+} were prepared by adding 0·5 mM EGTA to a solution without $CaCl_2$, together with a predetermined volume of a 1 M $CaCl_2$ solution, utilizing a computer program to calculate the free calcium concentration (Fabiato, 1989).

Tissue culture

Adult white rabbits were killed by gradual exposure to carbon dioxide followed by exsanguination. Care was taken to slowly increase the gas flow over several minutes to prevent any visual signs of distress. Tracheas were removed, and the ciliary epithelium was peeled off the cartilage rings and cut into small pieces. Two or three pieces were placed on a glass coverslip that was pre-incubated for 24 h in RPMI-1640 growth medium supplemented with 10% fetal calf serum, 20 units ml^{-1} penicillin, 2·5 units ml^{-1} nystatin and 20 μg ml^{-1} streptomycin. The glass coverslips were then placed inside 35 mm plastic Petri dishes with a few drops of growth medium and incubated at 37 °C under 5% CO_2. After 24 h growth medium was added, to a total volume of 0·5 ml. It was replaced every other day. The cells were used during their second week in culture; this was the time period at which the epithelial monolayers were big enough to be used in the experiments.

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

Simultaneous measurement of intracellular calcium and ciliary beating was carried out as previously described (Korngreen & Priel, 1994). Briefly, $[Ca^{2+}]_i$ was measured with the fluorescent indicator fura-2 (Grynkiewicz, Poenie & Tsien, 1985). Dye-loaded cells were epi-illuminated alternately at 340 and 380 nm. The fluorescence emitted at 510 nm, from an area of one to three cells, was collected and the 340/380 fluorescence ratio, averaged over the period of 1–2 s, was calculated. CBF was measured by trans-illuminating the same cells with light at 600 nm. Amplitude modulations, created by the light scattered from the beating cilia, were detected by a photomultiplier from a 1·25 μm diameter area on the cell surface, and then amplified and digitized.

Numerical analysis and data presentation

After subtraction of the DC component, the light scattering data were filtered with a 4 Hz 4th order digital Butterworth high-pass filter. The CBF was calculated by applying a fast Fourier transform (FFT) algorithm to 1 s segments of the light scattering data after their multiplication by a Hanning window. Since the Fourier transform was calculated on 1 s segments of the data the resolution of the calculated frequency was ± 1 Hz. Therefore, CBF traces are presented as discrete integer CBF values rather than as continuous lines. The discrete nature of the traces should not be taken to imply that the data are discontinuous.

Each experiment was carried out on cultured cells from at least two different animals. We have detected no variability in the results obtained from cultures that originated from different animals and therefore all the results, from a given experiment, were averaged together and displayed as means ± S.E.M. Since the experiments were performed on primary cultures from tissue explants only a single experiment was carried out on a single dish. The number of repetitions is given as the number of culture dishes and the number of animals used in preparing the cultures (for example, $n = 5$ (3) stands for 5 experiments carried out on cultures prepared from 3 animals). Statistical significance was determined using Student's t test, with $P < 0.05$ being considered significant. In the case of multiple comparisons, one-way analysis of variance was followed by Student's t test with Bonferroni's correction with $P < 0.05$ also being considered significant. Since the resolution of our system is 1 s we did not analyse, other than qualitatively, changes in CBF occurring on this time scale. Non-linear curve fitting was carried out with SigmaPlot (Jandel Scientific, San Rafael, CA, USA).

Experimental procedure

Cultures were incubated in 5 μM fura-2 AM in RPMI-1640 medium for 60 min at 37 °C in a rotating water-bath and then washed with physiological solution for 50–60 min at room temperature (23·5 ± 1 °C) to allow full equilibration and de-esterification of the dye in the cytoplasm. The coverslips with the cells were transferred to the lower part of a constant flow perfusion cell and affixed with a small amount of silicone grease. The chamber was closed with an upper part containing another coverslip as a window and kept at 37 °C by a water-heated plate. This cell allowed rapid exchange of solutions without a change in the flow rate which might mechanically stimulate the cilia (Sanderson *et al.* 1988). Experiments were carried out only on evenly fluorescent cells which did not display bright spots and had a steady CBF. Care was taken to measure only from areas that were completely covered with beating cilia and displayed steady CBF readings for more than 20 min. Moreover, measurements performed on several adjacent places on the same cell and on neighbouring cells showed similar values of the beat frequency. In some experiments the total concentration of the dye was monitored at 360 nm and a decrease in intensity of several percentage points was observed after half an hour of illumination. This decrease, which could be the result of minor photobleaching or fura-2 leakage from the cells, was small and did not affect the quality of the measurements.

RESULTS

Before stimulating the cells we sought to determine if there was any relationship between basal $[Ca^{2+}]_i$ values and basal CBF values. In the majority of the measured cells (~95%) both the CBF and the $[Ca^{2+}]_i$ displayed a stable basal value (S.D. $\leq$ 5%) for over 20 min of recording. In some cells (~5%), either the CBF or the $[Ca^{2+}]_i$ values were not steady but rather drifted slowly. No correlation was observed within these traces between the $[Ca^{2+}]_i$ and the CBF. These cells were discarded from the analysis. To assess the range of variation of the calcium concentration and CBF, averaging was performed on a 1 min segment from the resting period of each experiment. The resulting average basal CBF values are shown in Fig. 1 as a function of their corresponding average basal $[Ca^{2+}]_i$ values measured simultaneously for the same culture. Both values varied appreciably, the basal CBF ranging from 7 to 17 Hz and the basal $[Ca^{2+}]_i$ ranging from 80 to 260 nM (Fig. 1). The Pearson correlation coefficient for this plot was 0·1, indicating a complete lack of correlation between the average basal CBF and the average basal $[Ca^{2+}]_i$. Therefore, the basal values were averaged over all the experiments to give 167 ± 5 nM and 11·6 ± 0·3 Hz ($n = 70$ (19)). Moreover, the resting values of $[Ca^{2+}]_i$ and CBF were not affected by lowering the extracellular calcium concentration ($[Ca^{2+}]_o$) to 0·1 μM nor by application of the voltage-gated calcium channel blockers verapamil (10 μM) and diltiazem (100 μM). Furthermore, no difference was found between average basal CBF for cells loaded with fura-2 and cells not containing fura-2, both in control conditions and in solutions containing the above mentioned calcium channel blockers and low $[Ca^{2+}]_o$.

We then examined whether, on stimulation of the cells, there might be a relationship between $[Ca^{2+}]_i$ and CBF. To produce a wide range of cellular responses, experiments were carried out with extracellular ATP concentrations from 0·1 to 1000 μM. The correlation between the maximal rise in CBF and the maximal rise in $[Ca^{2+}]_i$ was determined. The increases in both $[Ca^{2+}]_i$ and CBF displayed a sigmoidal dependence on the concentration of ATP in the solution (Fig. 2*A* and *B*). Since the CBF dose–response curve reached saturation at the higher ATP concentrations we did not continue to obtain the full curve for $[Ca^{2+}]_i$. The data were fitted to the Hill equation. The Hill equation adequately describes the allosteric binding of a substrate to its enzyme. However, its application to data from a multistep signal transduction cascade might lead to misleading results. We therefore disregarded the Hill coefficient (n_H) representing the possible allosteric nature of the process and considered only the concentration of half-response (EC_{50}) characterizing the sensitivity of the process to the concentration of ATP. Fitting the curves with the Hill equation gave an EC_{50} of 12 ± 1 μM for the CBF plot (Fig. 2*B*; $n_H = 1·0 \pm 0·2$). Since we did not obtain a maximum value for the rise in $[Ca^{2+}]_i$ no EC_{50} could be extracted for Fig. 2*A*. Plotting the maximal rise of CBF *vs.* the maximal rise of $[Ca^{2+}]_i$ also resulted in a sigmoidal curve (Fig. 2*C*).

