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Date Nov. 26, 1986

CHARACTERIZATION OF THE CALCIUM-INDUCED INCREASE IN CILIARY FREQUEUNCY IN OUTGROWTHS OF RABBIT TRACHEAL EPITHELIUM

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Scott L. Bartusch

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I wish to thank Susan Austin for her continuous encouragement in completing this manuscript.

ABSTRACT

Studies show that ciliary beat frequency is regulated by the intracellular calcium concentration. To further study the calcium-induced frequency increase of individual cells, ciliated cells were exposed to various concentrations of extracellular calcium in the presence of calcium ionophore A23187, and the ciliary frequencies were then monitored continuously for up to 15 minutes. This method allowed for characterization of the calcium-induced ciliary increase in each cell over time.

Ciliated cells of rabbit tracheas were incubated for 30 minutes in calcium concentrations of 0.88 mM, 1.6 mM, 2.6 mM or 5.0mM. When calcium ionophore A23187 at 1×10^{-6} M was added, ciliary frequencies of individual cells began to increase, usually reaching a maximum frequency 10 minutes after addition of ionophore. After reaching their maximum frequencies, the frequencies began to decline. At 2.6 mM calcium, the calcium-induced frequency increase appeared to be related to the baseline frequency of individual cells. There was also evidence that the maximum frequency attained was directly related to the extracellular calcium concentration up to 2.6 mM calcium. The average calcium-induced frequency increase was greatest at 2.6 mM calcium, producing an average increase of 7.3 Hz. The average frequency increases at

0.88 mM and 1.6 mM were 5.6 Hz and 5.7 Hz respectively, and the average increase at 5.0 mM calcium was 4.6 Hz.

The results of this series of experiments can be used to further characterize normal ciliary activity. In 88% of the cells tested at 2.6 mM calcium, the maximum calcium-induced frequency was as high or higher than the frequency shift seen in the control recording of these cells. Only 7% of the cells with 0.88 mM calcium in the media reached maximum frequencies as high as the peak frequencies seen in their control recordings. A possible mechanism for this increase is suggested.

The results can also be used to describe metachronal waveforms and ciliary coordination between adjacent cells. Adjacent cells producing a metachronal wave appeared to maintain their coordination throughout the calcium-induced increase in ciliary frequency at all four of the calcium concentrations as observed by the swift, directed movement of particles in the media. The data shows that cells producing the wave were beating at different frequencies. Apparently mechanical coordination of adjacent cilia is not the only force determining ciliary frequency between adjacent cells. In the presence of A23187, several coordinated cells reached the same maximum calcium-induced frequency regardless of their baseline frequencies. This resulted in maintenance of the metachronal wave during the calcium-induced increase.

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I. INTRODUCTION

Cystic fibrosis is a lethal genetic disease which is characterized by poor mucociliary clearance. The stagnation of mucous has been partly attributed to a problem in the chloride secretion mechanisms in the tracheal epithelium of patients with cystic fibrosis (Welsh and Liedtke, 1986). Calcium absorption has been linked to chloride secretion and ciliary frequency modulation in normal tracheal epithelium (Smith, et al., 1982).

Calcium actively regulates ciliary function, yet the effects of calcium vary with the type of cell or tissue. Calcium produces ciliary inactivation in mussel gill lateral cilia, yet it produces activation of mussel gill abfrontal cilia (Stommel, 1984). Calcium induces reversal of ciliary beat direction in Paramecium (Naitoh and Kaneko, 1973). Increases in ciliary frequency in salamander oviduct (Eckert and Murakami, 1972), rabbit oviduct (Lee, et al., 1976), dog trachea (Kakuta, et al., 1985) and rabbit trachea (Girard and Kennedy, 1986) have all been linked to increases in cellular calcium levels.

The effects of calcium are well-documented, but the source of these changes in ciliary activity is unknown. Most of the theories dealing with ciliary frequency increases assert the role of calmodulin as an intermediate

that is some how affected by changes in calcium concentrations. Calcium binds to calmodulin and produces structural changes that may lead to an increase in ciliary frequency. Blum, et al., (1980) purified a dynein ATPase from cilia of Tetrahymena whose activity increased 10 fold in the presence of calmodulin and calcium versus calcium alone. Gordon, et al., (1982) suggested that the role of calmodulin is multifunctional, potentially controlling calcium channels and intracellular calcium concentrations which regulate the activation of dynein ATPase.

While the cellular concentration of free calcium appears to be 1×10^{-7} (Campbell, 1983), studies by Murakami and Eckert (1972) and Kakuta, et al., (1985) demonstrated that increases in ciliary frequency were related to increases in extracellular calcium at micromolar levels. Schultz, et al., (1986) found increasing cGMP levels with increasing extracellular calcium in micromolar amounts in Paramecium. He suggests that perhaps a sophisticated control loop involving calcium and cyclic nucleotides, as seen in other cells, is present in modulating ciliary activity. Girard and Kennedy (1986), using 1×10^{-5} M calcium ionophore A23187 and calcium concentrations of 0.8 mM, 2.75 mM, 5.4 mM and 12.5 mM, plotted the average frequency of 10 cells at various points in time for 35 minutes. They found that calcium concentrations in the range of 0.8 mM to 12.5 mM

produced ciliary frequency increases when calcium ionophore was present.

A recent study by Austin (1986, unpublished data) has described patterns of normal ciliary activity of individual cells as a function of time in rabbit tracheal outgrowths. Normal activity includes rapid, periodic increases in ciliary frequency (frequency shifts) which quickly subside, returning to the original frequency. These previously undescribed shifts in frequency were detected due to the sensitivity of the frequency acquistion system developed by Kennedy and Duckett (1981). The system allows for second by second updates of the frequency behavior of cilia. This same system can be applied in describing the frequency pattern in ciliated cells exposed to calcium and ionophore A23187.

In this study, we attempt to determine the effects of calcium on the frequency patterns of individual ciliated cells. This study is an extension of the studies of Girard and Kennedy (1986) and Austin (1986) in that the pattern of frequency increase of cilia of individual cells was followed over time. This study attempts to characterize the ciliary frequency increases induced by calcium ionophore A23187 at various calcium concentrations (0.8 mM, 1.6 mM, 2.6 mM and 5.0 mM) and compare these increases with the normal pattern of ciliary beat frequency. The pattern and rate of increase was compared

with the baseline frequency of the same cell obtained before calcium was added to the media. Characterizing the increase provides some information concerning the mechanisms which regulate the calcium-induced increase in frequency. It is also possible that the mechanisms behind frequency increases in normal ciliary patterns (frequency shifts) are similar to those producing the calcium-induced increases seen using calcium and calcium ionophore.

Studies (Tamm, 1973; Sanderson and Sleigh, 1981) have concluded that ciliary coordination between adjacent cells is due to mechanical forces of adjacent cilia. It has been suggested that coordinated activity originates from a single cilium which stimulates adjacent cilia due to the direct contact of each cell's cilia (Sanderson and Sleigh, 1981). This close coupling from direct contact propogates a coordinated wave. This study will examine the degree of frequency coordination between adjacent cells to determine if coordination is maintained during a calcium-induced increase in ciliary frequency. It is desirable to know if coordinated cells reach the same calcium-induced frequency, and if they retain their coordinated activity while increasing ciliary frequency.

II. MATERIALS AND METHODS

A. Tissue Preparation

Tracheas were obtained from rabbits of various breed and of both sexes. The rabbits were decapitated and the tracheas were obtained after exsanguination of the animal. The tracheas, extending from the cricoid cartilage to the bifurcation of the lungs, were cut into four longitudinal strips, and the epithelium was removed from the underlying connective tissue by microsurgical scissors. The epithelium was then cut into small pieces and incubated in culture dishes with Waymouth's MB 752/1 tissue culture medium, containing 0.8 mM calcium, with 10% Nu serum and antibiotics in a 95% air/5% CO_2 atmosphere. The tissue was in culture within 2 hours after slaughter. The culture medium was replaced every 2 days, and the cultures could be maintained for four weeks. As the tissue grows, cells migrate from the main tissue to form clumps of ciliated cells (outgrowths). Ciliary frequencies were obtained from ciliated outgrowths of the explants (Kennedy and Ranyard, 1983).

B. Frequency Quantitation

Ciliary frequencies were obtained using modifications of the method of Kennedy and Duckett (1981) as described by Duckett, et al., (1986). Culture dishes were placed on

the stage of a Nikon Diaphot-TMD inverted phase contrast microscope and maintained at 37°C with an air stream incubator. A field of ciliated cells was projected onto a television monitor and at the same time recorded on video tape by a video recorder. Ciliary frequencies were measured from the television screen using an Oriel photomultiplier detection system (Model 7070) and Fast Fourier transform analysis.

As a beating cilium passed in front of the aperature of the photomultiplier, changes in the amount of light received produced a signal from which a fundamental frequency was extracted by Fast Fourier analysis with a Hewlett-Packard spectrum analyzer (Model No. 3582A). The initial spectrum was an average of 10 seconds of data, and the frequency signal was updated every half second. The frequency spectrum consisted of spikes over a range of frequencies with the height of the spike proportional to the time cilia spent at that frequency.

C. Computer Acquisition of Frequency Data

Frequency signals from ciliated cells obtained as described above were stored on floppy discs using a DEC LSI/11-03 minicomputer. The computer program copied the Fourier transforms from the display buffer of the spectrum analyser via an IEEE buss and recorded them on floppy disk. Every other signal from the spectrum analyser was

transferred consecutively so as to give an accurate, second by second, description of the fundamental frequency over time.

