To the Graduate Council:

I am submitting herewith a dissertation written by Shaoyou Chu entitled "*In Vitro* Studies on the Regulatory Mechanisms of Ciliary Activity of Frog Palate Epithelium". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

John R. Kennedy, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of the Graduate School

IN VITRO STUDIES ON THE REGULATORY MECHANISMS OF CILIARY ACTIVITY OF FROG PALATE EPITHELIUM

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Shaoyou Chu

May 1993

DEDICATION

This dissertation is dedicated to my parents

Mr. Dayou Chu (褚大由)

and

Mrs. Huimo Zhang (張慧墨)

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ABSTRACT

The regulatory mechanisms of ciliary activity of frog palate epithelial cells are investigated in this study. Ciliary responses to electrical, mechanical and autonomic neurotransmitter are characterized with measuring ciliary frequency at single cell level. Ciliated cells of frog palate outgrowth cultures respond to electrical, mechanical and acetylcholine stimuli by an increase of ciliary beat frequency. The ciliary beat frequency shows dose response to electrical stimulation. Under effective electrical stimulation ciliary beat frequency increase is transmitted cell to cell from explant to outgrowth at a speed of 1.8 cells/second. Under mechanical stimulation ciliary response is transmitted in the same way as with electrical stimulation, but from stimulated cell to adjacent cells in all directions at a speed of 1.4 cells/second. Ciliated cells do not respond to epinephrine, but respond to acetylcholine in a dose dependent manner. The ciliary response to acetylcholine is inhibited specifically by atropine.

Direct innervation of frog palate ciliated cells has been demonstrated in this study with electron microscopy. Electrical stimulation of the palate outgrowth culture can induce ciliary beat frequency increase of ciliated cells in the culture. This ciliary response results from palatine nerve endings in the explant releasing acetylcholine in

response to the stimulation. Either removal of explant from a culture or atropine treatment inhibits outgrowth ciliated cells from responding to electrical stimulation.

The morphology and ciliary activity of the palate outgrowth cells over three weeks in culture are characterized. Outgrowth of the primary culture is formed by migration of ciliated epithelium from the explant. The outgrowth maintains stratified epithelial characteristics of the palate epithelium, but secretory granule in basal secretory cells are gradually lost in culture. As a coincidence ciliary beat frequency gradually increases. The ciliary beat pattern changes from frequent fluctuation to a relative constant frequency with culture age. It is suggested that the basal secretory cells of the palate may inhibit ciliary activity by releasing secretory granules.

Gap junctions are found between epithelial cells in both explants and outgrowths. Intercellular communication by gap junction provides an integrated control mechanism for regulation of the ciliary activity of large numbers of cells in the epithelium. Stimulation of a single cell or a few cells induces ciliary activity in many cells. Mechanical stimulation and gap junctional intercellular communication may represent a mechanism of local activation of mucociliary transport in response to exogenous particles. Electrical stimulation and gap junctional intercellular communication provides a mechanism of more general activation of mucociliary

transport mediated by the palatine nerve.

The effect of Ca^{2+} in mediation of ciliary response to stimulation is examined. It is found that extracellular Ca^{2+} is not necessary for initiation of ciliary response to a stimulation, but is important in maintaining the stimulated ciliary activity. It is suggested that mitochondria and nuclei are the major intracellular Ca^{2+} storage sites. Mobilization of Ca^{2+} from mitochondria and nuclei into the cytoplasm and cilia initiates ciliary beat frequency increase to various stimulations. Free Ca^{2+} or other signal molecules in stimulated cells passing through gap junctions into adjacent cells may cause the propagation of the ciliary response.

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PART I

CHARACTERIZATION OF RESPONSES OF FROG PALATE CILIATED CELLS *IN VITRO* TO ELECTRICAL AND MECHANICAL STIMULI AND AUTONOMIC NEUROTRANSMITTERS

I. INTRODUCTION

Many studies have shown that ciliated epithelia of animals are sensitive to various stimuli, such as mechanical stimuli (Spungin and Silberberg, 1984; Sanderson *et al.*, 1986, 1988), ß-adrenergic compounds (Blair and Woods, 1969; Santa *et al.*, 1974; Yeates *et al.*, 1975; Foster *et al.*, 1976, 1980; Iravani and Melville, 1976; Chopra *et al.*, 1979; Sanderson and Dirksen, 1989), cholinergic compounds (Kordik *et al.*, 1952; Albert *et al.*, 1973; Camner *et al.*, 1974; Wanner *et al.*, 1975; Slaughter and Aiello, 1982; Maruyama and Yamada, 1982; Aiello *et al.*, 1991) and the inflammatory agent substance P (Lindberg *et al.*, 1985, 1986a,b; Khan *et al.*, 1986; Karlsson *et al.*, 1986; Aiello *et al.*, 1991; Hernandez and Aiello, 1992). Frog palate epithelium has been one of the most studied ciliated epithelia. The regulation of ciliary activity of the epithelium has been investigated both *in vitro* and *in vivo* by measuring particle transport rate on the epithelial surface under various treatments (Lucas, 1935; Kordik *et al.*, 1952; Slaughter and Aiello, 1982; Maruyama *et al.*, 1983, 1984, 1985; Aiello *et al.*, 1991; Hernandez and Aiello, 1992). By measuring particle transport on epithelium, ciliary activity of a population of ciliated cells can be estimated. But the ciliary activity of a single ciliated cell and the relationship between adjacent ciliated cells can not be examined by measuring particle transport. Frog palate outgrowth cultures have been proven to be an ideal model to

study the regulation of ciliary activity at the single cell level. A single ciliated cell can be clearly differentiated from adjacent cells under the phase contrast light microscope, and the ciliary frequency can be measured in real time (Eshel *et al.*, 1985, 1987; Aiello *et al.*, 1991; Weiss *et al.*, 1992). Even with the availability of outgrowth cultures, few studies (Aiello *et al.*, 1991) have characterized ciliary response to various stimuli at the single cell level, especially the cooperative relationship between adjacent ciliated cells in response to a stimulus (Sanderson *et al.*, 1988).

In the present study, we have used a computer aided optical spectrum analyzer system (Kennedy and Duckett, 1981) and frog palate explant cultures (Aiello *et al.*, 1991) to investigate ciliary response (ciliary beat frequency change in response to stimulation), in real time, to various stimuli at the single cell level. Three different types of stimuli which were electrical and mechanical stimuli and neurotransmitter treatment, were chosen in this study since they mimic major physiological stimuli on the in situ frog palate ciliated epithelium. The mechanical stimulus is similar to particle or other local mechanical stimulation of palate ciliated cells. The electrical stimulus mimics a nerve mediated signal on palate ciliated epithelium. Neurotransmitters mimic neuronal or hormonal stimuli on the palate epithelium. Study of the effects of these stimuli at the single cell level may provide a better understanding of the mechanisms which regulate the

ciliary function of the frog palate epithelium.

II. MATERIALS AND METHODS

Tissue culture

Frogs (*Rana pipiens*) were sacrificed by pithing the brain and the spinal cord. Palate tissue was prepared for culture according to the method of Aiello *et al.*, (1991). Briefly, the palate mucosa was excised, rinsed with frog Ringer,s solution (133 mM NaCl, 2.4 mM $NaHCO_3$, 1.1 mM $CaCl_2$, 1.9 mM KCl, 11 mM Glucose and 10 mM HEPE, pH 7.2) to remove mucus and blood, and placed in amphibian culture medium (Wolf amphibian culture medium, GIBCO) supplemented with 10% Nu-serum (Collaborative Research), 50 units per ml of penicillin and 50 μg per ml of gentamicin. The palate was pinned onto a piece of dental wax and cut into 1 mm^2 pieces. One or two pieces of the palate explant were placed, connective tissue side down, in a 35 mm plastic culture dish (Falcon) with 0.8 ml of the culture medium (Wolf amphibian culture medium, GIBCO). The tissues were cultured in humidified air at 25°C. Epithelial outgrowths appeared in 24 hours. After secure attachment, the volume of culture medium was kept at 1.5 ml and changed every 48 hours. Tissue culture outgrowths between day 3 and day 7 were used in this study.

Optical spectrum analysis of single cell ciliary beat frequency and ciliary activity with time

Measurement of ciliary beat frequency on fresh frog palate and outgrowth cultures was performed with the optical spectrum analyzer system described by Kennedy and Duckett (1981).

Briefly, culture dishes were placed on the stage of an inverted microscope (Nikon, DIAPHOT-TMD) with a television camera (RCA, TC1005) positioned in parallel with the viewing eyepieces. The image of beating cilia was recorded with a video tape recorder (Panasonic, AG-1950) and observed on a TV monitor. A photoamplifier (Oriel model 2870), with a microscope objective lens used as a limiting aperture for the photocell, was positioned over the image on the monitor and the signal fed to a frequency spectrum analyzer (Hewlett-Packard model 358A) which displayed the spectrum on an oscilloscope screen. The major peak of the spectrum corresponds to the dominant ciliary beat frequency and is updated every half second. Measurements of ciliary frequency were made in real time at the single cell level. Every measurement lasted 10 seconds and the major peak was recorded as the ciliary frequency of the cell. Ciliated cells were measured randomly and the values averaged.

To observe ciliary frequency change in a single cell over time, an IBM PC (IBM, personal system/2, model 50Z) computer with a data acquisition and ciliary frequency analysis program installed was linked with the optical spectrum analyzer system. With this improved system, it was possible to measure a single cell for up to 400 seconds. For each second a spectrum corresponding to the ciliary beat was recorded by the computer, and the fundamental peak of the spectrum was selected as the ciliary frequency for the cell at that second.

In this manner, at the end of the measurement the computer produced a curve, second by second, showing the change in ciliary frequency pattern for the measured cell over time.

Ciliary response to electrical stimulation

Pieces of fresh palate tissue and outgrowth cultures were subjected electrical stimulation first, to determine the optimal electrical threshold for stimulation and second to determine the response of outgrowth cells to threshold stimulation. The ciliary frequency of ciliated cells was measured at the single cell level, in response to the stimulus. For the purpose of providing electrical stimulation, a pair of electrodes (platinum wire of 0.008 inch in diameter) was connected to a Grass stimulator (S6, Grass Instrument). Monophasic, rectangular wave pulses of single stimuli (10 ms in duration) at various voltages were used in these experiments. To stimulate fresh palate tissue, a piece of palate (connective tissue side down) was pinned to the bottom of 35 mm plastic culture dish and covered with 1 ml of frog Ringer's solution. The culture dish was attached to the stage of the inverted phase contrast microscope. The pair of electrodes, 5-10 mm apart, was submerged in the frog ringer's on either side of the piece of palate without directly contacting the tissue. Ciliary activity of the palate cells could be observed and measured by imaging on the edge of the palate explant. To stimulate the outgrowth cells, the culture

medium was replaced by frog Ringer's solution and the culture dish and electrodes were positioned on the microscope stage as described above for fresh palate tissue. Ciliated cell responses to the electrical stimuli were observed in real time and recorded on the video tape. Ciliary response (frequency increase) to the electrical stimulation was measured in real time or by repeat playing of the image from video tapes, and was analyzed with the computer aided optical spectrum analyzer.