As we have previously shown, increasing the $[Ca^{2+}]_i$ with the calcium ionophore ionomycin, at 1·5 mM Ca^{2+}_o, also caused a large rise in CBF (Korngreen & Priel, 1994). In order to compare these results, the average maximal rises in $[Ca^{2+}]_i$ and CBF induced by 1 μM ionomycin, at 1·5 mM Ca^{2+}_o, were added to the curve displayed in Fig. 2*C*. Another way of inducing a rise in $[Ca^{2+}]_i$ is by irreversibly depleting the cytoplasmic calcium stores with the sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (Lytton, Westlin & Hanley, 1991). The average maximal rises in CBF and $[Ca^{2+}]_i$ obtained by doing so were also added to Fig. 2*C*. All the data in this curve fitted well to the Hill equation resulting in an EC_{50} of 420 ± 30 nM ($n_H = 5·8 \pm 1·2$). Therefore, all the stages involved in the extracellular ATP ciliary stimulation process appear to require the presence of calcium. In other words, the findings suggest that a rise in CBF may serve as a measure of a rise in $[Ca^{2+}]_i$.

In order to bypass part of the signalling pathway induced by extracellular ATP, the rise in $[Ca^{2+}]_i$ and CBF induced by depletion of the calcium stores with thapsigargin was examined. Thapsigargin is known to induce calcium influxes by causing calcium store depletion (Randrimampita & Tsien, 1993). Hence, the cultures were pre-incubated for 10 min in a physiological solution with a $[Ca^{2+}]_o$ of 0·1 μM. At this $[Ca^{2+}]_o$, which is similar to the resting value of $[Ca^{2+}]_i$, the plasma membrane chemical gradient of the calcium ions was almost completely cancelled. Therefore a possible activation of calcium influx, if it exists, would influence the calcium concentration in the cytoplasm only to a minor extent and the observed changes in $[Ca^{2+}]_i$ could be safely attributed to calcium release from intracellular storage compartments.

After application of 1 μM thapsigargin, a rise both in $[Ca^{2+}]_i$ (Fig. 3*A*) and in CBF (Fig. 3*B*) was observed; they decayed back to prestimulatory values over 10 min. The CBF did not decay monotonically but rather to an intermediate stable value, at which it remained temporarily, before decaying further (Fig. 3*B*). This pattern, which was also observed in experiments in which the culture was stimulated with extracellular ATP, was seen in ~5% of all the experiments carried out in this study. The high concentration of thapsigargin used in these experiments should have caused a complete depletion of the calcium stores. Contrary to this expectation, application of 100 μM ATP after the $[Ca^{2+}]_i$ and CBF had decayed back to their initial values caused a small but pronounced rise in $[Ca^{2+}]_i$

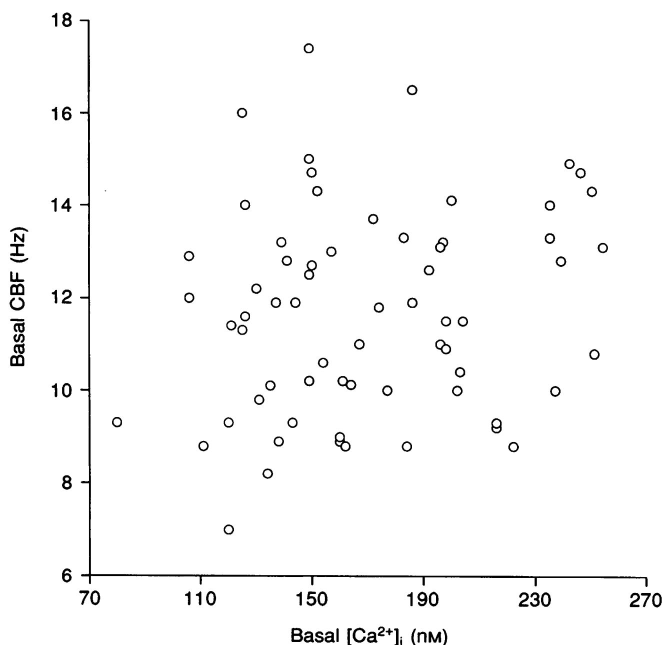

Figure 1. Lack of correlation between the basal levels of CBF and $[Ca^{2+}]_i$

Each point in the graph is a 1 min average of the resting stage of a simultaneous experiment (S.D. $\leqslant 5$%). Pearson product moment correlation showed that indeed there was no significant correlation between CBF and $[Ca^{2+}]_i$ ($P < 0·05$).

and CBF (Fig. 3A and B). Furthermore, the $[Ca^{2+}]_i$ trace presented in Fig. 3A continued to decay to basal levels after the small rise in $[Ca^{2+}]_i$ induced by extracellular ATP had decayed. Indeed we observed that addition of ATP at different times following store depletion with thapsigargin did not change the pattern of decay indicating that the thapsigargin-sensitive store was depleted well before the calcium concentration in the cytoplasm decayed back to basal values (data not shown). An enlarged time scale is shown in Fig. 3C. Close observation of the rise in CBF after the application of thapsigargin, in Fig. 3B, revealed that it consisted of a single step from its basal value to its maximal one. This was more clearly seen when the rise in the CBF was plotted as a function of corresponding $[Ca^{2+}]_i$ values