The length of time for acquiring the frequencies was limited by the storage space on the disk. A disk can hold up to 487 displays, and the rate of signal transfer for one transform is approximately 1.1 seconds; therefore, one disk can hold 8.9 minutes of frequency data. Frequencies were usually followed for 2 minutes during the control period and up to 16 minutes during the experimental period. Acquisition of data for the longer time intervals was accomplished by stopping the video tape when the first floppy disk was full and then restarting the video tape when a new disk was loaded and the program for acquiring data was reset.

The fundamental frequency of each transform appears as a spike with the height proportional to the frequency at which the cilium was beating. Once frequency transforms were acquired with the computer, a histogram (Fig. 1) of frequency versus time could be produced. This allows one to observe the ciliary beat frequency of a cell over an extended period of time. In this way changes in the frequency pattern can be analyzed.

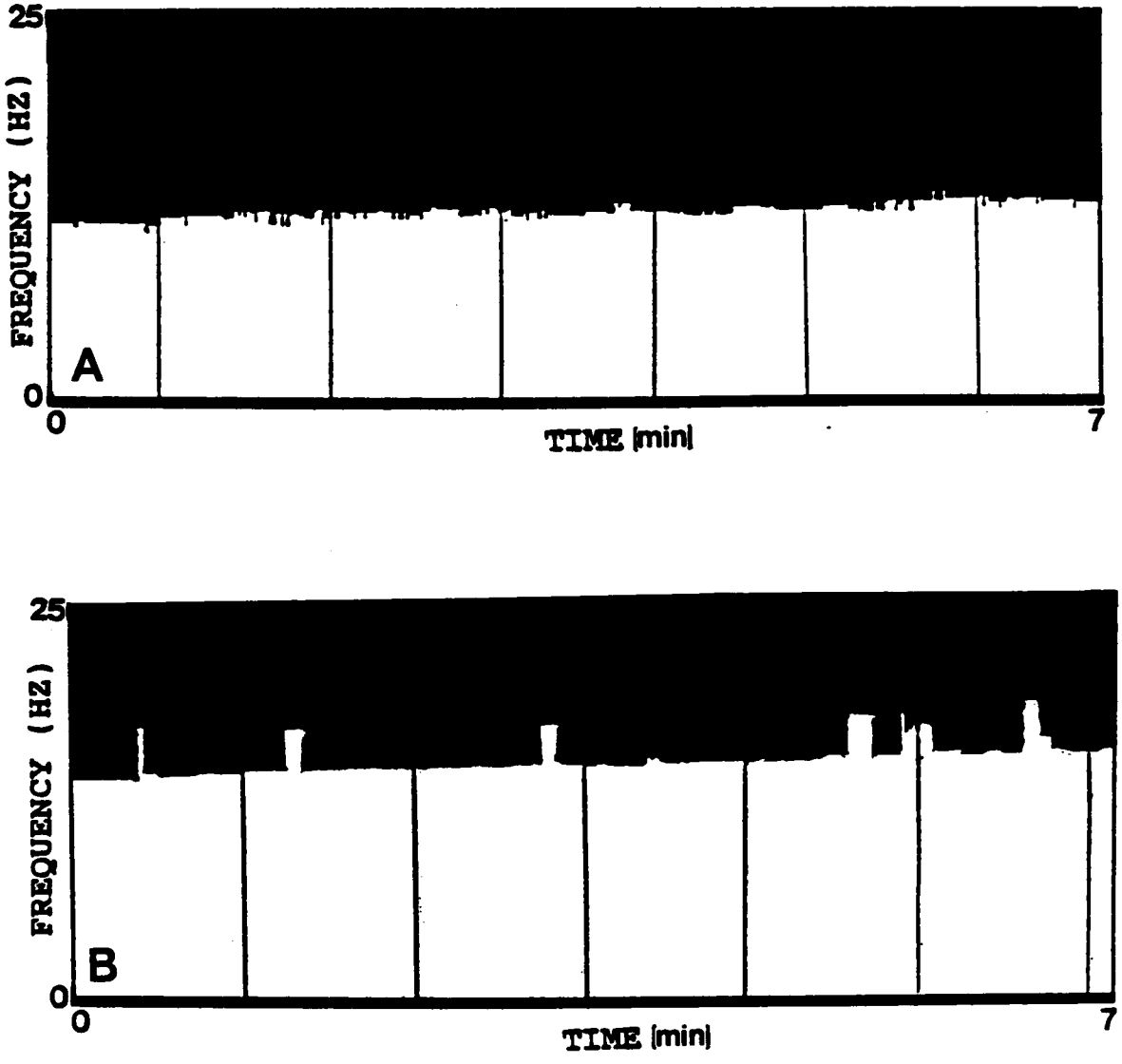


Fig. 1. Frequency histograms of two cells. A) exhibits a frequency pattern with no frequency shifts. B) exhibits a frequency pattern punctuated by frequency shifts.

D. Exposure to Ionophore

To test the effect of increasing calcium concentrations on the pattern of ciliary beat frequency for individual cells, cultures were exposed to media containing calcium concentrations of 0.88 mM, 1.6 mM, 2.6 mM and 5.0 mM. First, a 2 minute recording of the baseline frequency of a cell was obtained using the detection method described above. Cells were then exposed to the various calcium concentrations and calcium ionophore A23187, and ciliary frequencies were again recorded. This was done by removing growth medium plus calcium and replacing it with new medium containing the desired calcium and ionophore concentrations. The new medium was equilibrated for temperature and pCO_2 of the growth medium before addition to the culture dish.

It was desirable to use the lowest ionophore concentration that still produced a ciliary increase. A previous study used 1×10^{-5} M ionophore (Girard and Kennedy, 1986). Experiments using 1×10^{-7} M failed to produce increases in frequency, but 1×10^{-6} M did yield good results. Therefore, the ionophore concentration for this set of experiments was set at 1×10^{-6} M.

Whenever there was a change in medium, there was always a large drop in ciliary frequency of 5-7 Hz. Controls from which medium was removed and immediately returned to the culture dish also showed these drops in

frequency. In an attempt to eliminate this artifact and verify the findings of the first series of experiments, the following method was employed: Ciliated outgrowths were incubated for 5 minutes in medium at the desired calcium concentration. A mixture of medium and ionophore at 10 times the desired ionophore concentration was equilibrated for temperature and pCO_2 for 1 hour. A 2 minute recording of the baseline frequency was made. Then appropriate volumes of the ionophore and medium mixture were added by drop to the culture medium. To obtain the final concentration, volumes of 0.1 mls or 0.2 mls of medium plus ionophore at 10 times the desired concentration were added to culture dishes holding 0.9 mls or 1.8 mls of media respectively. It took no more than 30 seconds to add the solution by drop. This method eliminated the drop in frequency in 42% (n=59) of the cells tested. This form of addition reduces the physical agitation presumably responsible for the drop in frequency. The frequency drop in the remaining 58% of the cells examined was reduced to 2-3 Hz on average. The final frequencies obtained 10 minutes after addition of ionophore, however, were comparable using both methods.

III. RESULTS

A. Effect of Medium Change on Ciliary Frequency

As stated above, whenever medium was removed and replaced with fresh medium with calcium ionophore A23187 and calcium, there was a large drop in ciliary frequency of 5-7 Hz which was uncharacteristic of normal ciliary patterns (Fig. 1). To determine if the drop in frequency was related to either the A23187 or the concentration of calcium, recordings of ciliary frequency were made after removing the medium and replacing it with fresh medium with varying combinations of calcium and/or A23187. Equivalent drops in frequency were obtained when growth medium was replaced with new growth medium alone, with medium containing A23187, and with medium containing 0.88 mM, 1.6 mM, 2.6 mM or 5.0 mM calcium. This led to the conclusion that the drop in frequency was in some way due to the physical agitation or disruption of the cells environment, and not in any way connected to the contents of the medium added.

When a small volume (0.2 mls) of medium plus calcium ionophore A23187, at a concentration necessary to produce a final ionophore concentration of 1×10^{-6} M, was added by drop to media containing the appropriate concentration of calcium, 42% (n=59) of the cells tested showed no decrease in frequency. In these cases, the ciliated cells

continued their normal beating pattern for 30-60 seconds after A23187 introduction. By 90 seconds all of the cells showed an increase in frequency above the baseline. The pattern of increase was similar to the pattern of increase in those cells in which ionophore was added by complete media change.

When A23187 was added by drop, 58% (n=59) of the cells showed decreases in baseline frequency. In these cells the frequency dropped by an average of 2-3 Hz. The ciliary frequency would then begin to rise in a manner similar to those cells subjected to complete media change. Thus it appears that the drop in frequency was related to physical agitation of the cells' environment. In using the drop method, a smaller volume (0.2 mls) was added to the culture dishes in 30 seconds, and a smaller disruption was produced in the cells' environment thus producing a smaller change in the ciliary pattern. We also concluded that after the drop in frequency, the resulting pattern of ciliary frequency increase was similar using either method of ionophore addition. However, the time required to return to baseline was by 3-5 minutes when complete media change was employed.