Ciliary response to mechanical stimulation

Mechanical stimuli on ciliated cells in outgrowth cultures were performed with a micropipette (tip diameter was about 1 μm) controlled by a micromanipulator (Narishige, Japan). The micropipette was prepared with a vertical pipette puller (Model 700C, David Kopf Instruments) from 1 mm (diameter) capillary tubing (with extruded fiber, Frederick Haer & Co.) Outgrowth cultures were placed on the phase contrast microscope stage as above. The micropipette was touched to a ciliated cell by manually controlling the micromanipulator. Under the phase contrast microscope, the tip of the micropipette was located at a position just above the ciliated cell. Then a straight downward movement was made towards the cell until the cell membrane was touched. After the stimulus, the micropipette was immediately moved back to the ready position. In this way, one ciliated cell could be

specifically stimulated without disturbing adjacent cells. The ciliary activity of the ciliated cells before, during and after the stimulation was recorded on video tape which was used for optical spectrum analysis of the ciliary response to the stimulation.

Ciliary response to acetylcholine and epinephrine

Frog palate outgrowths were rinsed with the frog ringers three times to remove the mucus, and the cultures were maintained in 0.9 ml of the frog Ringer's solution. The cultures to be tested were placed on the stage of the inverted microscope for 10 minutes before treatment. Ciliary frequency of randomly selected ciliated cells were first measured as the control. Acetylcholine or epinephrine (0.1 ml at the appropriate concentration in frog ringers) was pipetted into the dish to produce the desired final concentration. Two minutes after the treatment, ciliary frequency of ciliated cells (which were the same cells measured in the control) were measured. Concentrations (10^{-9}M to 10^{-3}M) of acetylcholine and epinephrine, respectively, were tested for their effect on ciliary activity. For each concentration, four experiments were performed, and the ciliary frequency of thirty ciliated cells was recorded. To examine the effects of the neurotransmitters on ciliary frequency over time, the images of ciliated outgrowth cells before, during and after the treatment were analyzed with computer assisted optical

spectrum analysis or recorded on video tape for later analysis. In these experiments, acetylcholine (10^{-5}M), atropine (10^{-5}M) and epinephrine (10^{-3}M) were used. All experiments were repeated at least ten times. All experiments were carried out at 25°C.

III. RESULTS

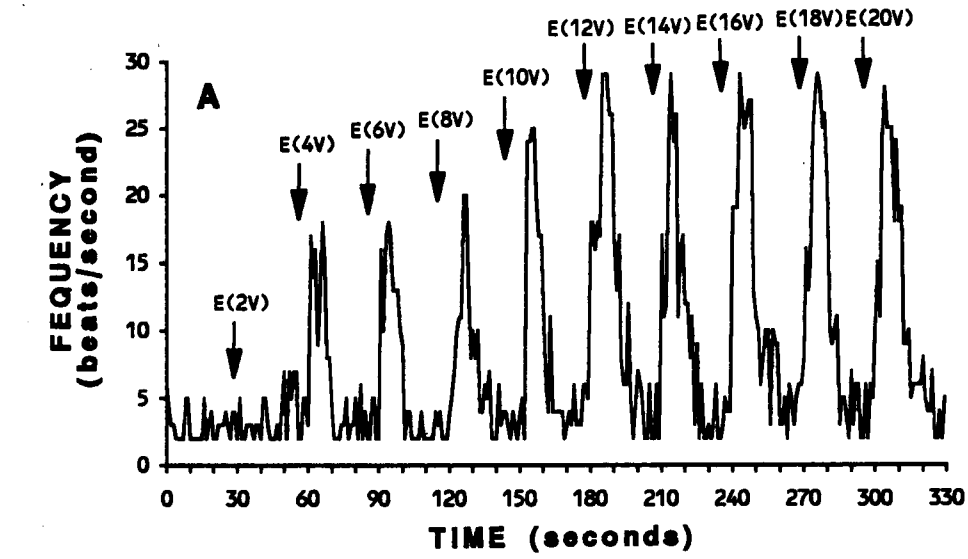
Ciliary response of frog palate explant cells to increasing electrical stimulation

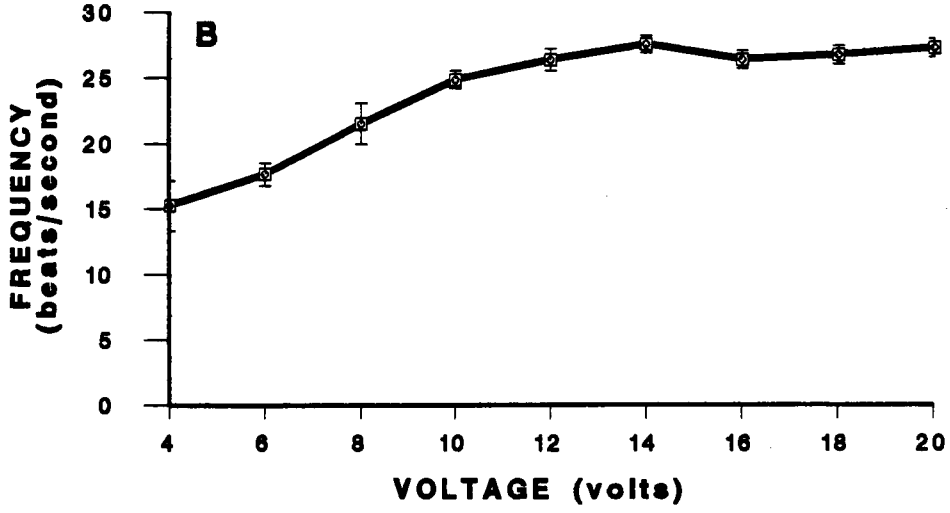
This experiment was performed to determine if frog palate explant cells could respond to electrical stimulation. Results showed that ciliated cells responded immediately to the stimulation by increasing ciliary beat frequency. Further analysis revealed that the increase of ciliary frequency in response to electrical stimulation was in a dose dependent manner (Fig.1A). Each of six experiments was analyzed to determine the threshold and maximal stimuli, the peak ciliary frequency and the duration of the increased ciliary activity. Electrical stimuli used in the experiments ranged from 2 V to 20 V. Four of the six experiments showed that 4 V was the threshold (Fig.1A), and the other two showed 6 V was the threshold. Dose response of stimulated ciliary activity was determined by measuring peak ciliary frequency and duration of the ciliary activity under each stimulus. Both the peak ciliary frequency (Fig. 1B) and duration of increased frequency (Fig. 1C) gradually increased when the stimulus increased from 4 V to 12 V. All six experiments showed 12 V as the maximal stimulus. Any stimulus stronger than 12 V did not induce higher ciliary activity either in terms of the peak ciliary frequency or duration (Fig.1B,C).

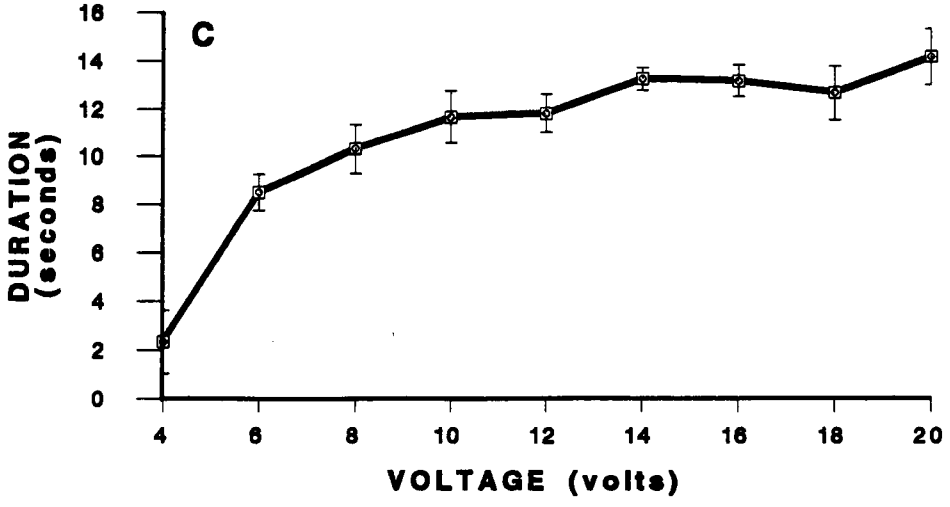
Effect of electrical stimulation on ciliary activity of outgrowth cells

An electrical stimulus above threshold always increased

Fig. 1. Ciliary beat frequency dose response to electrical stimulation on frog palate explant. Electrical stimuli ranged from 2 V to 20 V (10 ms, single stimulus) on fresh frog palate explant in frog ringer's at 30 second intervals. (A) A typical ciliary frequency dose response to electrical stimulation. (B) Ciliary frequency increase over time as a function of voltage. (C) Duration of frequency increase as a function of voltage. Each point in (B) and (C) represented mean±SE of six experiments.


A
E(2V)
E(4V)
E(6V)
E(8V)
E(10V)
E(12V)
E(14V)
E(16V)
E(18V)
E(20V)
FEQUENCY (beats/second)
TIME (seconds)


B
FREQUENCY (beats/second)
VOLTAGE (volts)


C
DURATION (seconds)
VOLTAGE (volts)

ciliary frequency of frog palate outgrowth cells. Ciliary response to the stimulation always started at the explant, then progressed to the ciliated cells in the outgrowth. Since the ciliary activity of the outgrowth (Fig. 2) could be studied at the single cell level with our system, the progression of increasing ciliary frequency between ciliated cells of outgrowth was examined. A single 10 V stimulus was used for each experiment. It was found that the ciliary response progression was always unidirectional, from the explant to outgrowth. The cell immediately adjacent to the explant (cell one (Fig. 3A) reached a frequency of 28 beats/second 3 seconds after stimulus. Three cells away (Fig. 3B) the response was 22 beats/second 4 seconds after stimulation. At a distance of 7 cells (Fig. 3C) the response was 20 beats/second 8 seconds post stimulation. Thus the closer a ciliated cell was to the explant the faster the cellular response, the higher ciliary frequency and the longer the duration of ciliary frequency increase (Fig. 3D).