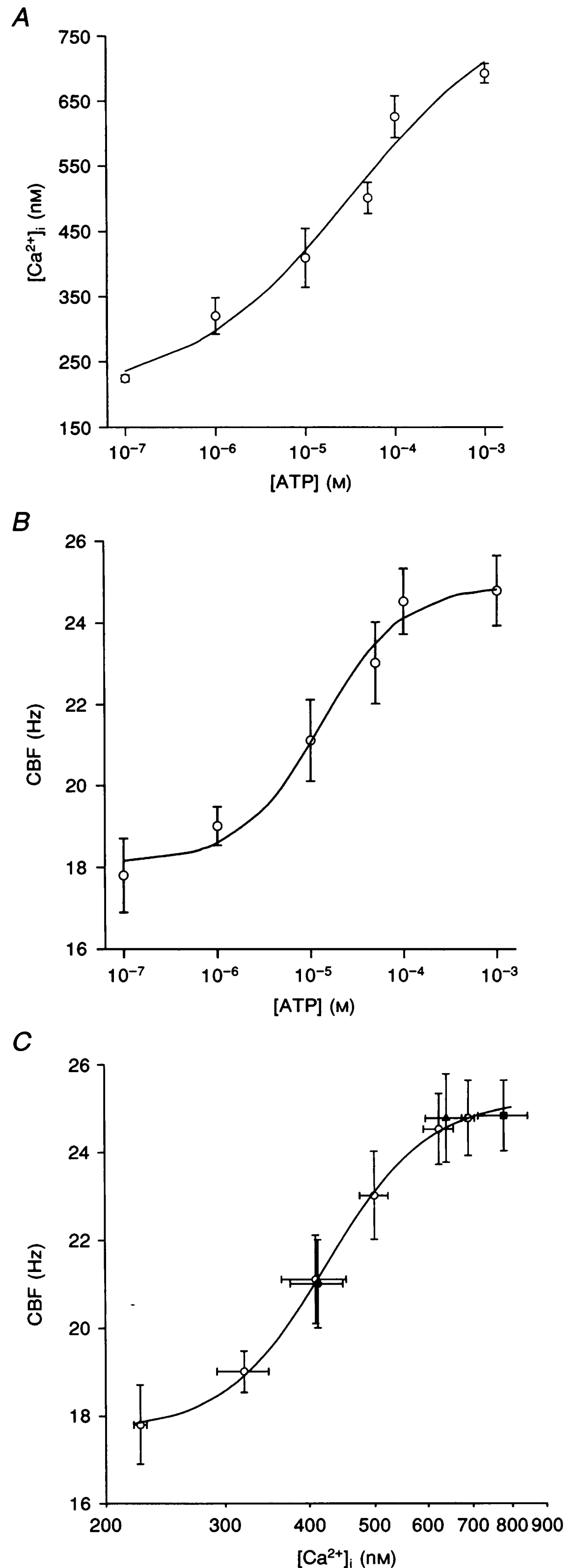

Figure 2. Dose–response relationships of maximal rise in $[Ca^{2+}]_i$ (A) and maximal rise in CBF (B) to given concentrations of extracellular ATP

The CBF *vs.* $[Ca^{2+}]_i$ plot (C) incorporates the average rises in $[Ca^{2+}]_i$ and CBF induced by the ATP concentrations used to construct the upper plots (○) together with rises induced by 1 μM thapsigargin (●), 100 μM extracellular ATP at 0·1 μM extracellular Ca^{2+} (▲), and 1 μM ionomycin at 1·5 mM extracellular Ca^{2+} (■). All the curves were fitted by a Hill equation (continuous line). Data are presented as means ± S.E.M.

(Fig. 3*D*). There was a rise in $[Ca^{2+}]_i$, from 120 to 300 nM, in this experiment, that lasted for several seconds without any significant change in CBF. Then the CBF leapt, within 1 s, from 11 to 21 Hz while the rise in $[Ca^{2+}]_i$ continued, reaching its maximum value after 50 ± 5 s. This single step activation of the CBF appeared in all the experiments carried out with thapsigargin. The rise in $[Ca^{2+}]_i$ prior to the leap in CBF was averaged for all the experiments giving 300 ± 50 nM ($n = 4$ (3)), which was significantly higher than the basal value of $[Ca^{2+}]_i$ ($P \leqslant 0{\cdot}01$). The residual ciliary activation induced by extracellular ATP also displayed a step rise in CBF (Fig. 3*C*). Contrary to the pattern observed after the application of thapsigargin, the jump in CBF preceded the rise in $[Ca^{2+}]_i$ (Fig. 3*C*). The data in Fig. 3*C* are presented as a function of time because the net rises in $[Ca^{2+}]_i$ and CBF were small and too close to the error margin of the instrument to be presented as a $[Ca^{2+}]_i$ *vs.* CBF plot. The net rises in CBF and $[Ca^{2+}]_i$ observed for these residual responses to extracellular ATP were $3{\cdot}2 \pm 0{\cdot}6$ Hz and 39 ± 5 nM ($n = 4$ (3)). Although the rise in $[Ca^{2+}]_i$ is not significantly different from the basal levels, the rise in CBF is significant ($P \leqslant 0{\cdot}05$). This may indicate that extracellular ATP triggers the release of calcium from a storage compartment that was not depleted by thapsigargin.

Another way to induce a rise in $[Ca^{2+}]_i$ from intracellular sources is with the calcium ionophore ionomycin. This agent can deplete intracellular calcium storage compartments by increasing the permeability of the compartments' membranes to calcium ions. This can be used to release calcium into the cytoplasm from more calcium stores than