B. Effects of Various Calcium Concentrations on the Pattern of Frequency Shift

To determine the effects of increasing calcium concentrations on the frequency shift pattern of normal cells, the calcium-induced frequency pattern was compared with the normal frequency pattern of each cell. Austin (1986, unpublished data) recently described extended time analysis (over 8 minutes of continuous recording) of ciliary activity in rabbit tracheal cells. Austin found that ciliated cells maintained a certain baseline frequency. Cilia would show small increases or decreases in frequency around the baseline, but most of the time was spent at this baseline frequency; moreover, baseline frequencies varied between cells. The normal ciliary pattern also exhibited rapid, large, instantaneous increases in ciliary frequency (frequency shifts) which quickly returned to baseline (Fig. 1B, page 17). These frequency shifts occured at random intervals and reached the same peak frequency each time (Austin, 1986, unpublished data).

Girard and Kennedy found that extracellular calcium did not alter normal ciliary patterns. Thus, a normal frequency pattern could be obtained from cells in medium with added calcium. This frequency pattern could then be compared to the pattern obtained after adding ionophore.

In order to establish whether A23187-stimulated cells maintained the frequency shift pattern during calcium-induced ciliary increase, ciliary frequencies of 117 cells exposed to one of the four calcium concentrations were followed from A23187 addition until they reached a maximum frequency (Fig. 2). Maximum frequencies were reached within 10-13 minutes after A23187 addition. Once maximum frequencies were attained, they could be maintained for 10-20 minutes but never exceeded this 10-13 minute level. The calcium concentrations employed were 0.88 mM, 1.6 mM, 2.6 mM and 5.0 mM.

Fig. 2 shows represenative patterns of increases at each calcium concentration. The first portion of each histogram represents 2 minutes of untreated control (baseline) frequency. The period after the gap (inserted to delineate between the two time periods) represents the change in frequency after addition of 10^{-6} A23187. Fig. 2A shows the pattern of frequency increase at 0.88 mM calcium. For this cell the baseline frequency was 13 Hz. Upon addition of ionophore, the ciliary frequency rose to 16 Hz in 110 seconds. The frequency appeared to increase in a graded fashion, reaching maximum increase by 4 minutes, and sustaining that frequency for the remaining 6 minutes of recording. Fig. 2B shows a cell's pattern of increase at 1.6 mM calcium. Again the pattern of frequency increase was slow and steady starting from a

Fig. 2. Representative frequency histograms of each of the four calcium concentrations used. A) 0.88 mM, B) 1.6 mM, C) 2.6 mM and D) 5.0 mM. The first part of each histogram is a 2 minute control recording of the baseline frequency pattern of the cell. The second part of each histogram (after the gap) represents the calcium-induced increase in frequency for 7 minutes after the frequency returns to baseline.

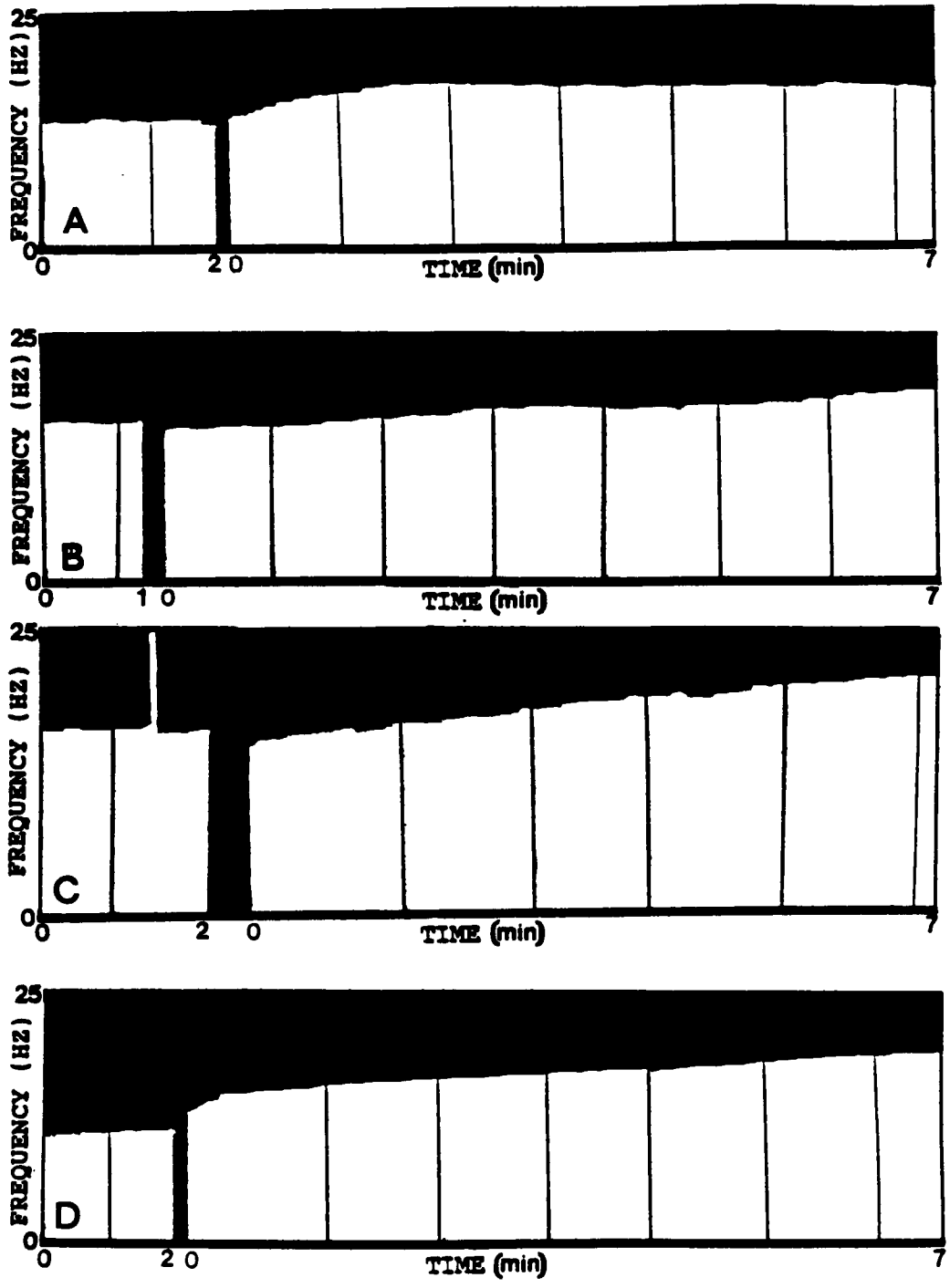

25
FREQUENCY (HZ)
0
A
0
20
TIME (min)
7
25
FREQUENCY (HZ)
0
B
0
10
TIME (min)
7
25
FREQUENCY (HZ)
0
C
0
20
TIME (min)
7
25
FREQUENCY (HZ)
0
D
0
20
TIME (min)
7

Fig. 2.

baseline frequency of 15 Hz and reaching a maximum frequency of 19 Hz with few frequency decreases. The histogram in Fig. 2C shows the frequency pattern of a cell exposed to 2.6 mM calcium. The baseline frequency was 16 Hz showing a frequency shift that reached a peak frequency of 24 Hz. The maximum frequency of 22 Hz was reached after 7.5 minutes. Fig 2D shows the pattern of frequency increase at 5.0 mM calcium. The baseline frequency was 11 Hz, but it reached a frequency of 19 Hz upon addition of A23187. Again the increase was smooth and consistent showing a slow progression in ciliary frequency increase.

As compared with the normal ciliary pattern seen in Figs. 1B (page 17), the calcium-induced patterns showed no transitory shifts in their ciliary beat frequencies. There were also no decreases in ciliary frequency after initiation of the frequency increase. The subsequent frequency increase was achieved in a graded fashion. These results suggest that a slow, steady increase in intracellular calcium produced a slow, steady increase in ciliary frequency. The frequency shifts observed in untreated cells may be due to rapid increases in cellular calcium levels followed by a rapid decrease either through storage or elimination of cellular calcium.

In order to determine whether increasing calcium concentrations resulted in the attainment of progressively higher frequency levels, the average frequency increase

produced at each calcium concentration was calculated. Fig. 3 is a plot of the relationship between the calcium concentration in the media and the maximum increase in frequency above the baseline. The data shows that 2.6 mM calcium produced the greatest average increase (7.3 Hz) in ciliary frequency above the baseline frequency. There was little difference between the increases produced by 0.88 mM and 1.6 mM calcium, and 5.0 mM calcium showed the smallest increase at an average of 4.6 Hz above baseline. The figures plotted (Table 1) were obtained by averaging the difference of the maximum frequency reached and the baseline frequency at each concentration.

As seen in Table 1, the standard error at each concentration was large, suggesting no real difference in the average frequency increases. An analysis of variance showed that a difference does exist ($p > 0.05$) in the averages of the calcium-induced changes in frequency from 0.88 - 5.0 mM; however, t-tests only showed a statistical difference between the average values at 2.6 mM and 5.0 mM calcium. This finding was questionable due to precipitate (probably calcium) in media with 5.0 mM calcium. Since there was precipitation, we were unsure as to how much calcium was still in solution in the media. The average frequency increase at 5.0 mM might also be skewed by the deleterious effects of having such an excess of calcium in the tissue media. Thus, it appears that the differences

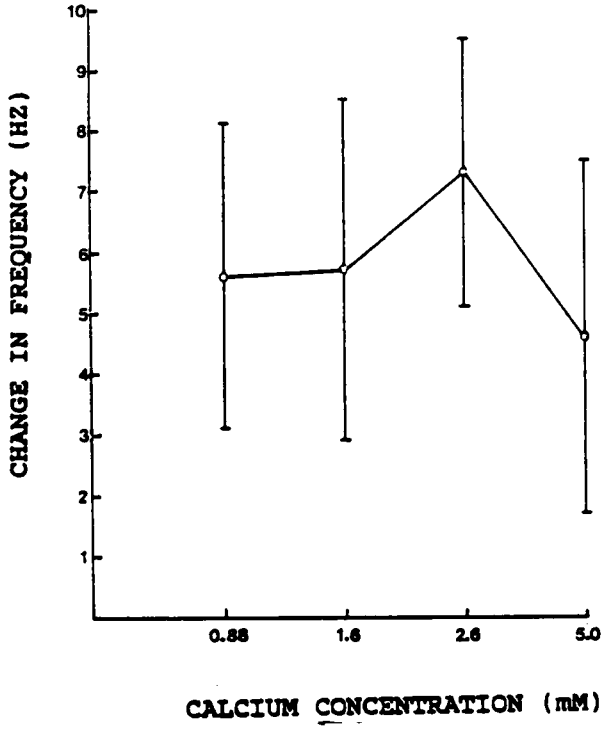


Fig. 3. Plot of the average frequency increase +/- standard error versus the calcium concentration of the media. The data was obtained from Table 1.