To characterize the pattern of frequency increase we followed the method of Sanderson *et al.*,(1988) (Fig. 4) to measure lag time (LG), the time from stimulus to frequency increase; rise time (RS), the time from frequency increase to 50% peak frequency which represented the speed of ciliary response; recovery time (RC), the time from the peak frequency to 50% frequency decrease which represented the major duration of the ciliary response, and response magnitude (RM), the

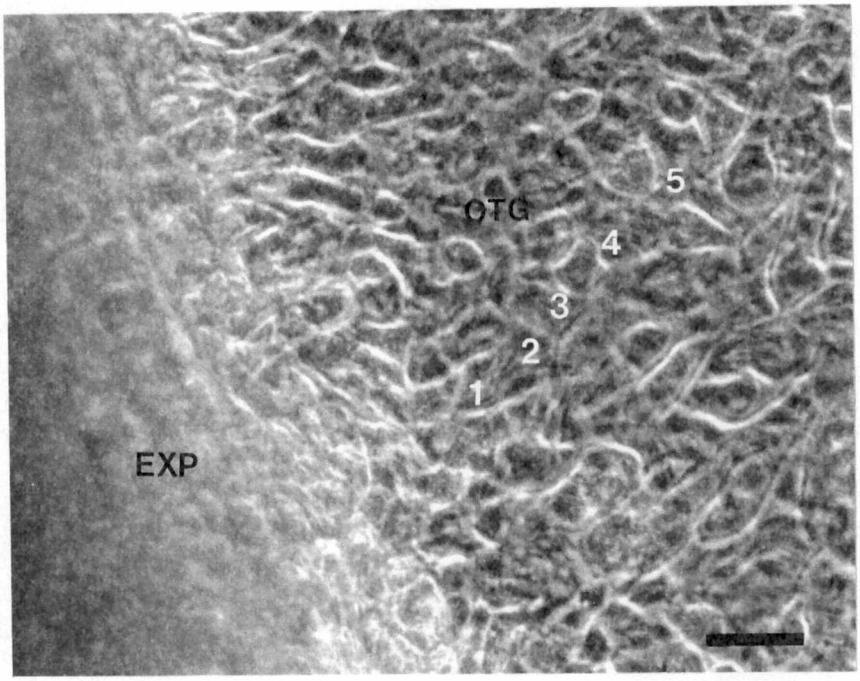
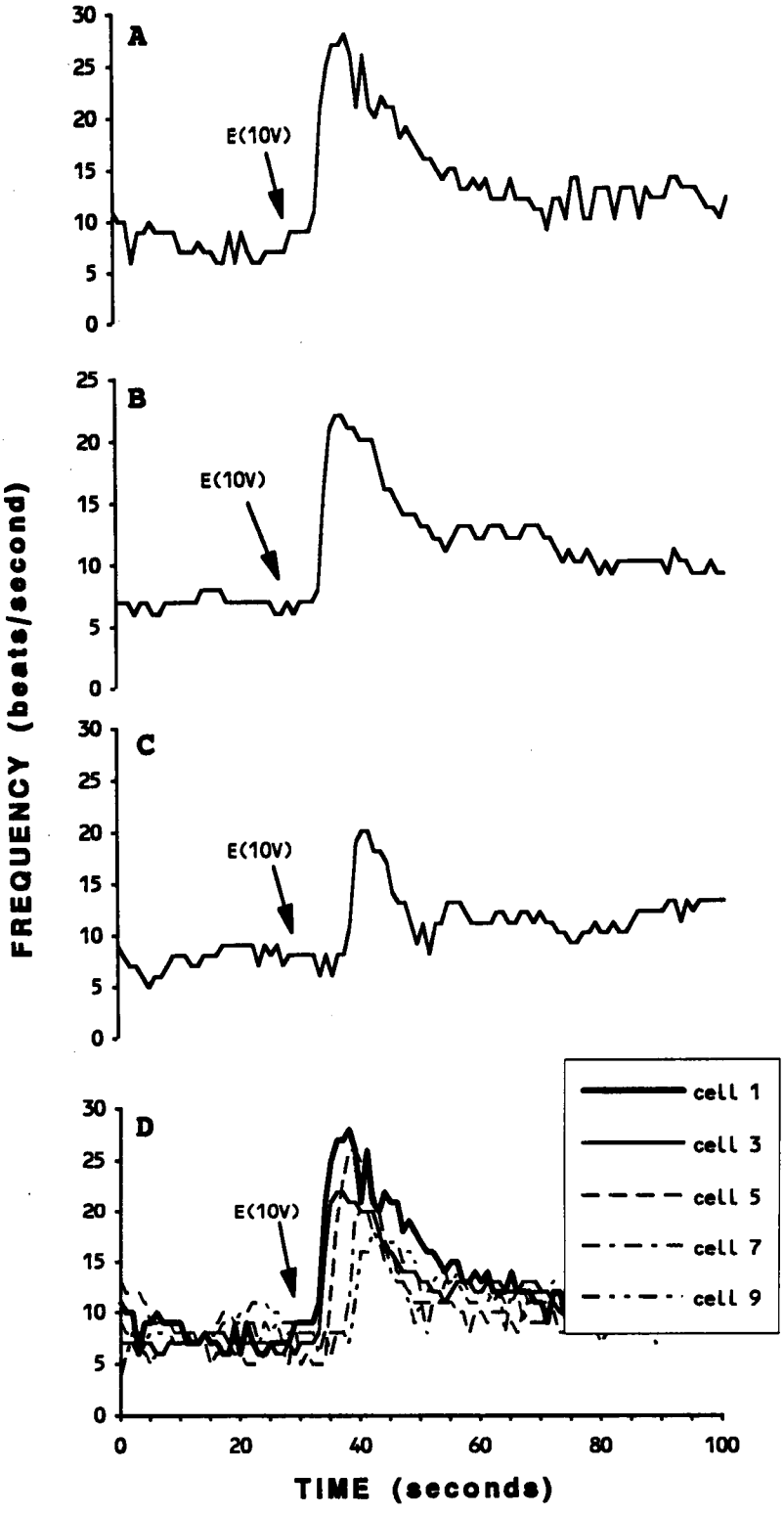


Fig. 2. Phase contrast micrograph of a frog palate explant and outgrowth subjected to electrical stimulation. The numbers (1-5) indicate cell distance (in terms of cells) of outgrowth (OTG) ciliated cells away from the explant (EXP). Bar = 50 μm.

Fig. 3. Typical ciliary beat frequency response and progression under electrical stimulation. The culture was stimulated by a single 10 V stimulus. Ciliary response of cells at different distance from the explant were measured. Ciliary response of the cell at the location of one cell (A), three cells (B) and seven cells (C) from the edge of the explant. (D) Ciliary responses of five ciliated cells at a distance of 1, 3, 5, 7 and 9 cells from the edge of the explant, plotted together.


A
B
C
D
E(10V)
FREQUENCY (beats/second)
TIME (seconds)
cell 1
cell 3
cell 5
cell 7
cell 9

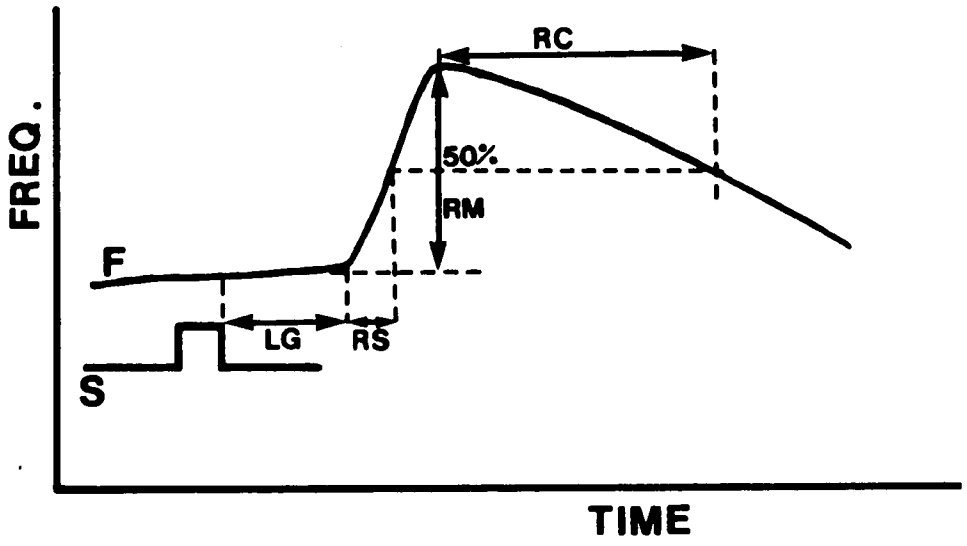


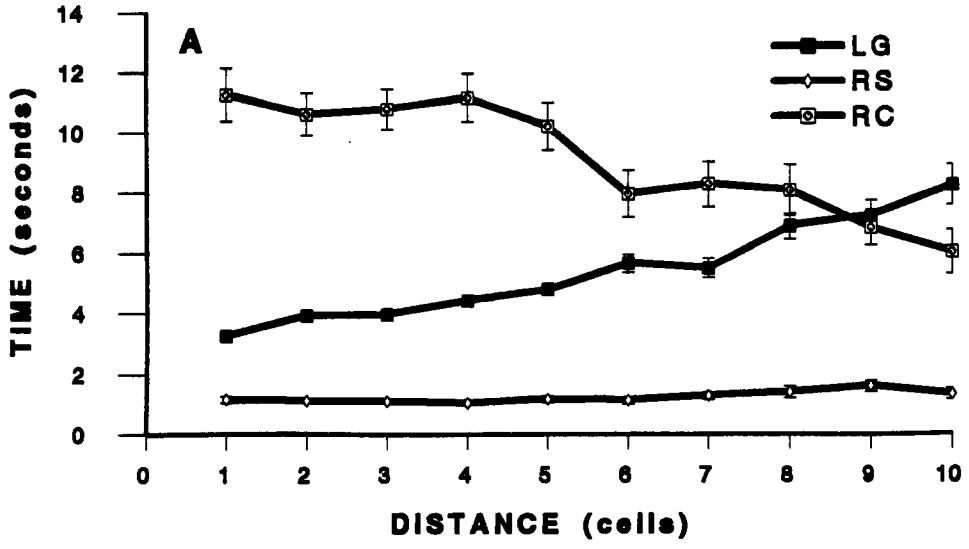
Fig. 4. Diagram showing definitions of parameters calculated from frequency response curves (F). LG, lag time; RS, rise time; RC, recovery time; RM, response magnitude; S, stimulus pulse. [Ref: Sanderson, M.J., Chow, I. and Dirksen, E.R. Am. J. Physiol. 254 (Cell Physiol. 23): C63-C74, 1988].