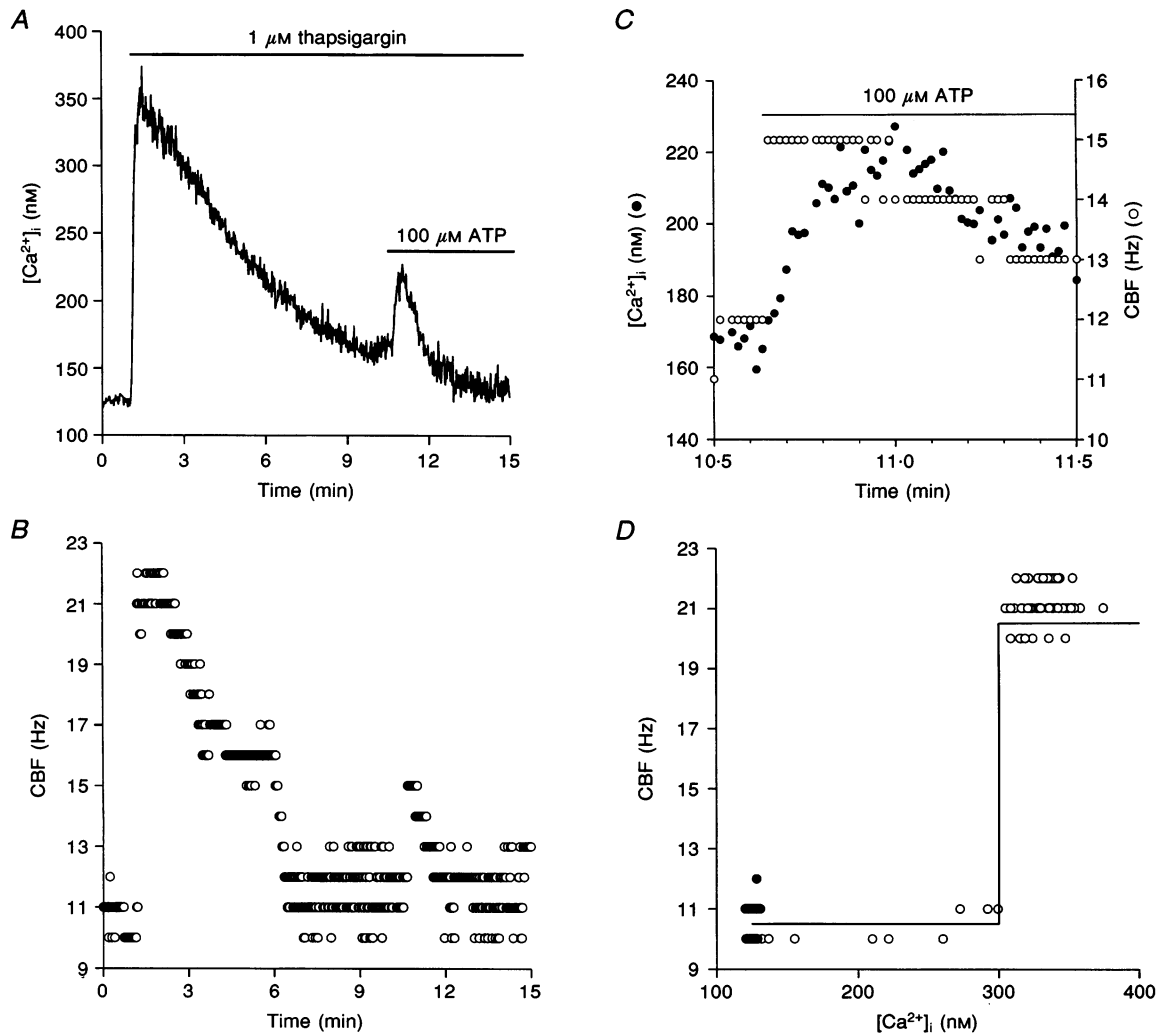

Figure 3. Calcium store depletion with 1 μM thapsigargin results in a rise in $[Ca^{2+}]_i$ (*A*) and CBF (*B*)
Further addition of 100 μM extracellular ATP resulted in a small but pronounced rise in $[Ca^{2+}]_i$ and CBF. The residual rises in CBF (○) and $[Ca^{2+}]_i$ (●) induced by extracellular ATP are presented on an enlarged time scale (*C*). The $[Ca^{2+}]_o$ was kept at 0·1 μM throughout the experiment. The time directly following the application of thapsigargin is displayed as a CBF *vs.* $[Ca^{2+}]_i$ plot (*D*) emphasizing the step activation of the cilia (●, control; ○, thapsigargin). The continuous line in *D* was drawn by hand as a visual aide. Experiments were carried out with a time resolution of 1 s ($n = 4$ (3)).

those depleted by thapsigargin. Carrying out the experiments at a $[Ca^{2+}]_o$ of 0·1 μM made certain that the intracellular calcium transients observed were not the result of calcium entry from the extracellular medium. The rise in $[Ca^{2+}]_i$ observed after internal store depletion by 1 μM ionomycin lasted 51 ± 10 s ($n = 3$ (2)), which is similar to the thapsigargin-induced rise. After reaching a maximum, the $[Ca^{2+}]_i$ decayed, in the experiment displayed in Fig. 4, past its initial value whereas the cilia reached a steady beating rate that was slightly higher than their basal beating frequency. This behaviour was in agreement with our observation that no correlation existed between the resting values of $[Ca^{2+}]_i$ and CBF (Fig. 1). During the excitation stage the CBF *vs.* $[Ca^{2+}]_i$ plot displayed a pattern other than that observed in the experiments with thapsigargin. Two distinct slopes, a steeper slope during the first seconds following the addition of the agonist, followed by a gentler slope for the rest of the rise phase (12 ± 2 and $39 \pm$

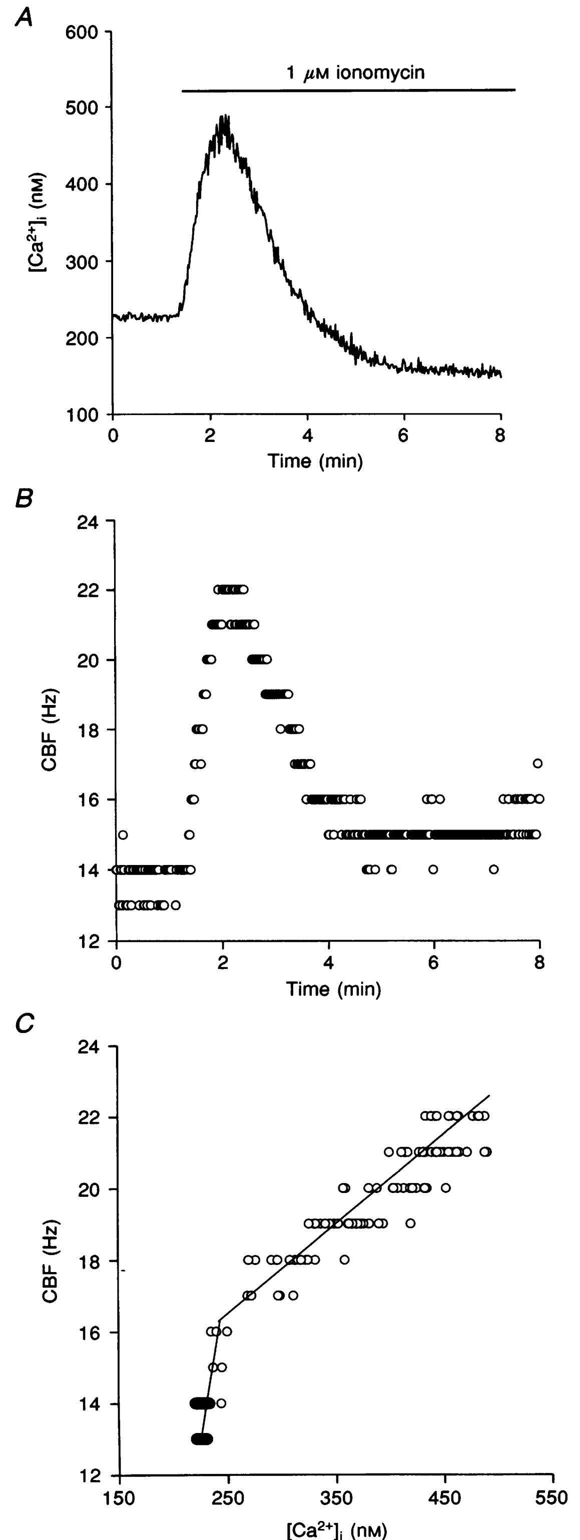

Figure 4. Calcium store depletion with 1 μM ionomycin results in a rise in $[Ca^{2+}]_i$ (*A*) and CBF (*B*)

The time directly following the application of ionomycin is displayed as a CBF *vs.* $[Ca^{2+}]_i$ plot (*C*) (●, control; ○, ionomycin). The continuous lines drawn through the data in *C* were fitted by linear regression to emphasize the two slopes that are apparent in this experiment. Experiments were carried out with a time resolution of 1 s ($n = 3$ (2)).

2 nM Hz^{-1}, respectively) were observed (Fig. 4C). Addition of 100 μM extracellular ATP to the cells, after both the CBF and the $[Ca^{2+}]_i$ had reached a steady resting value, did not cause any residual rise in CBF or $[Ca^{2+}]_i$ (data not shown).

We have observed that after reaching its maximum value, following the stimulation with extracellular ATP, the $[Ca^{2+}]_i$ decayed to a plateau value higher than the initial $[Ca^{2+}]_i$ (Korngreen & Priel, 1994). Elevated calcium plateaus, similar to those observed by us, have been observed in many systems and are now widely considered to be the manifestation of calcium influx (Rasmussen & Rasmussen, 1990). Since this elevated plateau was not as easy to detect as the initial rise in $[Ca^{2+}]_i$, its value was extracted by curve fitting. The decay phase of each experiment was fitted to an exponential decay of the form $[Ca^{2+}]_i = Ae^{-kt} + C$, where k is the rate of calcium decay, A is a proportionality constant, and C gives the value of the elevated calcium plateau. A typical example of such a curve fitting is displayed in Fig. 5A. After the rise generated by 100 μM extracellular ATP in the displayed experiment, $[Ca^{2+}]_i$ clearly decayed to a steady, higher than basal concentration that did not reach the initial calcium concentration even 12 min after the addition of the agonist. The decay fitted well to the equation chosen. The values obtained for the decay rate were verified by fitting a straight line to the logarithm of the decay phase. The values of the elevated plateau were identical to those obtained by averaging a segment of the elevated calcium plateau. This elevated calcium plateau appeared at all concentrations of extracellular ATP (Table 1). Furthermore, the elevated plateau and the rate of calcium decay displayed a dependence on the concentration of ATP in the solution (Table 1).