Table 1. Average frequency increases of all the cells tested at each calcium concentration. Also included are the standard error and size of each sample.

Calcium Concentration	Average Frequency Increase	+ - Standard Error	Sample Size
0.88 mM	5.6 Hz	2.5 Hz	37
1.6 mM	5.7 Hz	2.8 Hz	29
2.6 mM	7.3 Hz	2.2 Hz	28
5.0 mM	4.6 Hz	2.9 Hz	23

in the average frequency increases were not statistically real and no correlation between calcium concentration and induced frequency increase could be made. To find significance in the average calcium-induced frequency increase, the data had to be viewed from a different perspective.

In view of the large standard error for each of the means in Fig. 3, the baseline frequency of each cell was examined with respect to change in frequency to determine whether baseline frequency influenced the amount of increase a cell could achieve. Figs. 4-7 are bar graphs of the calcium-induced increases at each calcium concentration. On the abscissa is plotted the increase in ciliary frequency. Plotted on the ordinate is the number of cells reaching those increases. The numbers in the graph show the baseline frequency values for each cell.

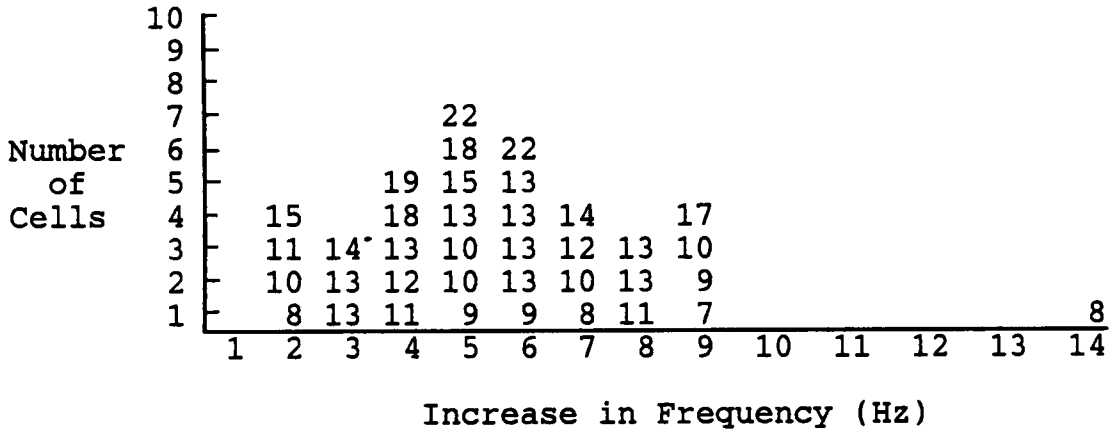
Figs. 4-7 show that the baseline frequency is not a factor in determining the maximum increase produced at calcium concentrations of 0.88 mM and 1.6 mM. At 0.88 mM calcium (Fig. 4), three cells with baseline frequencies of 8 Hz showed increases of 2, 7 and 13 Hz. Five cells with baseline frequencies of 13 Hz showed increases of 3 Hz, 4 Hz, 5 Hz, 6 Hz and 8 Hz. At 1.6 mM calcium, cells with baseline frequencies of 10 Hz showed increases of 1-2 Hz to as high as 12-14 Hz with substantial baseline variation between these values (Fig. 5). On the other hand, at 2.6

Fig 4. Bar graph of the range of increases at 0.88 mM calcium. Each point on the graph is the baseline frequency of a cell.

Fig. 5. Bar graph of the range of increases at 1.6 mM calcium. Each point on the graph is the baseline frequency of a cell.

Fig. 6. Bar graph of the range of increases at 2.6 mM calcium. Each point on the graph is the baseline frequency of a cell.

Fig. 7. Bar graph of the range of increases at 5.0 mM calcium. Each point on the graph is the baseline frequency of a cell.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8
7
6
5
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3
2
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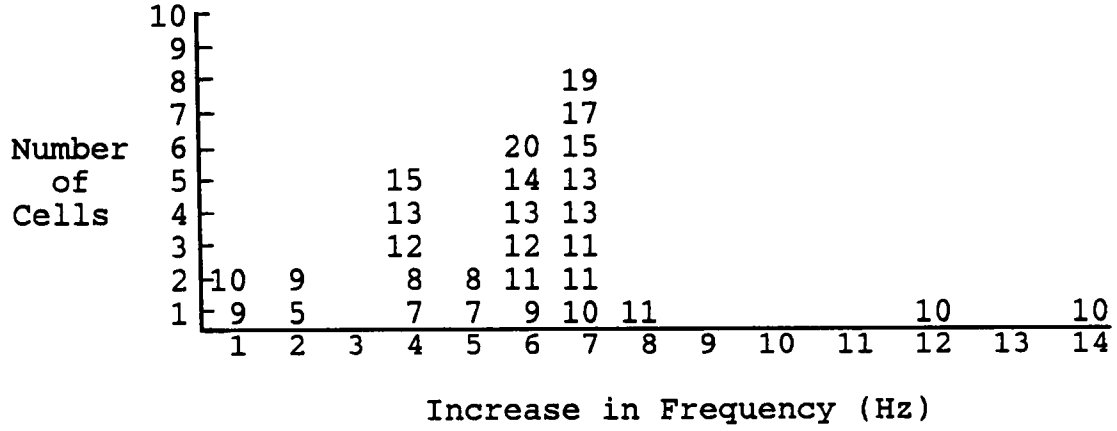
18 22
19 15 13
15 18 13 13 14 17
11 14 13 10 13 12 13 10
10 13 12 10 13 10 13 9
8 13 11 9 9 8 11 7 8
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Increase in Frequency (Hz)

Fig. 4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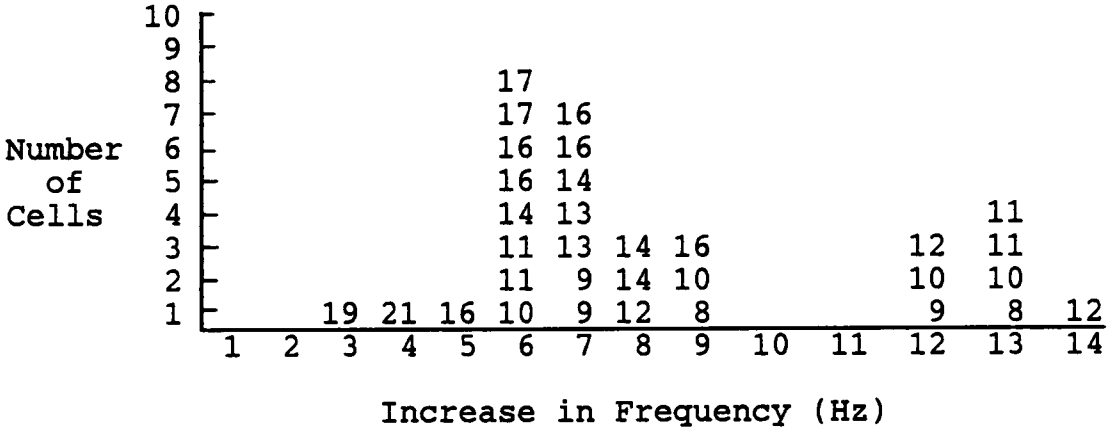
12 12 11
10 9 8 8 11 11
9 5 7 7 9 10 11 10 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Increase in Frequency (Hz)

Fig. 5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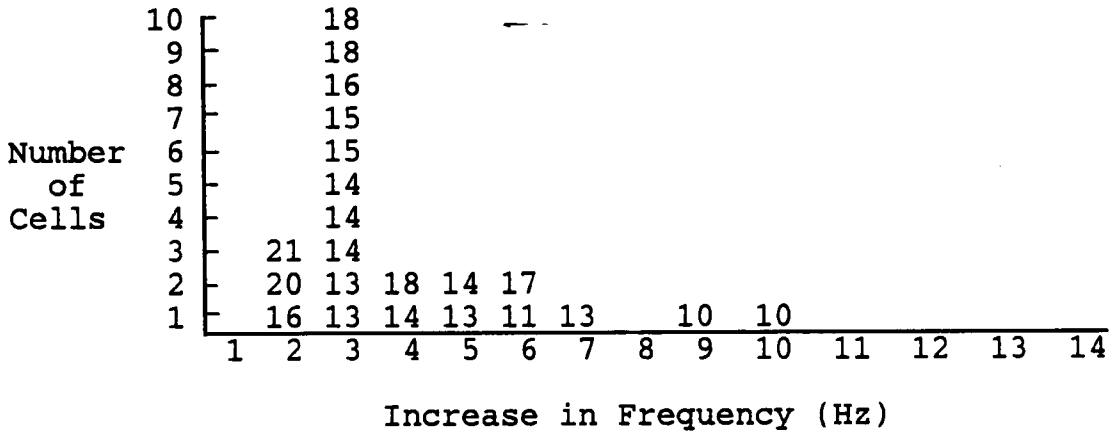
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Increase in Frequency (Hz)

Fig. 6


Number of Cells
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21 14
20 13 18 14 17
16 13 14 13 11 13 10 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Increase in Frequency (Hz)

Fig. 7

mM calcium (Fig. 6), cells with the highest baseline frequencies showed the smallest increase in frequency. Those with the lowest baseline showed the greatest increases in frequency. It is evident, however, that overlap does occur. At 5.0 mM (Fig. 7) all cells analyzed were at or above 10 Hz and very little increase in frequency was attained.