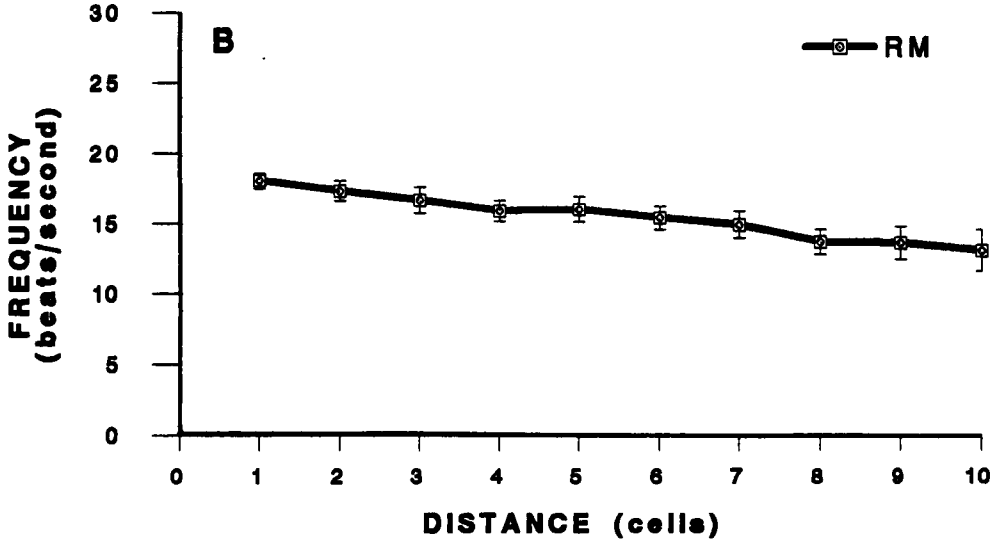
difference between peak and basal frequency which represented the stimulated frequency increase. Ciliated cells at distances of 1-10 cells to the edge of explant were measured in the same time frame by replaying the video tape in each experiment to obtain a ciliary frequency curve for each cell. After examining measurements from more than 200 ciliated cells in 11 experiments (Fig. 5), we found that the lag time (Fig. 5A-LG) of ciliated cells gradually increased with the cell distance from the explant. The lag time increase as a function of cell location was significant ($P<0.01$) by one way ANOVA. The rise time (Fig. 5A-RS) was not significantly different between the ciliated cells tested ($P>0.05$). The recovery time (Fig. 5A-RC) gradually decreased with increasing cell distance from the explant ($P<0.01$). The response magnitude (Fig. 5B-RM) also declined slightly as a function of the cell's distance from the explant ($P<0.01$). The average lag time between any two ciliated cells in the ten cell range was 0.55 ± 0.16 second (mean±SE) so that the velocity of the response progression was about 1.8 cells/second.

Effect of mechanical stimulation on ciliary activity of outgrowth cells

Ciliated outgrowth cells (Fig. 6) were employed to examine the effects of mechanical stimulation on ciliary frequency. A micropipette touched to the apical membrane of an outgrowth ciliated cell always induced immediate frequency increase (Fig. 7A). Ciliary responses of the mechanically stimulated

Fig. 5. Characterization of progression of ciliary response to electrical stimulation. Data shown here were collected from response curves of ciliary beat frequency as shown in Fig. 3. (A) Lag time (LG) and cell distance to explant, data from 255 cells in 11 experiments; rise time (RS) and cell distance to explant, data from 250 cells in 11 experiments, and recovery time (RC) and cell distance to explant, data from 201 cells in 11 experiments. (B) Response magnitude (RM) and cell distance to explant, data from 249 cells in 11 experiments. Each point was expressed by mean±SE.


A
LG
RS
RC
TIME (seconds)
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
7
8
9
10
DISTANCE (cells)


B
RM
FREQUENCY (beats/second)
0
5
10
15
20
25
30
0
1
2
3
4
5
6
7
8
9
10
DISTANCE (cells)

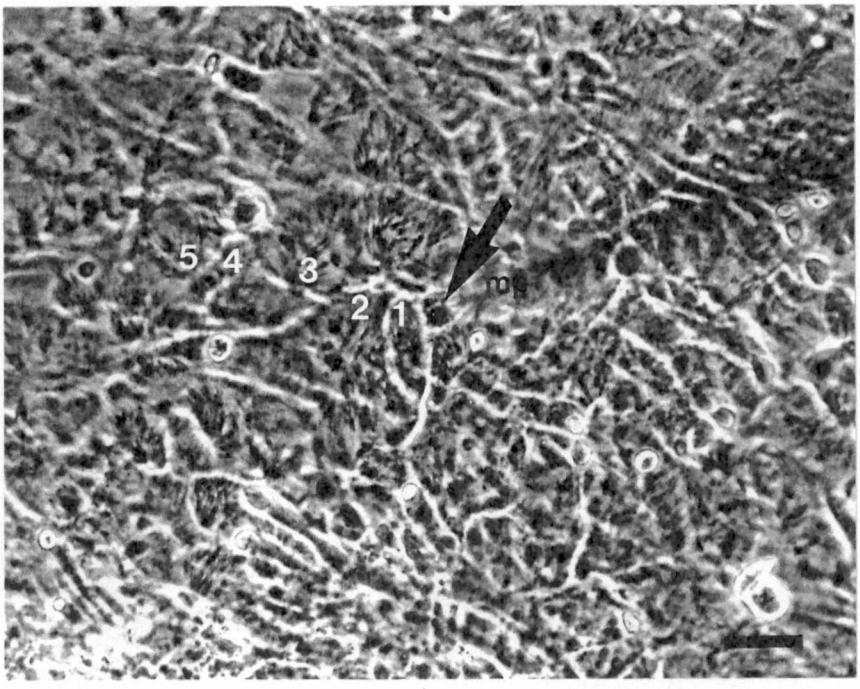
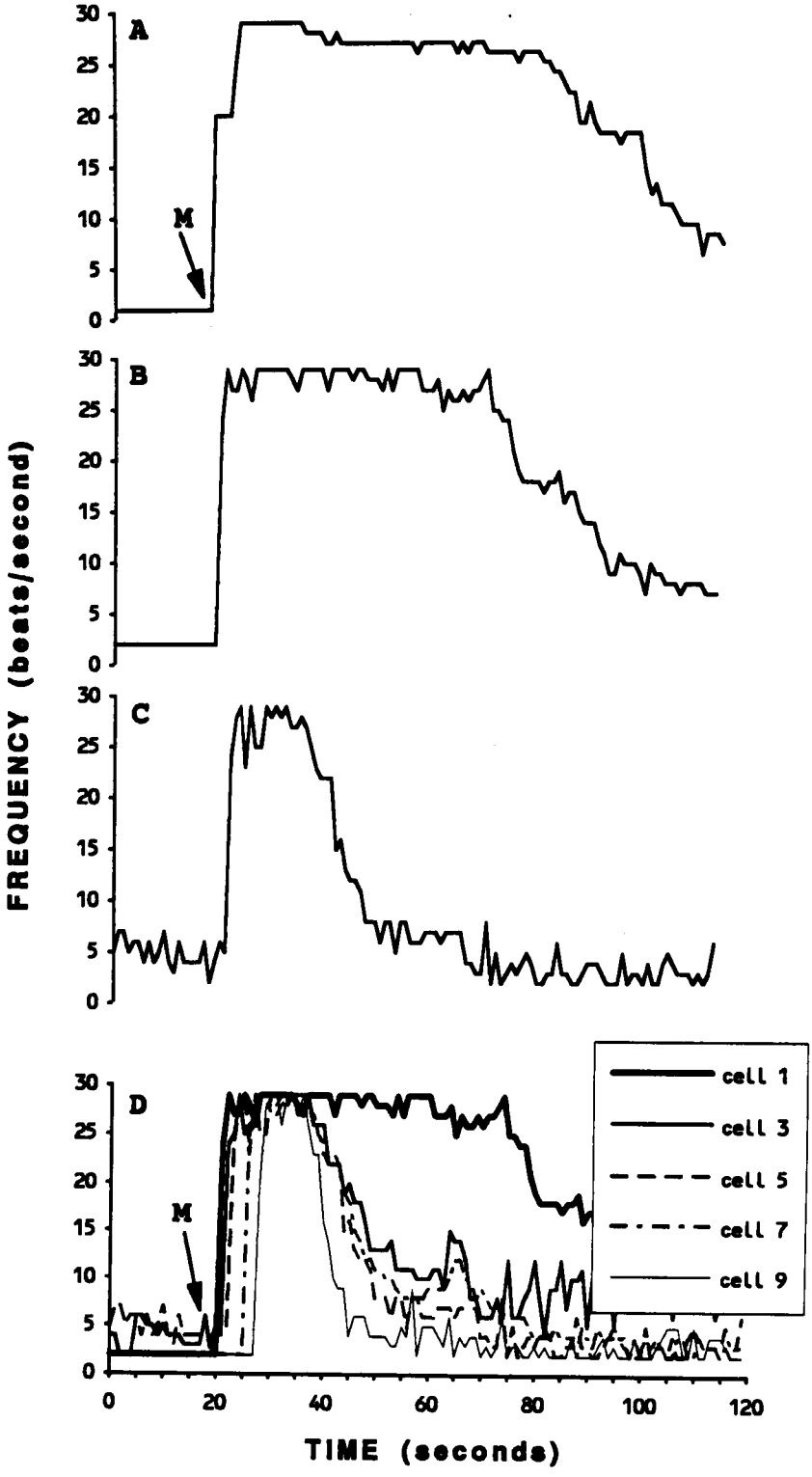


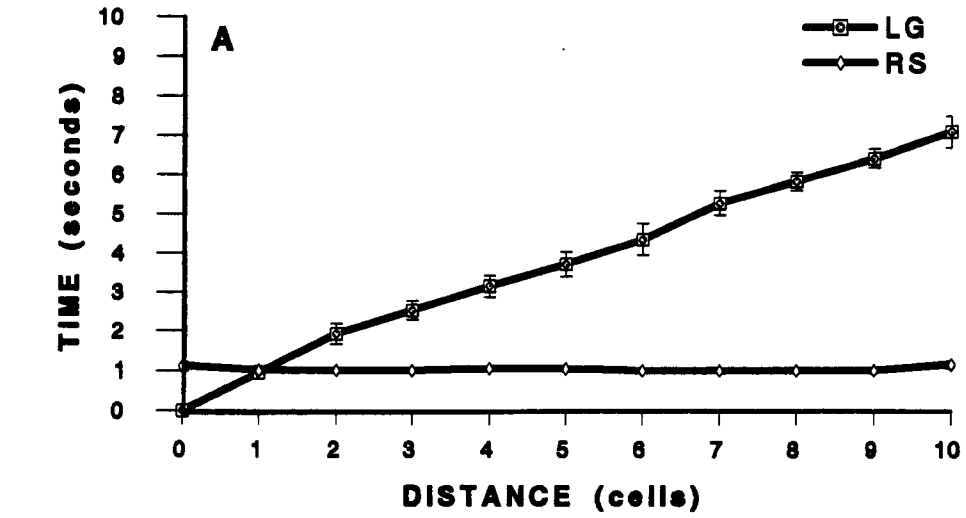
Fig. 6. Phase contrast micrograph of the outgrowth under mechanical stimulation. The micropipette (mp) is pointing at a ciliated cell (arrow). The numbers indicate distance (in terms of cells) to ciliated cells from the stimulated cell. Bar = 50 μm.