Calcium influx through voltage-gated calcium channels has been shown, in several studies, to be involved in the activation of the cilia (Villalon *et al.* 1989; Tamm & Terasaki, 1994). To examine whether this was happening in our system we employed the voltage-gated calcium channel blockers verapamil (10 μM) and diltiazem (100 μM) to the extracellular ATP-induced ciliary stimulation. These concentrations, which are at the upper limit of the inhibitors' specificities, did not cause any reduction in the magnitude of the rise in $[Ca^{2+}]_i$ (data not shown). Moreover, the elevated plateau of $[Ca^{2+}]_i$ was not affected (Table 1). Since no involvement of voltage-gated calcium channels was found, we tested whether any type of calcium influx was responsible for the elevated calcium plateau. The culture was stimulated with 100 μM extracellular ATP in a medium containing 0·1 μM Ca^{2+}_o (Fig. 5B). While the rise in $[Ca^{2+}]_i$ is similar to that observed at normal $[Ca^{2+}]_o$, the decay in $[Ca^{2+}]_i$ is clearly faster than the decay observed at normal $[Ca^{2+}]_o$ (Fig. 5A), and reached the initial calcium concentration (Table 1). The rate of calcium decay obtained by the exponential curve fitting was more than twice as fast as the decay rate obtained at normal $[Ca^{2+}]_o$ (Table 1).

Having quantified the components of the extracellular ATP-induced rise and decay of $[Ca^{2+}]_i$ we now turned to observe the corresponding excitation of the CBF. Corresponding with the rise in $[Ca^{2+}]_i$ (Fig. 6A), induced by the bath application of 100 μM ATP, there was a strong rise in CBF (Fig. 6B), consisting of two stages as previously described by us (Korngreen & Priel, 1994). While the calcium concentration decayed, as previously shown in Fig. 5A, to an elevated plateau above its resting value (Fig. 6A) the CBF decayed slightly to a high level of sustained activation

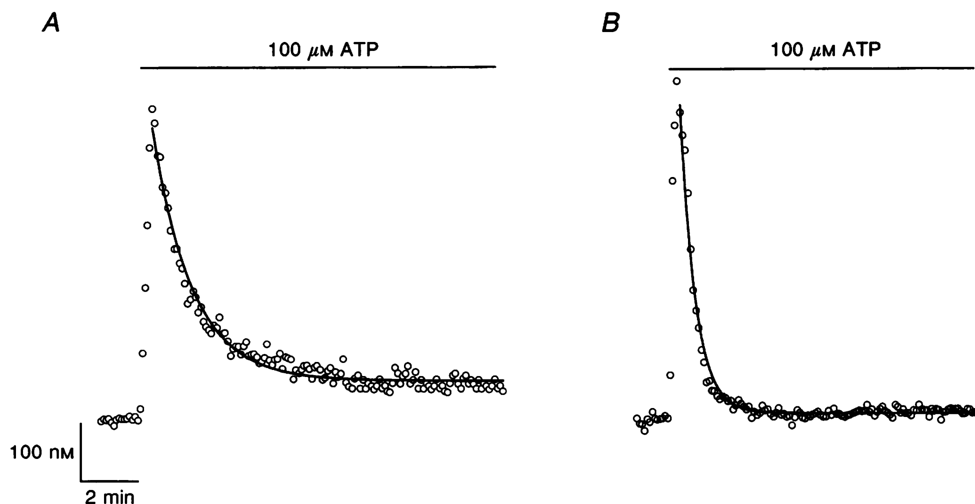

Figure 5. Time course of the effect of extracellular ATP on $[Ca^{2+}]_i$

A, time course of the effect induced by 100 μM extracellular ATP on $[Ca^{2+}]_i$ (○) and a fitted exponential decay (continuous line) in normal conditions (1·5 mM Ca^{2+}_o). *B*, time course of the effect induced by 100 μM extracellular ATP on $[Ca^{2+}]_i$ (○) and a fitted exponential decay (continuous line) in low $[Ca^{2+}]_o$ (0·1 μM). Both experiments were carried out with a resolution of 1 s but only every fifth point is displayed for clarity.

Table 1. Characteristics of the ATP-induced ciliary activation

Treatment [ATP] (μM)	Pretreatment	t_{Ca} (s)	$t_{1,CBF}$ (s)	$t_{2,CBF}$ (s)	$[Ca^{2+}]_i$ plateau (% of basal)	$[Ca^{2+}]_i$ rate of decay (s^{-1})	CBF plateau (% of basal)	n
0·1	—	18 ± 6	—	25 ± 4	109 ± 5	18 ± 3	115 ± 8	3 (2)
1	—	20 ± 4	2·6 ± 0·3	39 ± 8	114 ± 3	19 ± 4	139 ± 10*	4 (2)
10	—	18 ± 2	1·8 ± 0·2	36 ± 5	123 ± 3	22 ± 4	143 ± 6*	4 (2)
50	—	18 ± 3	2·6 ± 0·6	27 ± 3	145 ± 14*	23 ± 4	195 ± 10*	4 (3)
100	—	20 ± 3	2·3 ± 0·3	38 ± 4	147 ± 4*	33 ± 3	194 ± 10*	12 (6)
1000	—	19 ± 3	2·3 ± 0·3	33 ± 5	148 ± 6*	34 ± 4	200 ± 12*	3 (2)
100	0·1 μM Ca^{2+}_o	21 ± 2	2·1 ± 0·2	38 ± 3	96 ± 3†	80 ± 9†	97 ± 6†	16 (5)
100	100 μM diltiazem	25 ± 6	1·8 ± 0·2	42 ± 7	145 ± 5*	34 ± 3	193 ± 11*	3 (2)
100	10 μM verapamil	25 ± 3	1·7 ± 0·2	37 ± 6	144 ± 5*	32 ± 4	190 ± 10*	7 (3)

Average values of the time required for $[Ca^{2+}]_i$ to reach its maximal value (t_{Ca}), for the initial jump in CBF ($t_{1,CBF}$), and for the CBF to reach its maximal value ($t_{2,CBF}$) under the various conditions used in the research. Also presented are the values of the elevated calcium plateau, rate of calcium decay, and the sustained ciliary activation as the relative value above the basal value. Cultures were pretreated with the experimental solution for 10 min prior to the experiment. The concentration of ethanol used to dissolve verapamil and diltiazem was kept under 0·1 %. The total rise in CBF was small at 0·1 μM ATP; it was hard to observe the two-step rise clearly so no value was extracted. Since approximately half of our measurements were carried out at a time resolution of 2 s and the other half at a resolution of 1 s, the time of the initial jump in CBF should be taken as an upper limit. The data are presented as means ± S.E.M. The number of repetitions (*n*) is the number of culture dishes used for each experiment (given in parentheses are the numbers of animals these cultures were produced from). * Significantly different from basal values (ANOVA followed by Student's *t* test with Bonferroni's correction, $P < 0·05$). † Significantly different from the effect at normal conditions (Student's *t* test, $P < 0·05$).