Thus it appears that there is little relationship between baseline frequency and the level of resulting frequency increase at either 0.88 or 1.6 mM calcium. However, at 2.6 mM calcium, cells with lower baselines may be capable of reaching higher frequencies. Whether this pattern persists at 5.0 mM calcium is unclear in part because of the small number of cells with baselines at lower frequencies and in part because the majority of cells showed little (2-3 Hz) increase at this concentration.

Figures 4-7 also highlight the frequency increases that appear most often at each calcium concentration. The data shows an uneven distribution of increases at each calcium concentration. For instance, at 0.88 mM calcium a disproportionate number of the cells tested (18 of 37) exhibited increases between 4-6 Hz. At 1.6 mM and 2.6 mM calcium, most (29 of 57) of the cells were at the 6-7 Hz range. While at 5.0 mM, the majority of the cells (13 of 23) showed increases in the 2-3 Hz range. This data

suggests some correlation between calcium concentration and the resulting frequency increase.

The relative importance of the baseline frequency was realized when a comparison was made of the average calcium-induced increase as seen as a percentage of the baseline. Table 2 shows that cells with lower baseline frequencies were able to increase proportionately higher than cells with higher baseline frequencies at each calcium concentration. For instance, at 0.88 mM calcium, cells with a baseline frequency of 7 Hz showed an average increase of 129% of their baselines. Cells at a baseline frequency of 22 Hz exhibited an average increase of 25% of their baselines. Perhaps the effects of calcium are hidden due to restraints placed on the cell by its baseline frequency.

Austin (1986, unpublished data) described two types of frequency behaviour in rabbit tracheal ciliated cells. The first frequency pattern was that of a ciliated cell whose frequency was maintained with little variance for over 8 minutes. The cell maintained a baseline frequency with no frequency shifts and produced a histogram similar to that in Fig. 1A (page 17). The second pattern of behaviour exhibited a baseline frequency punctuated with frequency shifts. In some cases, these frequency shifts did not return back to the original baseline frequency but returned to establish a higher baseline frequency. Austin

Table 2. Table of average calcium-induced increases for all the cells at one baseline frequency as a percentage of the baseline frequency.

	Calcium-induced Increase Relative to the Baseline												
0.88 mM													
Baseline	7	8	9	10	11	12	13	14	15	17	18	19	22 Hz
Average Increase as a % of the baseline	129	91	74	56	42	44	42	35	23	53	25	21	25 %
1.6 mM													
Baseline	5	7	8	9	10	11	12	13	14	15	17	19	20 Hz
Average Increase as a % of the baseline	40	64	62	33	40	61	68	46	92	37	41	37	30 %
2.6 mM													
Baseline	8	9	10	11	12	13	14	16	17	19	21 Hz		
Average Increase as a % of the baseline	138	96	100	87	95	54	52	42	35	16	19 %		
5.0 mM													
Baseline	10	11	13	14	15	16	17	18	20	21 Hz			
Average Increase as a % of the baseline	95	55	35	26	20	16	35	19	10	9 %			

suggested that all ciliated cells showed both patterns of behavior. To see if there was any relationship between baseline ciliary frequency pattern and the calcium-induced increase, the two populations of cells, those exhibiting frequency shifts and those that did not, were examined independently. The population exhibiting frequency shifts was studied to determine whether any relationship existed between the peak frequency shifts and maximum calcium-induced frequencies (Fig. 8), a comparison was made between the maximum frequency reached by a cell in each experiment and the peak frequency attained during the frequency shift in that cell's baseline recording (Table 3). The comparison showed an increase in the percentage of cells that had a maximum frequency equal to or greater than the peak frequency shift as the calcium concentration increased. At 0.88 mM calcium, only 7% of the cells exhibiting frequency shifts attained a maximum calcium-induced frequency as high as the peak frequency shift. The proportion rises to 73% at 1.6 mM calcium, and at 2.6 mM calcium 88% of the cells tested reached a maximum calcium-induced frequency as high as their peak frequency shift. The percentage falls to 64% at 5.0 mM. This drop at 5.0 mM could be due to some adverse effects of precipitate in the media or due to calcium toxicity. This data suggests some validity to a concentration

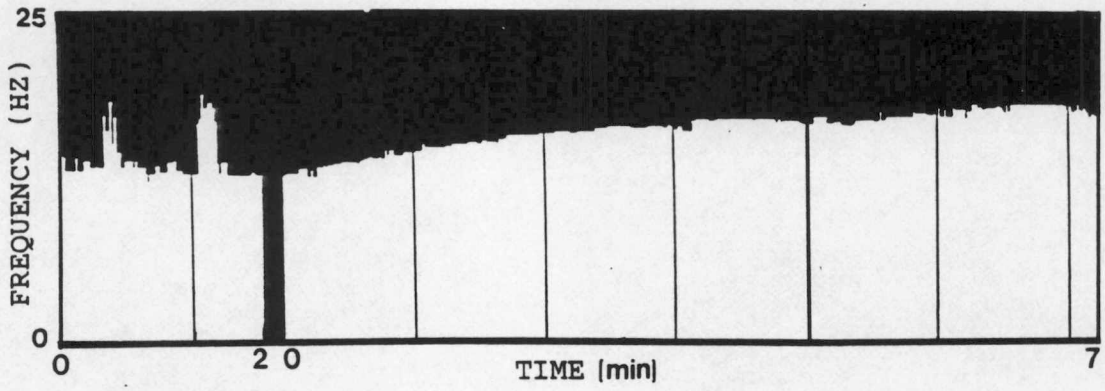

Fig. 8. Frequency histogram of a cell showing the maximum calcium-induced frequency equalling the peak frequency shift.

Table 3. Table of the percentage of cells whose maximum calcium-induced frequency was below or equal to and above their peak frequency shift. The size of the sample is included at each calcium concentration.

Calcium Concentration	Below Frequency Shift	Equal to or Above Frequency Shift
0.88 mM (n=15)	93%	7%
1.6 mM (n=12)	27%	73%
2.6 mM (n=17)	12%	88%
5.0 mM (n=14)	36%	64%

dependent calcium-induced increase in ciliary frequency (Fig.3).

Statistical analysis of the average frequency increase at each calcium concentration showed no statistical relationship between calcium concentration and calcium-induced frequency increase. Real differences in the average increases might be lost in the large variability in the baseline frequencies and the large variability in the frequency responses. Both of which produced a large standard error. A study comparing the response of cells at the same baseline to different calcium concentrations would eliminate part of this variability.

However, analysis of the cells exhibiting frequency shifts did suggest a positive correlation between the amount of calcium in the media, up to 2.6 mM, and the maximum calcium-induced frequency. While applying a specific frequency increase to a specific calcium concentration may be misleading, it appears that 2.6 mM calcium produces maximum frequency stimulation compared with the effects of 0.88 mM and 1.6 mM calcium. The effects of 5.0 mM are still questionable due to the uncertainty concerning how much calcium remained in the medium after precipitation.

As seen in Table 3, there was a relationship between the maximum frequency shift and the maximum

calcium-induced frequency attained. At 2.6 mM calcium, 88% of the cells that exhibited frequency shifts, reached a maximum frequency as high or higher than their peak frequency shift, compared with 73% at 1.6 mM calcium, 64% at 5.0 mM calcium and 7% at 0.88 mM calcium. Since increases in ciliary frequency may be due to increases in intracellular calcium, perhaps the frequency shifts of normal ciliary behavior are due to transient increases in intracellular calcium. The maximum inducible frequency may approximate the maximum of the normal frequency shift.

C. Rate of Increase

Frequency histograms provide good information regarding patterns of frequency increases in ciliated cells. The frequency histograms in Fig. 2 (page 22) show that patterns of increase vary between cells. The ciliary increase in Fig. 2D (page 22) showed a graded pattern of increase, while the frequency pattern of Fig. 2B (page 22) had an increase punctuated by quick drops in frequency. Because of this irregularity in the pattern of increase, it is difficult to assign one value as the slope. Observation of the ciliary frequency increase suggests that the rate of increase was a characteristic of each cell. It is apparent that this type of frequency increase is not characteristic of the normal pattern of ciliary activity.

Ciliary frequencies began to rise upon addition of ionophore A23187 to the culture media. Assuming that calcium is in some way responsible for the increase in frequency, then the rate of increase should be related to one, or possibly more of the following: a) the rate of calcium's entry into the cell, b) the rate of ATP production by a pathway that uses calcium as an intermediate, c) the rate of calcium release by intracellular pools.