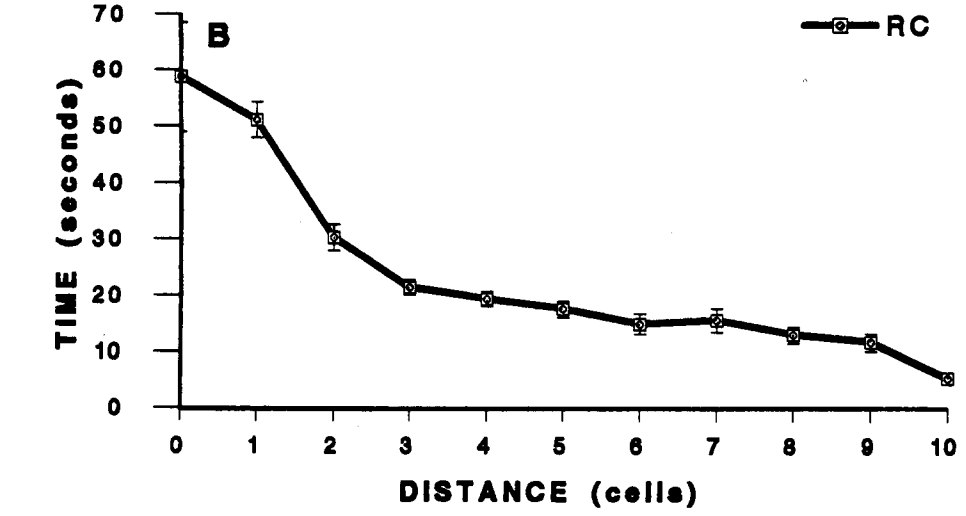
Fig. 7. Typical ciliary beat frequency response and progression under mechanical stimulation. Response curves of ciliary beat frequency of the stimulated cell (A), cells located at a distance of one cell (B) and five cells (C) from the stimulated cell. (D) Ciliary response of five ciliated cells at a distance of 1, 3, 5, 7 and 9 cells from the stimulated cell, plotted together.

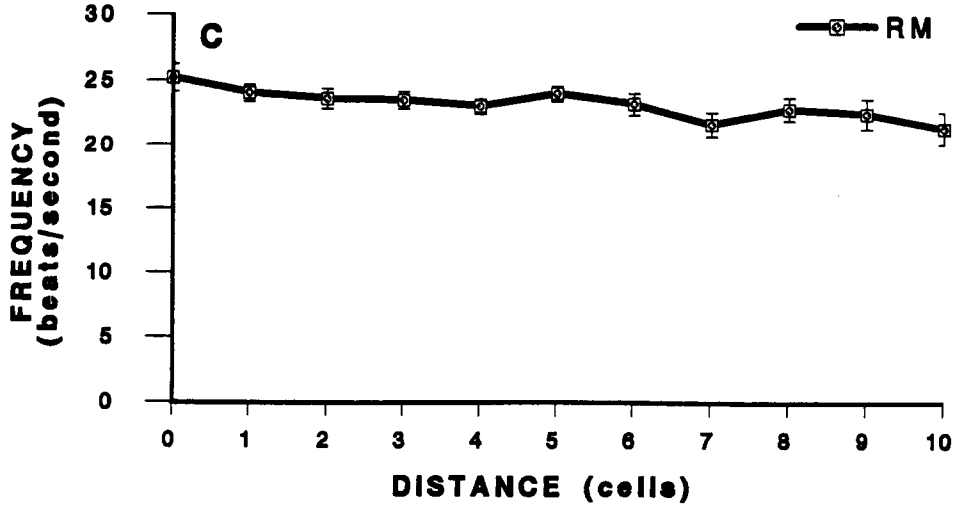

A
B
C
D
M
M
FREQUENCY (beats/second)
TIME (seconds)
0
5
10
15
20
25
30
0
20
40
60
80
100
120
cell 1
cell 3
cell 5
cell 7
cell 9

ciliated cells and adjacent cells (1-10 from the stimulated cell) were measured in the same way as with the electrical stimulation experiments above. The ciliary response was propagated in all directions from the stimulated cell. Except for the onset of ciliary response, the progression was similar to that of electrically stimulated ciliary response, in the manner of cell by cell from adjacent to more distal ciliated cells of the outgrowth. It was also evident that the closer the location of a ciliated cell to the stimulated cell, the stronger the induced ciliary response (Fig. 7B,C & D). About 200 ciliary beat frequency response curves were obtained from ten experiments. These ciliary response curves were further analyzed for lag time, rise time, recovery time and response magnitude as described for electrical stimulation. Results showed that the lag time (Fig. 8A-LG) of induced ciliary response was gradually increased as cell distance increased ($P<0.01$, one way ANOVA). The rise time (Fig. 8A-RS) was not significantly different between ciliated cells in the 10 cell range from the stimulated cell ($P>0.05$). The recovery time (Fig. 8B-RC) gradually declined with increasing distance from the stimulated cell ($P<0.01$). The response magnitude (Fig. 8C-RM) was not significantly different between ciliated cells in 10 cell range ($P>0.05$). The average lag time between any two adjacent ciliated cells in the 10 cell range was 0.71 ± 0.05 second (mean±SE) so that the velocity of the response progression was about 1.4 cells/second.

Fig. 8. Characterization of progression of ciliary response to mechanical stimulation. Data shown here was collected from ciliary response curves as shown in Fig. 6. (A) Lag time (LG) and cell distance to the stimulated cell, data from 205 cells in the 10 experiments; rise time (RS) and cell distance to the stimulated cell, data from 210 cells in the 10 experiments. (B) Recovery time (RC) and cell distance to the stimulated cell, data from 194 cells in the 10 experiments. (C) Response magnitude (RM) and cell distance to the stimulated cell, data from 201 cells in the 10 experiments. Each point was expressed as mean±SE.


A
LG
RS
TIME (seconds)
0
1
2
3
4
5
6
7
8
9
10
DISTANCE (cells)


B
RC
TIME (seconds)
0
10
20
30
40
50
60
70
DISTANCE (cells)


C
RM
FREQUENCY (beats/second)
0
5
10
15
20
25
30
DISTANCE (cells)

Effect of acetylcholine and epinephrine on ciliary activity of outgrowth cells

Ciliated cells in frog palate outgrowth cultures responded to acetylcholine with an increase of ciliary beat frequency. The dose response curve of ciliary beat frequency to acetylcholine (Fig. 9-Ach) showed that acetylcholine started to increase ciliary beat frequency at 10^{-7}M, and the maximal ciliary beat frequency was reached at 10^{-5}M. The specificity of acetylcholine on ciliated cells was verified by the fact that atropine was able to block acetylcholine induced frequency increase whether added before (Fig. 10A) or together with (Fig. 10B) acetylcholine. Atropine also blocked ciliary response when added after an increase had already been induced with acetylcholine (Fig. 10C).

Epinephrine, the sympathetic neurotransmitter, did not increase ciliary beat frequency. In four experiments, we observed that ciliary beat frequency of ciliated outgrowth cells did not respond to epinephrine treatment either at low (10^{-9}M) or high (10^{-3}M) concentrations (Fig. 9-Ep). But, those ciliated cells which did not respond to 10^{-3}M epinephrine were still able to respond to 10^{-5}M acetylcholine treatment (Fig. 10D).