(Fig. 6*B*). This sustained ciliary activation appeared at all concentrations of extracellular ATP. One-minute segments of stable CBF, which were clearly after the $[Ca^{2+}]_i$ had levelled at the elevated plateau, were averaged (Table 1). These sustained CBF activation values displayed a dependence on the concentration of ATP in the solution similar to that of the elevated calcium plateau. Removal of the ATP from the solution resulted in a decay in $[Ca^{2+}]_i$ to initial values, and a slower decay in CBF, to the basal beating frequency (Fig. 6*A* and *B*).

The biphasic rise in CBF seen in Fig. 6*B* was even more evident when the rise phase of CBF was plotted as a function of corresponding $[Ca^{2+}]_i$ values (Fig. 6*C*). During the initial jump in CBF, which lasted for 1–2 s (Table 1), only a small increase in $[Ca^{2+}]_i$ was observed. Since this initial rise in CBF and $[Ca^{2+}]_i$ occurred within the error margins of our instrument we did not quantify it other than to determine its approximate time scale (Table 1). Following this jump the rate of the CBF ascent slowed down. This resulted in the CBF reaching its maximal value after the $[Ca^{2+}]_i$ had already begun to decay. This was emphasized in Fig. 6*C* by fitting a smooth curve through the data points. The time course of the primary and secondary rises in CBF and the time required for the $[Ca^{2+}]_i$ to reach its maximal value were independent of the concentration of extracellular ATP used (Table 1).

No effect was observed on the initial biphasic rise pattern of CBF by lowering the $[Ca^{2+}]_o$ to 0·1 μM (Fig. 6*F* and Table 1). The magnitude of the CBF rise was also not affected and the average rises induced by 100 μM ATP under these conditions fitted well with the CBF *vs.* $[Ca^{2+}]_i$ plot obtained at normal (1·5 mM) $[Ca^{2+}]_o$ (Fig. 2*C*). The sustained ciliary activation was, however, completely eliminated (Fig. 6*E* and Table 1). Furthermore, the CBF decayed, in the displayed experiment, past its initial value (Fig. 6*E*) without a corresponding decay in the $[Ca^{2+}]_i$ (Fig. 6*D*). This observation, as in the ionomycin experiment (Fig. 4), agreed with the finding that the CBF was not correlated with the $[Ca^{2+}]_i$ in the resting state (Fig. 1). Finally, returning the calcium concentration in the solution to 1·5 mM, in the presence of ATP in the solution, caused a rise in $[Ca^{2+}]_i$ to a plateau and a marked rise in CBF (Fig. 6*D* and *E*). Washing the ATP from the solution at this stage resulted in a decay in $[Ca^{2+}]_i$ and CBF back to basal values (data not shown). Increasing the $[Ca^{2+}]_o$ from 0·1 μM to 1·5 mM prior to the addition of ATP to the solution did not cause any change in the CBF or in the $[Ca^{2+}]_i$. This set of experiments leads us to conclude that an influx of calcium is responsible for sustaining the cilia at a high level of activation for the duration of the stimulus. Since the initial pattern of ciliary activation was not influenced by reducing $[Ca^{2+}]_o$ it would appear that it was the result of intracellular events.

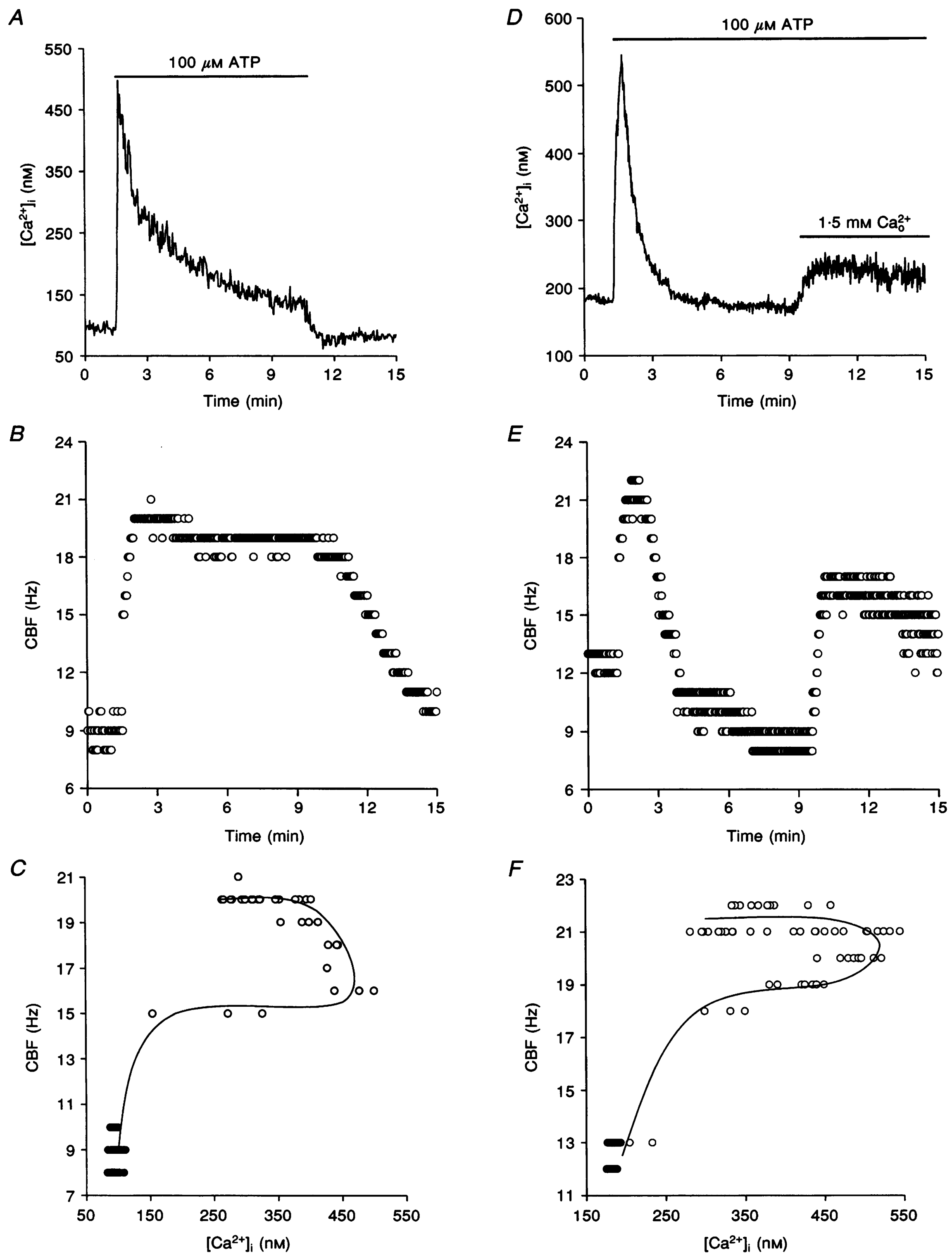

Figure 6. The effect of ATP on $[Ca^{2+}]_i$ and CBF

The rises in $[Ca^{2+}]_i$ (*A*) and CBF (*B*) induced by 100 μM extracellular ATP at 1·5 mM Ca^{2+}_o. Application and removal of the ATP from the solution are indicated by the bar. The biphasic rise is emphasized in the CBF *vs.* $[Ca^{2+}]_i$ plot of the control period and the time directly following the addition of ATP (*C*). ●, control; ○, ATP. The time between adjacent points in this experiment was 2 s. When the same experiment was carried out at 0·1 μM extracellular Ca^{2+} the rise in $[Ca^{2+}]_i$ (*D*) and CBF (*E*) retained its biphasic pattern (*F*). ●, control; ○, ATP. Horizontal bars represent the times of addition of 100 μM ATP and 1·5 mM Ca^{2+}_o. The time between adjacent points, in this experiment, was 1 s. The continuous lines in the CBF *vs.* $[Ca^{2+}]_i$ plots (*C* and *F*) were drawn by hand as a visual aid for the time flow in the experiments.