The rate of frequency increase was calculated at each calcium concentration using the Least Squares Fit equation (Agresti and Agresti, 1979) for estimating the slope of the linear portion of the curve. Ciliary frequency values obtained immediately after ionophore addition by either the drop method or complete change in media were used for the zero time value. The time for the maximum ciliary frequency was determined by observing at what time after ionophore addition the ciliary frequency reached its maximum value. Using this method for estimating the rate of increase, the slope at 0.88 mM calcium was 0.59 Hz/min. (n=12). At 1.6 mM calcium the slope was 0.70 Hz/min. (n=10). At 2.6 mM and 5.0 mM calcium, the slopes were 0.89 Hz/min. (n=8) and 0.26 Hz/min. (n=10) respectively. These slopes appear to increase with increasing calcium concentration up to 2.6 mM. However, at 5.0 mM the slope decreases, possibly due to precipitation of calcium in the

culture media at this concentration. Thus in a pattern that parallels the average frequency increases at each concentration, the rates of increase peak at 2.6 mM.

D. Adjacent Cells

When functioning in the trachea, groups of ciliated cells will beat in a manner which produces a steady, directed stream of movement of the mucous layer. The movement of this mucous layer lying in contact with the tips of the cilia is thought to be achieved by coordinating the beat pattern of the cilia of several cells (Sanderson and Sleigh, 1981). Cilia of adjacent cells would beat at the same frequency, but the cilia of one cell would be just a half beat behind the cilia of the cell before it forming a metachronal wave. This slight staggering of ciliary beating would produce a waveform that could rapidly transport mucous and particles in a posterior-anterior direction (Sanderson and Sleigh, 1981).

To determine whether coordinated cells beat at the same frequency and maintained their coordination during calcium stimulation, groups of cells were studied. The cells chosen not only touched at their borders, but in most cases their cilia were also in contact. These patches of cells were best observed as the cilia of several cells produced a metachronal waveform that moved particles directionally. Ciliary frequencies of adjacent

cells were recorded in the same time frame for a 2 minute control period and then for 12 minutes after addition of A23187 plus either 2.6 or 5.0 mM calcium to the culture medium.

Area 1 (Fig. 9) represents a group of four cells in which the cilia overlapped in the beating pattern. The beat pattern of the four cells was coordinated, but it did not form a smooth flow of particle movement. It appeared that the cilia beat at the same time but in directions perpendicular to their the neighboring cells. The arrow indicates the predominant direction of particle movement. Table 4 shows the baseline frequencies of these cells which ranged from 10 Hz to 13 Hz. At 2.6 mM calcium, the average increase of the cells was 7.75 Hz . After 12 minutes three of the cells were beating at 20 Hz, and one was beating at 19 Hz. These cells maintained coordinated beating throughout the calcium-induced frequency increase; however, the ciliary frequencies did not increase simultaneously.

Area 2 (Fig. 10) represents another area of coordinated activity. Table 5 shows both the baseline frequencies of each of the seven cells which ranged from 11-19 Hz, and the maximum frequency attained upon addition of ionophore A23187. Upon addition of 2.6 mM calcium, five cells increased their ciliary frequencies by 6 Hz while one cell, with the highest baseline frequency of 19

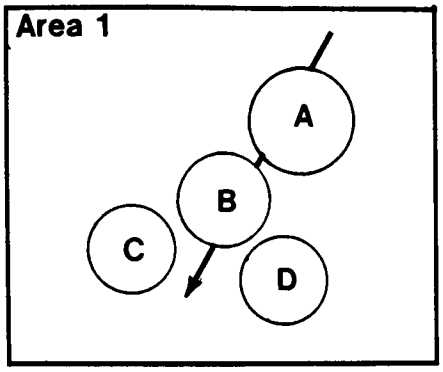

Fig. 9. Diagram of the cells recorded in Area 1. The arrow indicates the direction of particle movement.

Table 4. Shows the response of the coordinated cells in Area 1 to calcium ionophore and 2.6 mM calcium.

Cell	Baseline Frequency	Maximum Frequency	Change in Frequency
A	13 Hz	20 Hz	7 Hz
B	10 Hz	19 Hz	9 Hz
C	12 Hz	20 Hz	8 Hz
D	13 Hz	20 Hz	7 Hz

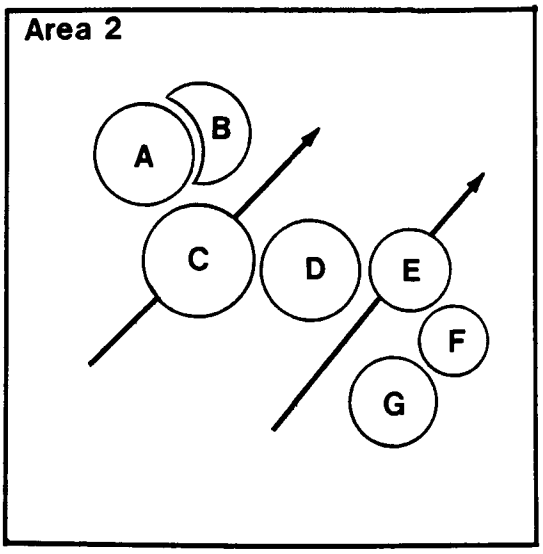

Fig. 10. Diagram of the cells recorded in Area 2. The arrows indicate the direction of particle movement.

Table 5. Shows the response of the coordinated cells in Area 2 to calcium ionophore and 2.6 mM calcium.

Cell	Baseline Frequency	Maximum Frequency	Change in Frequency
A	11 Hz	17 Hz	6 Hz
B	11 Hz	17 Hz	6 Hz
C	16 Hz	22 Hz	6 Hz
D	16 Hz	22 Hz	6 Hz
E	19 Hz	22 Hz	3 Hz
F	17 Hz	23 Hz	6 Hz
G	16 Hz	23 Hz	7 Hz

Hz, increased its frequency by 3 Hz. Cell G increased its frequency by 7 Hz. After the increases, three of the cells were beating at 22 Hz, two cells were at 23 Hz, and cells A and B, which were adjacent, were both beating at 17 Hz. The coordinated activity that was producing the metachronal wave was maintained throughout the increase in ciliary frequency. The direction of the wave was determined by following the path of small particles in the media that were carried by the wave.

If the direction of the wave were viewed as moving from bottom left to upper right as seen in Fig. 10, adjacent cells appear to exhibit some coordination in their ciliary frequencies. Cells A and B started at the same frequency and reached the same calcium-induced frequency. Cells C, D, and E exhibitied baselines of 16 Hz, 16 Hz, and 19 Hz respectively, and they all attained 22 Hz in 2.6 mM calcium. Cells F and G both reached a calcium-induced frequency of 23 Hz. It appears that cells A and B were coupled and cells C, D, and E were coupled in their frequency patterns. Cells F and G also showed coordination in reaching the same maximum frequency.

The results from these first two cell areas were suprising. As seen in Area 1, the four cells were beating at three different ciliary frequencies during the control period; however, the range of values was only 3 Hz. The cells in Area 2 had baseline frequency values from 11-19

Hz. These results showed that adjacent cells did not necessarily have to beat at the same frequency to maintain a metachronal wave. However, if cells F and G are seen as peripheral to the stream of flow, then cells C, D and E exhibit adjacent cell coordination in attaining the same frequency and carrying the wave. It is interesting to note that as one moves in a perpendicular direction from the wave, the cells show less coordination.

The rates of increase of the ciliary frequencies between some adjacent cells were similar. While cilia of adjacent cells may not have been beating at the same frequency, it appears that the changes in frequency between adjacent cells were related in some cases. For instance, Table 6 shows the frequency of each of the cells from Area 1 at 30 second intervals during the calcium-induced increase. Cells A and B appear to slightly staggered but synchronous in their frequency increases. Cells C and D, though, somewhat lateral to the direction of wave movement and somewhat higher in frequency than cell B, parallel the responses of cells B and A respectively. Nonetheless, all cells increased in a similar fashion and maintained a frequency which was not greater than 1 Hz difference to maintain the metachronal wave integrity.

Table 7 shows the frequency over time for each cell in Area 2. This data suggests a staggering of ciliary

Table 6. Table of the frequencies (in Hz) of each cell in Area 1 at 30 second intervals after addition of ionophore A23187. The cells were exposed to 2.6 mM calcium.

	Minutes After Addition of A23187								
Cell	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5
A	14	14	16	17	17	18	18	19	19
B	13	14	15	16	17	17	17	18	18
C	15	15	16	17	18	18	19	19	19
D	12	13	14	15	15	16	17	17	18
Cell	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5	10.0
A	19	20	20	20	20	20	20	20	20
B	18	19	19	19	19	19	20	19	20
C	19	19	20	20	20	20	20	20	20
D	18	18	19	19	20	20	20	19	19

Table 7. Table of the frequencies (in Hz) at 30 second intervals during the calcium-induced increase for each cell in Area 2. The cells were exposed to 2.6 mM calcium.

	Minutes After Addition of A23187											
Cell	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
A	12	12	12	13	13	13	14	15	15	15	16	17
B	13	13	13	14	14	14	14	15	16	16	16	17
C	13	14	15	15	16	17	18	19	20	21	21	22
D	13	14	15	15	16	17	18	19	20	20	21	22
E	16	17	17	18	18	19	19	20	21	21	21	22
F	14	15	16	17	18	19	19	20	21	21	21	22
G	16	16	16	17	18	18	19	20	21	21	22	23

frequency increases between cells forming a metachronal wave. At 5.5 minutes, cells A, B, E, F and G showed frequency increases. At 7.0-7.5 minutes, all 7 of the cells showed increases in ciliary frequency. Cells C and D showed parallel frequency patterns throughout the frequency increase.