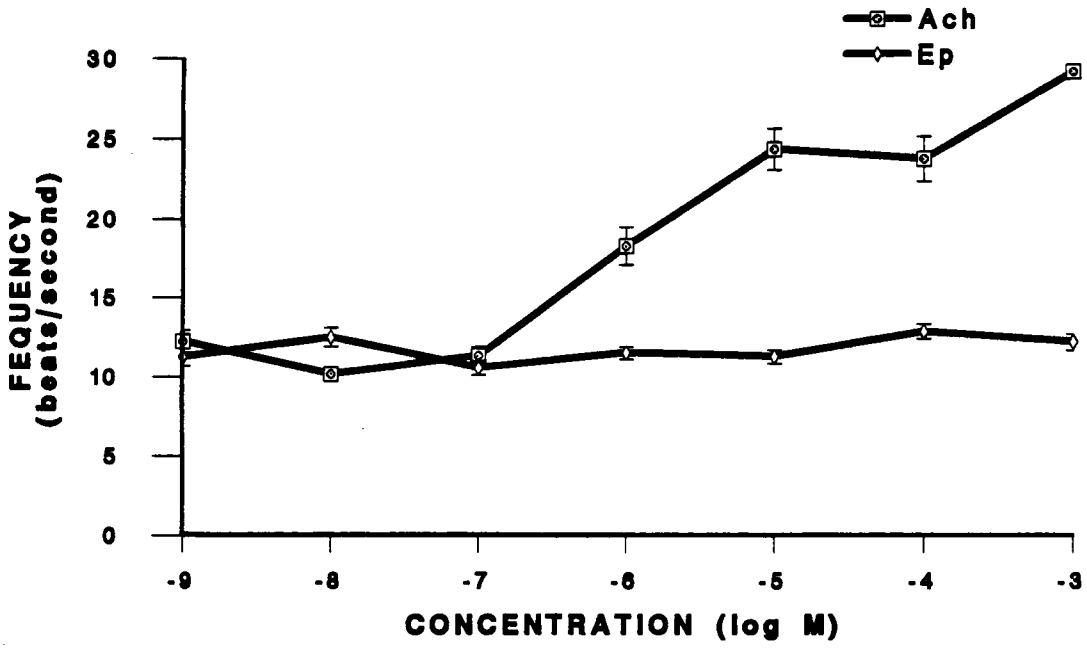
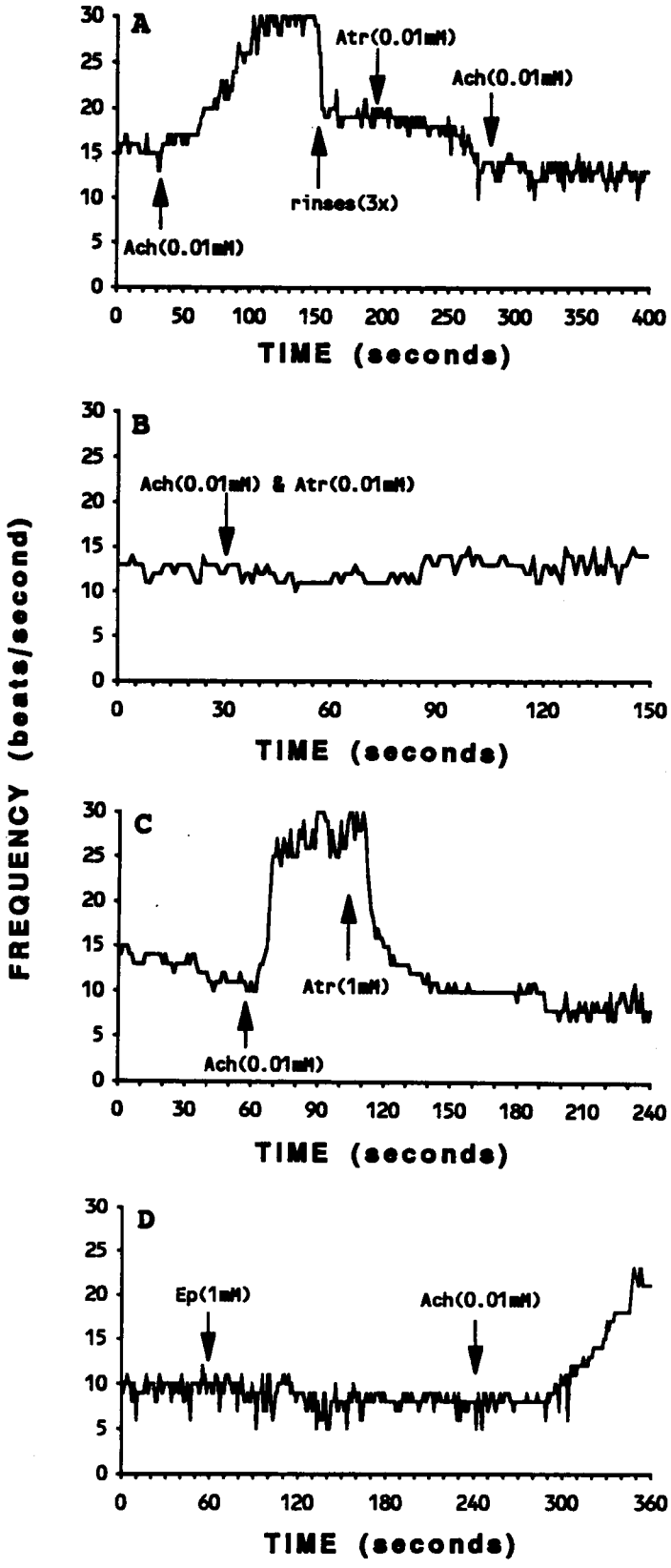


Fig. 9. Ciliary frequency dose response to acetylcholine and epinephrine. Frog palate outgrowth ciliated cells showed dose response to 10^{-9}M -- 10^{-3}M acetylcholine (Ach) by frequency increase. The ciliated cells did not response to 10^{-9}M -- 10^{-3}M epinephrine (Ep). Each point represented mean±SE of ciliary frequency from 30 ciliated cells in 4 different experiments.

Fig. 10. Atropine inhibited ciliated cells response to acetylcholine. (A) Atropine (Atr) added prior acetylcholine (Ach) prevented the ciliated cell from responding to Ach. (B) A mixture of Ach and atropine added together inhibited the ciliary response. (C) Ciliary response to Ach was reversed by atropine. (D) The ciliated cell did not respond to epinephrine (Ep), but did respond to acetylcholine (Ach).


A
Atr(0.01mM)
Ach(0.01mM)
rinses(3x)
Ach(0.01mM)
B
Ach(0.01mM) & Atr(0.01mM)
C
Atr(1mM)
Ach(0.01mM)
D
Ep(1mM)
Ach(0.01mM)
FREQUENCY (beats/second)
TIME (seconds)

IV. DISCUSSION

In the present study, we observed that ciliated cells of frog palate outgrowth cultures responded to direct electrical and mechanical stimuli and acetylcholine by increasing ciliary beat frequency. Although all three stimuli induced increased frequency of ciliary beat, there were recognized differences between the ciliary responses to these stimuli. The most significant difference was the point of onset of frequency increase under these stimuli. The response always started at the explant, and was gradually propagated to the outgrowth with electrical stimuli. With mechanical stimuli the response started at the stimulated ciliated cell, then propagated to neighboring ciliated cells. Under acetylcholine treatment, many ciliated cells responded simultaneously, and did not show any regular progression pattern. These differences suggested that ciliary activity was under multiple controls involving different mechanisms.

The fact that ciliary activity can be stimulated by autonomic neurotransmitters has been observed for some time. In mammalian airway ciliated epithelia, both parasympathetic (acetylcholine and its agonist) and sympathetic (agonists for beta adrenergic receptors) neurotransmitters stimulate ciliary activity (Vanas, 1974; Verdugo *et al.*, 1980; Wong *et al.*, 1988a, 1988b; Sanderson and Dirksen, 1989). Both muscarinic receptors and beta-adrenergic receptors have been studied in the mammalian respiratory tract with regard to both smooth

muscle and epithelial cells (Barnes *et al.*, 1983; Basbaum *et al.*, 1984; Carstairs *et al.*, 1985; Cheung and Townley, 1982). In frog palate epithelium ciliary activity appears to be controlled by the palatine nerve (Seo, 1931; Lucas, 1935; Slaughter and Aiello, 1982; Maruyarma *et al.*,1983). Acetylcholine stimulation of ciliary activity has also been observed (Kordik *et al.*, 1952; Burn and Robbins, 1958; Carson and Robbins, 1974; Slaughter and Aiello, 1982; Maruyama and Yamada, 1982; Aiello *et al.*, 1991). However, the effect of adrenergic agonists on the ciliary activity of frog palate is still controversial. Maruyama (1984) reported that concentrations of noradrenaline below 10^{-7}M suppressed ciliary movement, and ciliary movement was markedly accelerated by concentrations of noradrenaline over $2x10^{-6}$M. The experiments were performed on the Japanese bullfrog with measurements of particle transport rate on the palate. With similar methods, Hernadez and Aiello (1992) reported that with *Rana pipiens* noradrenaline did not affect particle transport although it did cause vasoconstriction. The present study on frog palate ciliated cells in culture with direct measurement of ciliary beat frequency at the single cell level showed that epinephrine (agonist for both α- and ß-adrenergic receptors) did not affect the ciliary beat frequency at concentrations of 10^{-9}M -- 10^{-3}M. However, the ciliary beat frequency of cells in culture showed dose response reaction to acetylcholine treatment; with a threshold of about 10^{-7}M and

maximal stimulation at 10^{-5}M. Furthermore, five minutes in 10^{-3}M epinephrine did not affect ciliary frequency, but a follow up treatment with 10^{-5}M of acetylcholine induced significant ciliary frequency increase. These results suggested that ciliary beat frequency in the frog (*Rana pipiens*) palate is in part under parasympathetic but not sympathetic nervous control.

The question remains as to whether electrical stimulation of explant and outgrowth ciliated cell frequency acts directly on the cell through change in membrane potential or only through release of acetylcholine from nerve endings or by both mechanisms. Ciliary activity change under electrical stimulus has been studied in *Paramecium*. Both frequency and orientation of ciliary beat can be changed by introducing an electric current into the medium in which *Paramecium* is grown (Eckert, 1972). Electrical stimulation affected electrotonic current, specifically Ca^{2+}, across the cell membrane changing intracellular Ca^{2+} which induced ciliary activity change (Eckert, 1972; Brehm and Eckert, 1978; De Peyer and Machemer, 1982a, b; Andrivon, 1988; Machemer, 1990). Using electrical stimulation on the palatine nerve of frog, Slaughter and Aiello (1982) found that mucus transport rate was greatly but temporarily increased both *in vivo* and *in vitro*, and the effect was inhibited by atropine. These results suggested that ciliary activity was under cholinergic nervous and muscarinic receptor control. But the effect of direct

electrical stimulus on palate epithelium in the absence of the nerve was not examined. We found, in this study, that the ciliary frequency of frog palate epithelium showed immediate increase to direct electrical stimuli. Both ciliary frequency and duration of frequency increase were expressed as a dose response to direct electrical stimuli.

Although the basis of this response needs further study, possible mechanisms include: (1) ciliated cells could respond to the stimulus directly, similar to *Paramecium*'s response to the electrical stimulus, changing membrane permeability to Ca^{2+}. The resultant higher intracellular concentrations of free Ca^{2+} could then induce ciliary frequency increase. (2) The ciliary response could be mediated by the palatine nerve through release of a neurotransmitter resulting in the frequency increase. In these studies increased ciliary activity always started at the explant, then progressed to the outgrowth, which suggested that the explant was more sensitive to the electrical stimulus than the outgrowth. If ciliated cells of the palate responded to electrical stimuli directly as with *Paramecium*, all the ciliated cells would have responded to the stimuli simultaneously. Thus, it appears that frog palate ciliated cells in culture do not respond to the electrical stimulus directly but through nerve endings in the explant.

The presence of palatine nerve branches in the frog palate is well established (Seo, 1931; Lucas, 1935). Electrical

stimulation of the palatine nerve causes particle transport increase on the palate epithelium which is believed to be a result of increased ciliary beat (Slaughter and Aiello, 1982). The dose response pattern observed with increased voltages suggests that the stronger the stimulus, the more the number of nerve endings releasing neurotransmitter (acetylcholine) thus causing stronger ciliary response. With a stimulus weaker than threshold, no nerve endings would be stimulated resulting in no ciliary response. On the other hand, with the maximal stimulus, all the nerve endings may release neurotransmitter so that the response was the strongest. Any stimulus stronger than the maximal would not induce increased response since all of the nerve endings would already have been involved in the response at the maximal stimulus. Thus it appears that direct electrical stimulation of the outgrowth culture causes the nerve endings in the explant to release acetylcholine, just as electrical stimulation of the palatine nerve increases ciliary frequency. The signal may then be propagated through an intercellular communication mechanism to neighboring cells as reported for rabbit tracheal epithelial cells (Sanderson *et al.*, 1988).