DISCUSSION

To gain a better understanding of the role played by calcium ions in the regulation of mucus-transporting cilia, both at rest and following stimulation, the concentration of this ion within the cilium should be measured. Such measurements have not as yet been performed. Thus the tool most widely used to investigate the role of calcium in this system has been, and still is, the measurement of the average cytoplasmic calcium concentration (Dirksen & Sanderson, 1989; Villalon *et al.* 1989; Di Benedetto *et al.* 1991; Lansly *et al.* 1992; Hansen *et al.* 1993, 1995; Salathe & Bookman, 1995; Tarasiuk *et al.* 1995). When interpreting the results obtained from such measurements one must keep in mind that the average cytoplasmic calcium concentration may not fully represent the calcium concentration in the vicinity of the cilium. Our approach, the simultaneous measurement of both $[Ca^{2+}]_i$ and CBF from the same area in the ciliary culture, provides an additional intrinsic probe of the physiology of the cell in the form of the CBF. As we have qualitatively shown (Korngreen & Priel, 1994), this 'double labelling' provides more information than would have been obtained by measuring CBF and $[Ca^{2+}]_i$ separately.

CBF activation by calcium ions

By simultaneous measurements of CBF and $[Ca^{2+}]_i$ it was established that the resting values of CBF and the apparent $[Ca^{2+}]_i$ measured by fura-2 did not correlate (Fig. 1). Possibly this indicates that the calcium ions are not involved in ciliary regulation at rest. On the other hand, a more attractive assumption is that the calcium concentration is not homogeneous throughout the cell. This hypothesis complies with the high degree of correlation observed between the maximal rise in CBF and the maximal rise in $[Ca^{2+}]_i$ following stimulation (Fig. 2). The strong correlation between CBF and $[Ca^{2+}]_i$ was observed with a number of agonists (Fig. 2*C*) including extracellular ATP, thapsigargin and ionomycin. This correlation suggested that the maximal rise in $[Ca^{2+}]_i$ measured for the entire cell could be correlated with the calcium concentration in the vicinity of the cilia when the CBF reaches its maximal value after stimulation. Furthermore, this correlation included experiments (Figs 3 and 4) in which a rise in $[Ca^{2+}]_i$ was induced without triggering the signalling pathway activated by extracellular ATP. This independence of the agonist leads us to conclude that all the biochemical and biophysical steps that occur between the rise in the concentration of calcium and the enhancement of the CBF to its maximum value appear to be regulated by calcium ions. There are many possible candidates for such steps, such as the action of calcium ions on the axoneme (Satir, 1985), calcium-dependent phosphorylation and calcium-activated ion channels (Weiss *et al.* 1992; Tarasiuk *et al.* 1995).

CBF activation by calcium from internal stores

Depletion of the cytoplasmic calcium stores with a high concentration of thapsigargin (1 μM) almost completely eliminated the rise in $[Ca^{2+}]_i$ and in CBF induced by extracellular ATP (Fig. 3). Similarly, following depletion of intracellular calcium storage compartments with ionomycin no effect of extracellular ATP on $[Ca^{2+}]_i$ or on CBF was observed. Furthermore, removal of the calcium ion chemical gradient across the plasma membrane, by lowering the $[Ca^{2+}]_o$ to 0·1 μM, did not affect the magnitude of the extracellular ATP-induced rise in $[Ca^{2+}]_i$ and in CBF (Figs 2*C*, 5 and 6). These experiments strongly indicate that extracellular ATP releases calcium from intracellular calcium stores in rabbit ciliated airway epithelium. Finally, depletion of these extracellular ATP-triggered calcium stores abolished not only the rise in $[Ca^{2+}]_i$ but also the rise in CBF (Figs 3 and 4), providing another indication that calcium release was probably responsible for the increase in CBF.

High concentrations of thapsigargin (1 μM) did not completely abolish the rise in $[Ca^{2+}]_i$ and CBF induced by extracellular ATP (Fig. 3). A small but pronounced rise in $[Ca^{2+}]_i$ and CBF was apparent after the application of extracellular ATP. Similar residual rises in $[Ca^{2+}]_i$ were observed in several cell types with different agonists and are now believed to be the result of calcium release from thapsigargin-insensitive internal calcium stores (Tribe, Borin & Blaustein, 1995). Although thapsigargin-insensitive calcium stores were identified recently in many cell types, the physiological rationale for the existence of multiple calcium sources remains unclear (Pozzan, Rizzuto, Volpe & Meldolesi, 1994). We would like to suggest that the thapsigargin-insensitive store in rabbit airway epithelium is adjacent to the cilia and its discharge causes the initial rapid rise in CBF following the application of extracellular ATP (Fig. 6*C* and *F*).

This hypothesis is supported by several findings. Discharging the thapsigargin-insensitive calcium store should cause the CBF to rise prior to the rise in the average $[Ca^{2+}]_i$, as indeed is shown in Fig. 3*C*. The rise in CBF detected by discharging this store after the thapsigargin-sensitive store was depleted was smaller than the initial jump in CBF induced by extracellular ATP (Fig. 6). This discrepancy could be the result of a partial depletion of the thapsigargin-insensitive store caused by the leakage of calcium during the prolonged incubation of the cells with the physiological solution that contained 0·1 μM Ca^{2+}_o. Since the calcium source proposed to be adjacent to the cilia would not be triggered by the application of thapsigargin, there should not be an immediate jump in the CBF following the application of the agonist. Indeed, a delay of several seconds was observed during which the calcium emanating from the thapsigargin-sensitive store filled the cytoplasm and only later flooded the cilia, causing their activation (Fig. 3*D*). This activation was carried out in a single jump in CBF (Fig. 3*D*) to its maximal value and not in two stages as in the ATP excitation (Fig. 6*C* and *F*). Following depletion of the intracellular calcium storage compartments with ionomycin, the rise in CBF displayed two distinct slopes when plotted as a function of $[Ca^{2+}]_i$ (Fig. 4*C*). The initial steep slope could be

attributed to the ionomycin-enhanced leakage of calcium from the calcium store adjacent to the cilia, which first affects the cilia and only subsequently causes a rise in the average $[Ca^{2+}]_i$; the gentler slope could result from enhanced calcium leakage from the cytoplasmic thapsigargin-sensitive store. Since no residual rise in $[Ca^{2+}]_i$ or in CBF was observed when ATP was applied to the cells after ionomycin, it is safe to assume that the thapsigargin-insensitive calcium store was indeed discharged by ionomycin.