It appears that there may be some type of coordination in the frequency increases, but the time coupling between increases by adjacent cells was not close enough to assume that overlapping cilia were responsible for synchronous frequency increases between adjacent ciliated cells. Analysis of these two areas suggests that adjacent cells did not have to beat at the same frequency to maintain a metachronal wave.

To determine whether adjacent cells responded in a coordinated manner during normal functional patterns, the baseline frequeny histograms were compared. This comparison examined whether there was a relationship between adjacent cells exhibiting frequency shifts. Area 3 (Fig. 11) shows a group of six cells that form a large wave pattern. The baseline frequencies of these cells varied from 11 Hz to 21 Hz. Table 8 shows that six of the seven cells exhibited frequency shifts during the 2 minute control period. Cells A and B shifted only 3 seconds apart, and cells E and F shifted only 3 seconds apart.

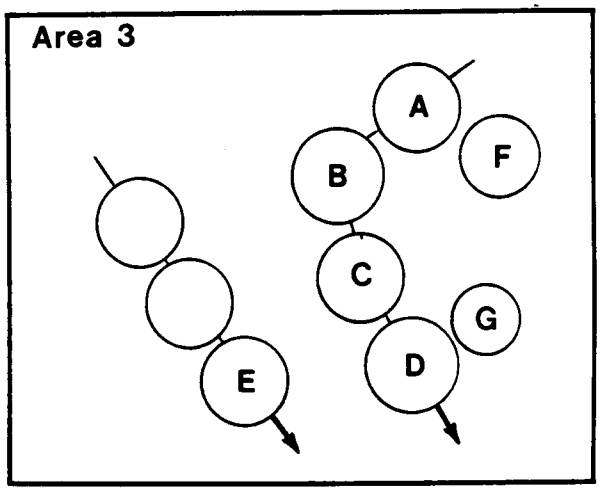

Fig. 11. Diagram of the cells recorded in Area 3. The arrows indicate the direction of particle movement.

Table 8. Comparison of each cell in Area 3 during the 2 minute control period showing baseline frequencies and frequency shifts.

Cell	Baseline Frequency	Frequency Shift	Time of Frequency Shift
A	17 Hz	23 Hz	64 secs
B	16 Hz	21 Hz	61 secs
C	21 Hz	25 Hz	22 secs
D	14 Hz	None	--
E	11 Hz	20 Hz	32 secs
F	17 Hz	24 Hz	35 secs
G	14 Hz	18 Hz	71 secs

All six cells shifted at different frequencies ranging from 18-25 Hz.

At 2.6 mM calcium all the cells, except cell G, had reached a maximum frequency between 22 Hz and 25 Hz (Table 9). While the baselines of these cells differed markedly, it appears that each cell was stimulated by calcium to attain an average frequency of 23.7 Hz. With the exception of one cell, this increase was within a 3 Hz range. Table 10 shows the frequency of each of the cells in Area 3 over the increase. Cells B and C show some similarity in the ranges of frequency covered. Cell D, while at the end of the flow, had the lowest range of frequency increase only attaining a final frequency of 22 Hz by 5.5 minutes. Cells A, E and F, having started at baseline frequencies of 17 Hz, 11 Hz and 17 Hz, respectively, all reached 23 Hz in less than 3 minutes.

Area 4 (Fig. 12) represents another area of coordinated activity with two clusters of three cells each. The clusters appeared to be coordinated in producing a waveform. Table 11 shows the baseline frequencies, frequency shifts and time of frequency shift during the 2 minute control period. The table shows shifts at 18-19 seconds for cells D, E and F. Cells A, B and C had different frequency shifts at different times. Cells D and E exhibited a second frequency shift at exactly 47 seconds.

Table 9. Shows the maximum calcium-induced frequencies of each cell in Area 3. The cells were exposed to 2.6 mM calcium.

Cell	Baseline Frequency	Maximum Frequency	Change in Frequency
A	17 Hz	23 Hz	6 Hz
B	16 Hz	25 Hz	9 Hz
C	21 Hz	25 Hz	4 Hz
D	14 Hz	22 Hz	8 Hz
E	11 Hz	24 Hz	13 Hz
F	17 Hz	23 Hz	6 Hz
G	14 Hz	20 Hz	6 Hz

Table 10. Table of the frequencies (in Hz) at 30 second intervals during the calcium-induced increase for each cell in Area 3. The cells were exposed to 2.6 mM calcium.

	Minutes After Addition of A23187										
Cell	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5
A	20	21	22	22	22	23	23	23	23	23	23
B	21	22	22	23	23	24	24	24	24	25	25
C	22	23	23	23	24	24	24	24	24	25	25
D	18	18	19	20	21	21	22	22	22	22	22
E	19	21	22	22	22	23	23	23	23	24	24
F	20	22	22	23	23	23	23	23	23	23	23
G	15	16	16	16	17	18	19	20	20	20	20

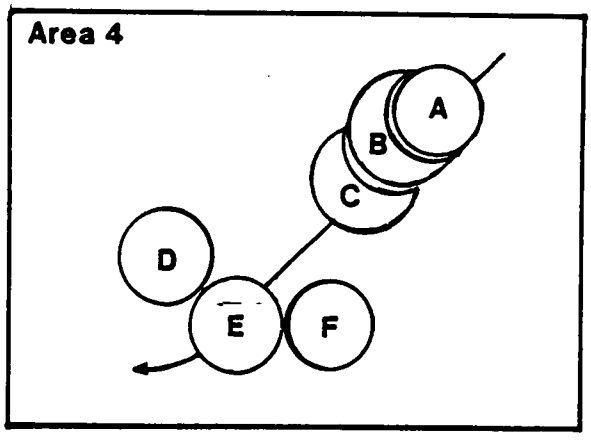

Fig. 12. Diagram of the cells recorded in Area 4. The arrows indicate the direction of particle movement.

Table 11. Comparison of each cell in Area 4 during the 2 minute control period showing baseline frequencies and frequency shifts.

Cell	Baseline Frequency	Frequency Shift	Time of Frequency Shift
A	14 Hz	18 Hz	13 secs
B	14 Hz	16 Hz	21 secs
C	14 Hz	17 Hz	22 secs
D	16 Hz	20 Hz/20 Hz	18/47 secs
E	15 Hz	19 Hz/18 Hz	19/47 secs
F	15 Hz	18 Hz	18 secs

Table 12 shows that in 5 mM calcium, cells A, D, E and F increased ciliary frequency to 18 Hz. Cells B and C increased their ciliary frequencies to 17 Hz. Four of the cells increased ciliary frequency by 3 Hz, while the other two cells increased by 2 Hz and 4 Hz. Table 13 shows the time of frequency increases of the cells in Area 4. Cells B and C were just a few seconds apart in their increases. Cells E and F were very close in their patterns of frequency increase.

We concluded from these four cell areas that adjacent cells do not necessarily have to beat at the same frequency to maintain a metachronal wave. Rounding errors of 1-2 Hz may have occured in frequency acquisition which could conceal tighter frequency coupling of some adjacent cells, but differences of 8 Hz and 4 Hz, as seen in the baseline frequencies of Areas 2 and 3 respectively, suggest that a tight coupling of ciliary frequency is not present. We also found that some adjacent cells will shift frequency at the same time as their neighbors, while other cells will not. Perhaps this has something to do with which cells are in the main flow of the current versus those cells on the periphery of the coordinated area.

These four cell areas exhibited three types of cell-cell coordination: 1) Some cell clusters showed continuity in their baseline frequencies. 2) Others showed

Table 12. Shows the maximum calcium-induced frequencies of each cell in Area 4. The cells were exposed to 5.0 mM calcium.

Cell	Baseline Frequency	Maximum Frequency	Change in Frequency
A	14 Hz	18 Hz	4 Hz
B	14 Hz	17 Hz	3 Hz
C	14 Hz	17 Hz	3 Hz
D	16 Hz	18 Hz	2 Hz
E	15 Hz	18 Hz	3 Hz
F	15 Hz	18 Hz	3 Hz

Table 13. Table showing the frequencies (in Hz) of each cell in Area 4 followed over 30 second intervals during the calcium-induced frequency increase. The cells were exposed to 5.0 mM calcium.

	Minutes After Addition of A23187											
Cell	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0
A	14	15	16	16	17	17	17	17	17	18	18	18
B	14	15	15	15	16	16	16	16	17	17	17	18
C	14	15	15	15	16	16	16	16	16	17	17	17
D	14	15	16	16	17	17	17	18	18	18	18	18
E	14	14	15	15	16	16	17	17	18	18	18	18
F	14	14	15	15	16	16	17	17	18	18	18	18

a continuity in that each cell attained the same maximum calcium-induced frequency. 3) There were also those that showed the exact same change in frequency due to calcium. These types of coordination have previously been explained by the physical contact of one cell's cilia with another cell's cilia. Mechanical coordination due to overlapping cilia would best explain similar baseline frequencies and similar calcium-induced frequencies, but the data from this study suggest that there may be an additional mechanism producing this coordination.

The datum was inconclusive as to how several cells beating at different frequencies can achieve a metachronal wave, unless ciliary frequencies decrease as one moves laterally from the wave. It was evident on the videotape that the current produced by the coordinated activity was maintained during the calcium-induced increase. Austin (1986,unpublished data) proposed that all the cilia on a cell beat at the same frequency. If this is true, then metachronal waves are being produced by some other mechanism than mechanical coordination of adjacent cilia.