The pattern of progression of ciliary response between outgrowth cells after either electrical or mechanical stimulus may provide information about whether intercellular communication is involved. To analyze the progression we used the four parameters reported by Sanderson *et al.*, (1988).

With the exception that ciliary response was initiated in different ways, the progression of the response induced by both electrical and mechanical stimuli were similar. The values for rise time (RS) from both electrical and mechanical induced frequency increase did not show significant differences between ciliated cells in the 10 cell range. This result suggests that with a signal stronger than threshold, once a frequency increase is induced in a ciliated cell, rise time is constant and not cell distance related. Both lag time (LG) and recovery time (RC) showed very significant difference between ciliated cells in the 10 cell range. Lag time (LG) gradually increased and recovery time (RC) gradually declined as a function of cell distance relative to stimulation points. From the lag time (LG) data, the speed of progression of the signal between the 10 cells was calculated. It was 1.8 cells/second for electrical stimulation and 1.4 cells/second for mechanical stimulation. These speeds were similar to the progression speed of ciliary response for rabbit tracheal ciliated cells in culture, 1.54 cells/sec at 37°C (Sanderson *et al.*, 1988). Response magnitude (RM) which represents magnitude of ciliary response significantly declined as a function of cell distance in the 10 cell range with electrical stimuli. On the other hand, the response magnitude (RM) did not show significant change in the 10 cell range under mechanical stimulation. This response magnitude (RM) difference may be a result of the different strengths between

electrical stimulus and the mechanical stimulus. Sanderson *et al.*, (1988) also found the response magnitude did not decline in a 7 cell range under mechanical stimulation of cultured mammalian tracheal ciliated cells. Our analysis of lag time, recovery time and response magnitude showed that the progression of ciliary response was a relatively slow and cell distance dependent event. The closer to the stimulation point, the faster and stronger the ciliary response would be.

Since the cilia of adjacent cells in the outgrowth do not touch each other, mechanical transduction of the ciliary response between ciliated cells could not contribute to the response progression in the culture. One possible explanation is that the progression is mediated by an intercellular signal transduction mechanism through gap junctional intercellular communication. The gap junctional intercellular communication has been studied in ciliated epithelium of mammalian airway (Sanderson *et al.*, 1988). It has also been shown that Ca^{2+} and cAMP, as second messengers, increase ciliary frequency in ciliated epithelia (Verdugo, 1980; Kakuta *et al.*, 1985; Girard and Kennedy, 1986; Tamaoki *et al.*, 1989; Villaón *et al.*, 1989; Sanderson *et al.*, 1990, 1992). Recently, it has been reported that IP_3 mediates Ca^{2+} release from intracellular store sites causing free Ca^{2+} increase in the cytoplasm. This results in stimulation of ciliary frequency in rabbit tracheal ciliated epithelium (Boitano *et al.*, 1992). All of these signal molecules are small enough to pass through gap junctions to

work as intercellular signal transducers. Future study on the gap junctional intercellular communication and change in the intracellular signal molecule level in the frog palate ciliated epithelium may produce more information about the machnisms of propagation of ciliary response between ciliated cells. These studies are currently in progress.

In summary, (1) ciliated cells of frog palate in culture respond to electrical, mechanical and acetylcholine stimuli by an increase of ciliary beat frequency. (2) Direct electrical stimulation on the palate culture is found to be a quantitative and reproducible method to induce ciliary beat frequency increase of frog palate ciliated cells in outgrowth culture. (3) Ciliary activity of frog palate ciliated cells in culture is under cholinergic but not adrenergic nervous control. (4) The ciliary response is transmitted from cell to cell, which may be mediated by an intercellular signal transduction mechanism with intracellular signal molecules and gap junction structures. This possibility is currently under investigation.

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PART II

INTRA-EPITHELIAL PALATINE NERVE ENDINGS AND THEIR REGULATION OF CILIARY ACTIVITY OF FROG PALATE EPITHELIUM

I. INTRODUCTION

The main function of mucociliary epithelium in the respiratory system is to keep the airway clear. Ciliary beating provides the mechanical force for mucus transport out of the airway. Dysfunction of ciliated epithelium will cause serious respiratory diseases (Afzelius, 1979; Pysher and Neustein, 1984). The oral cavity of frog is part of the respiratory airway, and has mucociliary epithelium on the palate. Morphologically, the palate ciliated epithelium is similar to the ciliated epithelium of mammalian trachea (Puchelle *et al.*,1984; Zahm *et al.*, 1986). However, two distinct features of the frog palate differentiate its ciliated epithelium from mammalian tracheal ciliated epithelium. First, the ciliary beat frequency pattern of frog palate ciliated cells shows significant fluctuations with time including sudden stopping and starting (Aiello *et al.*, 1991; Yager *et al.*, 1978; Zahm *et al.*, 1986). On the contrary, ciliary beating in mammalian trachea is fairly constant, as observed in human bronchia (Yager *et al.*, 1978), rabbit trachea in culture (Verdugo *et al.*, 1980; Kennedy and Duckett, 1981; Sanderson *et al.*, 1988), and rat trachea in situ (Dahlamm, 1960) and in culture (Gabridge, 1975). Second, there is evidence that suggests that ciliary activity of the frog palate is under nerve influence. Seo (1931) has reported a cilioexcitatory reflex for the frog palate. Lucas (1935) has observed that electrical stimulation on the

parasympathetic nerve to the palate increases mucus transport, and suggests that the palatine nerve originates from the seventh cranial nerve. Slaughter and Aiello (1982) have confirmed these findings and provided more pharmacological evidence about the cholinergic nerve stimulating ciliary activity on the palate of frog *Rana pipiens*. In addition, it has also been reported that substance P stimulates mucociliary transport in the frog palate (Aiello *et al.*, 1991; Hernandez and Aiello, 1992). On the other hand, it has been found that there is dopaminergic innervation in the palate of Japanese bull frog, which inhibits mucociliary transport on the palate (Maruyama *et al.*, 1983). Even with these findings, the mechanisms of ciliary activity control in the frog palate are still not clear. Specifically, the dominant effect of nerves on palate ciliary activity, and the distribution of the palatine nerve in detail needs more studies.

The objectives of the present study are to determine (1) if there is any direct contact between the nerves and the palate ciliated cells of *Rana pipiens*, (2) what the direct effect of the palatine nerve on the ciliary activity (stimulation or inhibition) is and (3) if the ciliary activity of the frog palate is directly electrically excitable. The possible mechanism of nerve control of palate epithelial ciliary activity will be discussed.

II. MATERIALS AND METHODS

Tissue preparation and tissue culture

Frogs (*Rana pipiens*) were sacrificed by pithing of the brain and spinal cord. The palate was exposed, and the palate mucosa was cut along its edge. The palatine nerve was carefully exposed, and cut so as to retain as much nerve as possible with the palate. The dissected palate was rinsed with frog Ringer's solution (NaCl 133 mM, $NaHCO_3$ 2.4 mM, $CaCl_2$ 1.1 mM, KCl 1.9 mM, Glucose 11 mM and HEPE 10 mM, pH 7.2) to remove blood and some mucus. A piece of the palate with palatine nerve was maintained in amphibian culture medium (GIBCO) for experiments on nerve stimulation. The remainder of the palate mucosa was cut into 1 mm^2 pieces and placed into 35 mm Falcon plastic culture dishes, ciliated surface up, with 0.8 ml of amphibian culture medium supplemented with 10% Nu-serum (Collaborative Research), 50 units/ml of penicillin and 50 μg/ml of gentamicin. Two or three pieces of the palate explant were placed in each dish. The tissues were cultured in humidified air at 25°C. Ciliated epithelial outgrowths appeared in 24 - 48 hours. After secure attachment, the culture medium was kept at 1.5 ml and changed every 48 hours. The cultures used for experiments were 2-7 days old.

Optical spectrum analysis of single cell ciliary beat frequency pattern over time

The frog palate explant cultures were subjected to ciliary beat frequency measurement with the optical spectrum analyzer

system described by Kennedy and Duckett (1981). Culture dishes were placed on the stage of an inverted phase-contrast microscope (Nikon DIAPHOTO-TMD) and a television camera (RCA TC1005) was positioned in parallel with the viewing eyepieces. The image of beating cilia was recorded with a video tape recorder (Panasonic AG-1950) for later analysis and also observed on a television monitor. A photoamplifier (Oriel model 2870) with a microscope objective used as a limiting aperture in front of the photo cell was positioned over the ciliary image on the monitor and the signal fed to a frequency spectrum analyzer (Hewlett-Parkard model 3582A) which displayed the spectrum on an oscilloscope screen. To acquire a ciliary beat frequency spectrum continually, an IBM PC (PS/2, model 50Z) computer with a data acquisition and ciliary beat frequency analysis program installed was linked with the optical spectrum analyzer system. With this system, a single cell was measured for a period of time (up to 400 seconds), and for each second a ciliary beat frequency spectrum was recorded. The peak value of the spectrum was picked as the ciliary beat frequency of the cell in that second. In this manner the computer plotted a curve representing second by second ciliary beat frequency change in real time.

Electrical stimulation of palatine nerves and explant cultures

It has been known that electrical stimulation (1.5 V, 10 Hz with 0.8 ms duration for 2-5 sec) of the palatine nerve can

increase mucociliary transport rate both *in vivo* and *in vitro* (Slaughter and Aiello, 1982). But the effect of electrical stimulation of the palatine nerve on ciliary activity change at the single cell level has not been examined. In this study, we used weaker (80-200 mV) and shorter (10 ms) electrical stimuli than those used by others (Slaughter and Aiello, 1982; Lindberg and Mercke, 1986) to stimulate the palatine nerve. The ciliary beat frequency of single ciliated cells was measured as a cellular response to the stimulation. In addition, an experimental system of electrical stimulation of frog palate outgrowth cultures was employed. The ciliary frequency of individual outgrowth cells was measured as a response to the electrical stimulation.