To try to explain the difference between the biphasic rise in CBF induced by extracellular ATP and the step rise induced by thapsigargin, we examined the time course of the diffusion of calcium in the cilium. The cilium was assumed to be a straight tube, 5 μm in length, in which calcium diffusion was linear and only along the major axis (Crank, 1975). The calcium store was modelled as a point source of 10 μM calcium, 0·1 μm from the lower end (base) of the cilium. As seen in Fig. 7, which displays the change in calcium concentrations above its basal value at several places along the length of the cilium, less than 1 s after the discharge of the store the calcium concentration reaches a maximum at the tip of the cilium and after 2 s the whole cilium is awash with approximately the same calcium concentration. This highly approximate calculation reveals that the diffusion of calcium in the cilium can provide a good explanation for single-step excitation of the cilia as induced by thapsigargin but fails to account for biphasic ciliary activation as induced by extracellular ATP.

This biphasic rise could be explained, however, if the actions of the calcium store proposed to be adjacent to the cilia are taken into account. If this calcium store, after discharge, sequesters the initial calcium wave emanating from the thapsigargin-sensitive store, the rate of calcium rise near the cilia will be attenuated resulting in the prolonged second stage of the ciliary excitation. A similar 'barrier' in the pathway of calcium has been suggested in smooth muscle cells (van Breeman, 1977). In several ciliary systems, including the tracheas of several mammals, a ring of membrane-associated organelles at the base of each cilium has been observed by electron microscopy and labelled the 'ciliary necklace' (Gilula & Satir, 1972). This ciliary necklace possesses negatively charged residues associated with calcium binding sites, and has therefore been hypothesized to take part in the mechanism controlling ciliary beat by calcium ions, though no conclusive evidence has been presented. An attractive possibility is, therefore, that similar specialized structures form the calcium source responsible for the initial ciliary stimulation in rabbit trachea.

CBF activation by calcium influx

Although the maximal rises in $[Ca^{2+}]_i$ and CBF were not affected by drastically decreasing the calcium gradient across the plasma membrane, the $[Ca^{2+}]_i$-elevated plateau (Figs 5*A* and 6*A*), and the sustained activation of the cilia (Fig. 6*B*) were eliminated (Figs 5*B*, 6*D* and *E* and Table 1). Moreover the rate of $[Ca^{2+}]_i$ decay was over twofold faster at low $[Ca^{2+}]_o$ (Fig. 5*B* and Table 1) than at normal $[Ca^{2+}]_o$ (Fig. 5*A* and Table 1). The sustained CBF, elevated $[Ca^{2+}]_i$, and the rate of $[Ca^{2+}]_i$ decay displayed a similar dependence on the concentration of ATP in the solution (Table 1). Both the $[Ca^{2+}]_i$ and the CBF decayed back to their initial values when the ATP was removed. Finally, increasing the $[Ca^{2+}]_o$ back to normal values in the presence of ATP in the solution caused a rise in $[Ca^{2+}]_i$ to an elevated plateau and an increase in CBF (Fig. 6*D* and *F*). This strongly indicates that a calcium influx may be required for prolonged stimulation of the cilia.

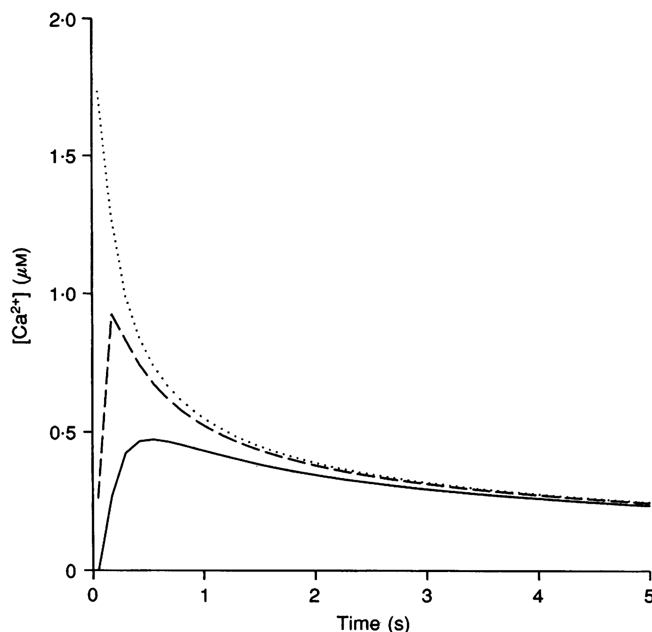

Figure 7. Calculated calcium transients at several locations along the cilia

The time course of calcium diffusion discharged from a point source containing 10 μM calcium positioned 0·1 μm below the base of the cilia. The diffusion coefficient of calcium was taken to be 20 $\mu m^2 s^{-1}$. The calcium concentration is displayed at the base of the cilium (dotted line), at 2·5 μm along the cilium (dashed line), and at the tip of the cilium which was set at 5 μm (continuous line).

The elevated plateau of calcium and the sustained ciliary activation were insensitive to the voltage-gated calcium channel blockers verapamil and diltiazem (Table 1). Therefore, it is likely that calcium enters the cell via receptor-operated calcium channels (Benham & Tsien, 1987) or through second messenger-operated channels (Lückhoff & Clapham, 1992; Randrimampita & Tsien, 1993). It has been hypothesized that calcium influx causes a rise in the calcium concentration in local domains near the plasma membrane, facilitating the activation of membrane-associated proteins in those domains without flooding the entire cell with calcium ions for a prolonged period of time (Rasmussen & Rasmussen, 1990). Therefore, the ciliary axoneme, being proximal to the plasma membrane, would be subjected to a higher calcium concentration than the average $[Ca^{2+}]_i$ measured by fura-2. Calcium influx was shown to mediate prolonged cellular responses in many systems such as aldosterone secretion and smooth muscle contraction (Rasmussen & Rasmussen, 1990). However, to the best of our knowledge, this is the first time that calcium influx has been suggested to participate in prolonged ciliary activation.

In summary, we propose that the biphasic rise in CBF, following stimulation with extracellular ATP, is due to the release of calcium from two distinct calcium stores – a small thapsigargin-insensitive store situated in close proximity to the cilia and a larger thapsigargin-sensitive store within the cell cytoplasm. The initial rapid rise in CBF is attributed to calcium release from the proximal calcium source, while calcium emanating from the cytoplasmic store is responsible for the rise in CBF to its maximal level. After the cilia have reached their maximal beating rate, the system is maintained at a high level of activation by an influx of calcium. This proposed sequence of events is by nature highly simplified as it does not account for the biochemical events leading from the rise in the calcium concentration in the vicinity of the cilia to the increase in the CBF. However, the suggestion that multiple calcium sources regulate CBF is in line with the properties required from the mucociliary clearance system. The cilia should respond both rapidly and for prolonged periods of time in order to transport mucus efficiently, thus clearing the airways. Our proposal thus accounts for the rapid initiation and prolonged propagation of the ciliary activity and provides a specific physiological role for pharmacologicaly distinct intracellular calcium stores.

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