IV. DISCUSSION

Calcium's modulation of ciliary frequency is gaining importance in studying the effects of cystic fibrosis. Cystic fibrosis (CF) is characterized by decreased membrane permeability in sweat gland ducts and in tracheal epithelium. The respiratory tract mucous of CF patients hinders normal ciliary activity due to its low water content and high viscocity. Recent evidence suggests that lack of chloride permeablility in the apical membrane of the tracheal epithelium is responsible for the abnormal respiratory tract fluid in CF patients (Knowles, et al., 1983; Welsh and Liedtke, 1986) , and in normal trachea, chloride secretion is linked with calcium uptake (Smith, et al., 1982).

A study of secretion in dog tracheal epithelium found that increases in cellular levels of cAMP and calcium were linked to chloride secretion (Smith, et al., 1982). Frizzell, et al., (1986) showed that the mechanisms for calcium-induced chloride secretion were present in CF patients, but they suggested that the cAMP mechanisms were defective. A calcium-induced increase in ciliary frequency would be instrumental in moving increased fluids present in the respiratory tract. The absence of such an interactive mechanism in CF patients would further add to mucous stagnation.

The purpose of this study was to find the effects of increasing cellular calcium on normal ciliary frequency patterns of individual cells. Present findings indicate that changes in extracellular calcium concentration (0.88 mM-5.0 mM) in the presence of calcium ionophore A23187 did alter the normal pattern of ciliary beat frequency in rabbit tracheal epithelium as shown in Fig. 2 (page 22). As described by Austin (1986, unpublished data) the normal pattern of ciliary frequency consists of rapid, large frequency shifts around a baseline frequency. Our measurements of the ciliary frequency of individual cells exposed to calcium ionophore A23187 showed a pattern of graded frequency increase. The increase was slow compared to the frequency shifts seen in normal frequency patterns. The maximum calcium-induced frequencies were reached by 10 minutes after calcium ionophore A23187 addition, and this maximum frequency could be maintained for several minutes.

These results agree with those of Girard and Kennedy (1986) who showed the effects of calcium ionophore A23187 and extracellular calcium (0.8-12.5 mM) on rabbit ciliary frequency. They reported the average frequency of ten cells at varying intervals for up to 120 minutes. Our study examined calcium-induced frequency changes of individual cells second by second for up to 16 minutes. This encompasses the time period reported by Girard and Kennedy (1986) to reach maximum frequency.

The results of this study differ from those of Walter and Satir (1978) which showed ciliary arrest in mussel gill cells exposed to calcium and ionophore. Walter and Satir concluded that the ciliary arrest was due to increasing cytoplasmic calcium. Stommel (1984) found that models of mussel gill lateral cilia were activated by submicromolar levels of calcium but inactivated by micromolar levels. Stommel (1984) also showed that models of mussel gill abfrontal cilia were quiescent at submicromolar calcium levels, but they were activated at 20 micromol/l. Perhaps there are two calcium-sensitive systems which modulate ciliary frequency in mussel gills, and the same system in abfrontal cilia is present in rabbit tracheal cilia.

In this study 0.88 mM, 1.6 mM and 2.6 mM calcium appeared to produce concentration-dependent increases in ciliary frequency as seen in Fig. 3. The 5.0 mM calcium concentration produced the smallest average increase in ciliary frequency. This pattern agrees with that found by Lee, et al., (1976) in which rabbit oviducts were exposed to different extracellular calcium concentrations. Lee found similar increases at 1.0 mM and 1.5 mM calcium, the highest increase at 2.5 mM calcium and the smallest increase at 5.0 mM calcium.

The small increase produced at 5.0 mM calcium could be do to a number of factors. There was precipitate,

presumably calcium, in the media with 5.0 mM calcium. This would lower the amount of free calcium available for entry into the cell, but it was unclear how much calcium remained in the media. There was also the possibility that calcium concentrations this high would have detrimental effects on the cell. If calmodulin were involved, 5.0 mM calcium could saturate all 4 calcium binding sites on calmodulin and possibly produce an adverse effect on ciliary frequeny (Cox, et al., 1981).

This concentration-dependent pattern of frequency increase agrees with the studies by Murakami and Eckert (1972) using salamander oviducts and Kakuta, et al., (1985) using demembranated dog tracheal cilia. Both of these studies showed increasing ciliary frequency with increasing calcium concentrations. While Kakuta, et al., (1985) used saponin-treated models exposed to calcium, and Murakami and Eckert (1972) used mechanical stimulation to elicit a calcium influx, in both cases ciliary frequencies increased with increasing calcium concentrations within physiological ranges (pCa 8-5).

Statistical analysis of the average frequency increase produced by each of the four calcium concentrations found no relationship between the extracellular calcium concentration and the resulting frequency increase. The large standard error in average frequency increase, produced by the large range in

baseline frequencies and maximum frequencies, was presumably responsible for the absence of any statistically significant differences. However, a comparison of the maximum level of frequency shift, exhibited in normal frequency patterns, with the maximum calcium-induced frequency suggested a positive correlation between the extracellular calcium concentration and the maximum calcium-induced frequency.

Eckert and Murakami (1972) suggested that ciliary frequency was directly related to the availability of ATP and indirectly related to the calcium concentration. The indirect dependence of ciliary frequency on calcium concentration was due to the control exerted by calcium on pathways leading to ATP synthesis. If the observed calcium-induced increase in ciliary frequency was due to calcium control over ATP-generating pathways, an increase in calcium could explain the frequency shifts seen in normal ciliary patterns.

The frequency shifts observed in the normal pattern of ciliary beating might be due to the release of calcium from intracellular pools possibly from the endoplasmic reticulum or mitochondria. Somlyo (1984) suggested that the endoplasmic reticulum was the site of cellular calcium regulation, and evidence suggests that at least 2 mM calcium is sequestered within the cell storage sites (Campbell, 1983). Calcium uptake by the endoplasmic

reticulum has been shown in liver cells (Moore, et al., 1975) and adipocytes (Bruns, et al., 1976). Release of calcium from the endoplasmic reticulum has been shown in peritoneal macrophages (Hirata, 1983). The release of these intracellular pools of calcium due to some stimulus might provide an energy-producing pathway with the calcium needed in an intermediary step to synthesize ATP.

The use of ionophore A23187 to slowly produce a rise in intracellular calcium (Girard and Kennedy, 1986) might slowly produce increasing levels of ATP which would raise the ciliary beat frequency. The pattern of increase at each calcium concentration was relatively similar. This suggests that the concentration of ionophore, which was held constant, regulated the entry of calcium. A steady entry of calcium would produce a steady increase in frequency. If the calcium were released quickly inside the cell, perhaps the increase would be sharper.

The ciliated cell frequency shift, observed in normal cells, might proceed in the following manner. First, a stimulus, whether mechanical, hydrodynamic, chemical or something intracellular, produces membrane depolarization. This depolarization would then open voltage-gated calcium channels and allow for a small increase in intracellular calcium. Murakami and Eckert (1972) showed that mechanical stimulation produced a depolarizing current which produced a calcium influx leading to increases in

ciliary frequency in salamander oviducts. Schultz, et al., (1986) showed that membrane depolarization opened voltage-gated calcium channels in Paramecium.

This entry of calcium might then induce a calcium release by intracellular calcium pools. The endoplasmic reticulum appears the most likely candidate due to its close proximity (about 10-20 nm) to the plasma membrane (Somlyo, 1984). Perhaps the membrane depolarization alone is enough to cause calcium release. The release of this calcium could then provide the intermediary structure leading to ATP synthesis.

Researchers feel that metachronal coordination between adjacent cells is due to mechanical agitation of adjacent cilia. Tamm (1973) suggested this type of coordination in ctenophores, and Sanderson and Sleigh (1981) described it in rabbit tracheal epithelium. It appears that clusters of cells follow the lead of one cell. The power strokes of the lead cell move the cilia of the next cell which move the cilia of the next cell, and so on. There was no similarity in ciliary frequency between two cells if only their borders touched. There was also the requirement that the cilia come in contact.

Extended time analysis of adjacent ciliated cells showed that adjacent cells forming a metachronal wave do not necessarily have to beat at the same frequency. A metachronal wave was maintained by several cells beating

at different frequencies. The cilia of the adjacent cells did touch, but apparently this was not the stimulus behind the establishment of the baseline ciliary frequency of these cells.

During the calcium-induced frequency increase, some cells in a metachronal wave pattern showed the same rate of increase as their neighboring cells. The similarities in frequency increase were not identical, but they were close enough to show some coordination in the pattern of frequency increase. There were other cells, however, that increased at rates markedly different from their neighboring cells' rates of increase.

It is unclear as to how the metachronal wave can be produced or maintained with cilia beating at different frequencies. However, particle movement demonstrates that a directional current was established. It is possible that all the cilia on a cell do not beat at exactly the same frequency. This could account for small variations between cilia of the same cell and between cilia of adjacent cells.

It is also possible that frequency coordination declines between cells located on the periphery of the wave. In summary, it appears that calcium is not only involved in ciliary frequency increases, but that the concentration of calcium affects the maximum frequency attained by individual cells in rabbit tracheal epithelium. The rapid

release of intracellular pools of calcium could explain the frequency shifts seen in normal ciliary patterns. It is apparent that coordinated activity between ciliated cells does not occur exclusively from mechanical agitation of adjacent cilia. Apparently there is another mechanism which controls the ciliary frequency of adjacent cells to form a metachronal wave.

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