Fresh palate with the palatine nerve, 2 day cultures of the palate explant with the nerve, and outgrowth cultures up to 7 days old were subjected to electrical stimulation. Ciliary beat frequency of the ciliated cells was recorded as the cellular response to the stimuli. A pair of electrodes (platinum wire of 0.008 inch in diameter) was connected to a Grass stimulator (S6, Grass Instrument). Stimuli used in these experiments were 10 ms in duration and were rectangular wave pulses. Single stimuli with various voltages (80-200 mV for nerve stimulation, 6-12 V for outgrowth culture stimulation) were used in different tests. To stimulate the palatine nerve in fresh tissue, palate tissue (connective tissue side down) was partially rested on a piece of filter

paper to prevent the palate from moving in the plastic culture dish. The culture dish was attached to the stage of the inverted microscope. The palate tissue was kept wet with culture media. The nerve was connected to the negative electrode above the culture medium. The ground electrode was submerged in culture medium adjacent to the tissue. To stimulate the palatine nerve in tissue culture, the same procedure was used except that the explant was allowed to attach to the plastic dish so that no filter paper was necessary. In experiments involving electrical stimulation of outgrowth cultures, a culture dish was placed on the stage of the microscope. The pair of electrodes (5 mm separation from each other) was submerged in the culture medium around the tissue, and the cells close to the negative electrode were observed. Before the electrical stimulation, the culture medium was removed from the culture dishes, and replaced by 1-2 ml of frog Ringer's solution. Experiments were repeated at least 5 times unless otherwise indicated.

Light and transmission electron microscopy

Fresh frog palate mucosa was rinsed twice with ice cold phosphate buffer (0.1 M, pH 7.4) for 5 min. The tissue was fixed for 1 hour in ice bath in a mixed fixative containing 1 part 2.5% glutaraldehyde in phosphate buffer and 1 part 1.66% OsO_4 in phosphate buffer. Dehydration was with a graded ethanol series, and tissue was embedded in EM bed-812

(Electron Microscopy Sciences). For light microscopy, 1 μm sections were prepared and stained with toluidine blue. For electron microscopy, sections were stained with uranyl acetate and lead citrate, and examined with an Hitachi H-600 electron microscope operated at 75 kV.

III. RESULTS

Morphological observations of the palatine nerve and intra-epithelial nerve endings

The frog palate is composed of a mucociliary epithelium and underlying connective tissue (Fig. 1A). The mucociliary epithelium is a stratified epithelium. The superficial layer contains ciliated cells and mucus cells, and the lower layers contain basal cells (Fig. 1A). In the connective tissue, there are palatine nerve branches. The fine structure of a nerve in connective tissue is shown in Fig. 1B, which contains several nerve fibers. In these nerve fibers, small neurotransmitter vesicles, mitochondria and glycogen particles are evident. Nerve branches in deep connective tissue are bigger and contain more nerve fibers than nerves close to the superficial parts of the connective tissue (Fig. 1A), which suggests innervation of the epithelium. But, it is not clear with light microscopy whether the nerves directly contact the epithelial cells.

Electron microscopy shows that the superficial layer of the epithelium is composed of ciliated cells and mucus cells (Fig. 2A,B). Beneath the superficial layer, there is at least one layer, but mostly two or three layers, of basal cells. Some of the basal cells contain electron dense secretory granules (Fig. 2A,B) in the apical half of the cytoplasm. Only basal cells rest on the basement membrane (Fig. 2A). Nerve endings can contact ciliated cells directly. Fig. 2B shows a ciliated cell which is directly innervated by two

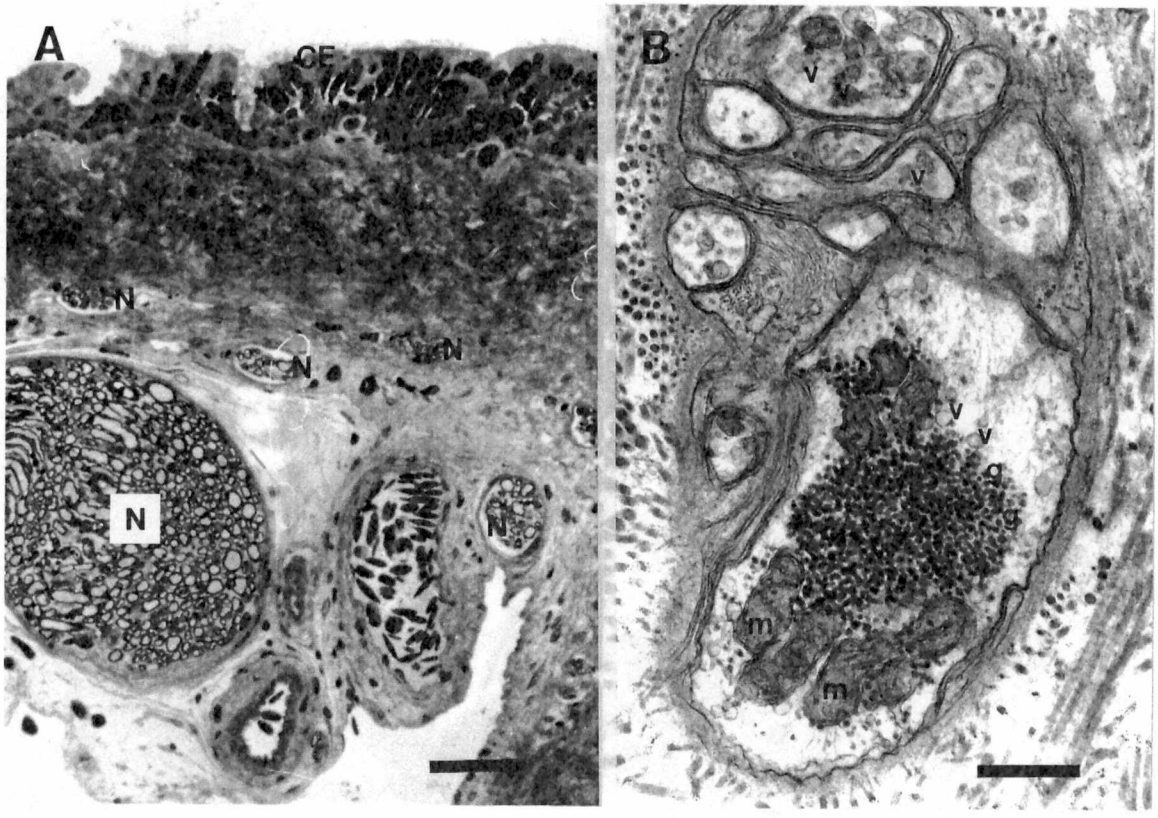
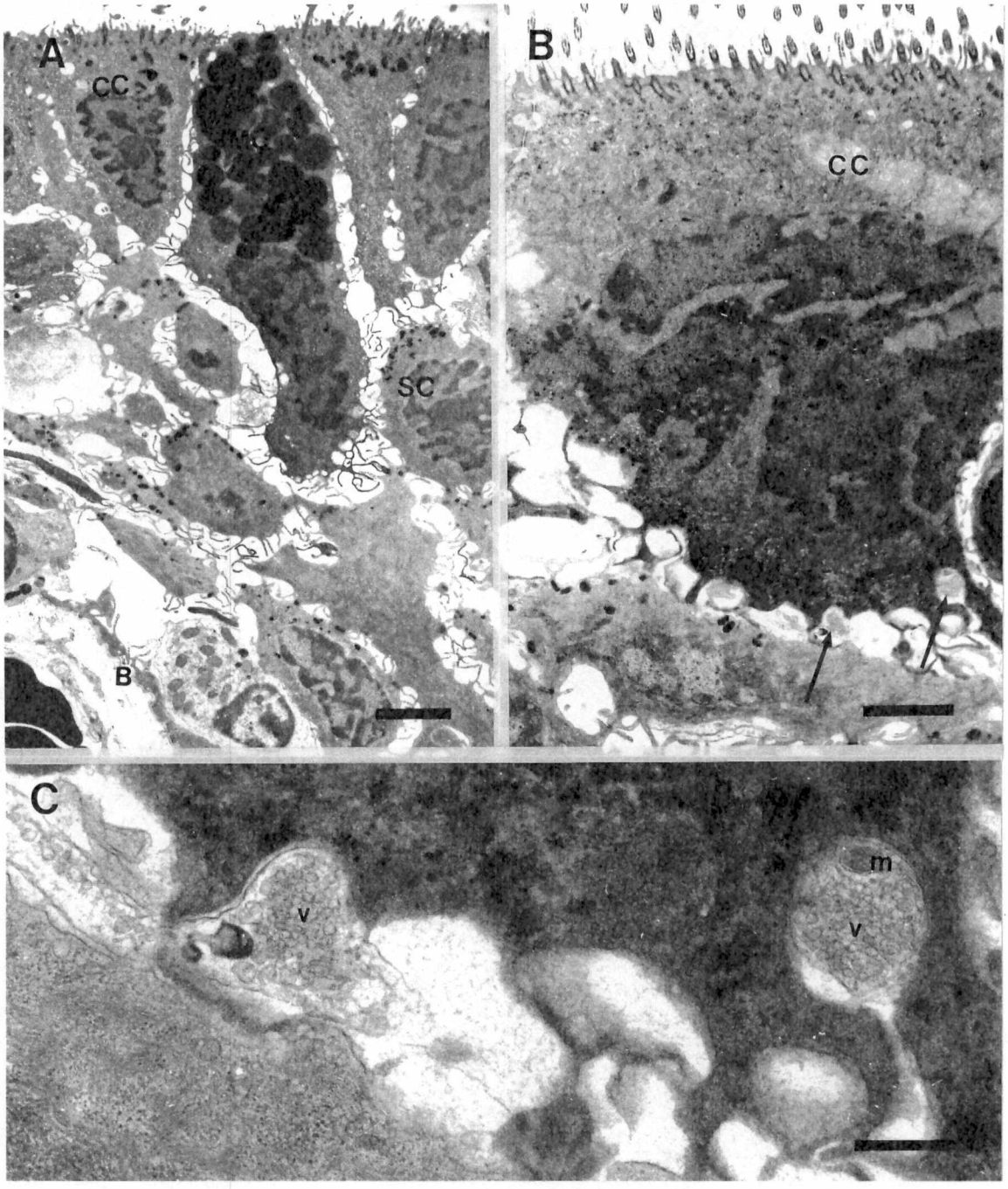


Fig. 1. Micrographs of the frog palate and palatine nerve. (A). A light micrograph of the frog palate shows the ciliated epithelium (CE) and palatine nerve branches of different size (N). Bar = 100 μm. (B). Electron micrograph of a nerve branch in the connective tissue. Small neurotransmitter vesicles (v), mitochondria (m) and glycogen particles (g) are shown in nerve fibers. Bar = 5 μm.

Fig. 2. Electron micrographs of frog palate epithelium and intra-epithelium nerve endings. (A). Fine structures of the epithelium. Ciliated cells (CC), a mucus cell (MC), basal secretory cells (SC) and basal lamina are shown. Bar = 5 μm. (B). A ciliated cell (CC) with two nerve endings (arrows) on its basal surface. Bar = 2 μm. (C). Enlargement of nerve endings shown in (B). Numerus small neurotransmitter vesicles (v) and a mitochondrion (m) are shown in the nerve endings. Bar = 0.5 μm.

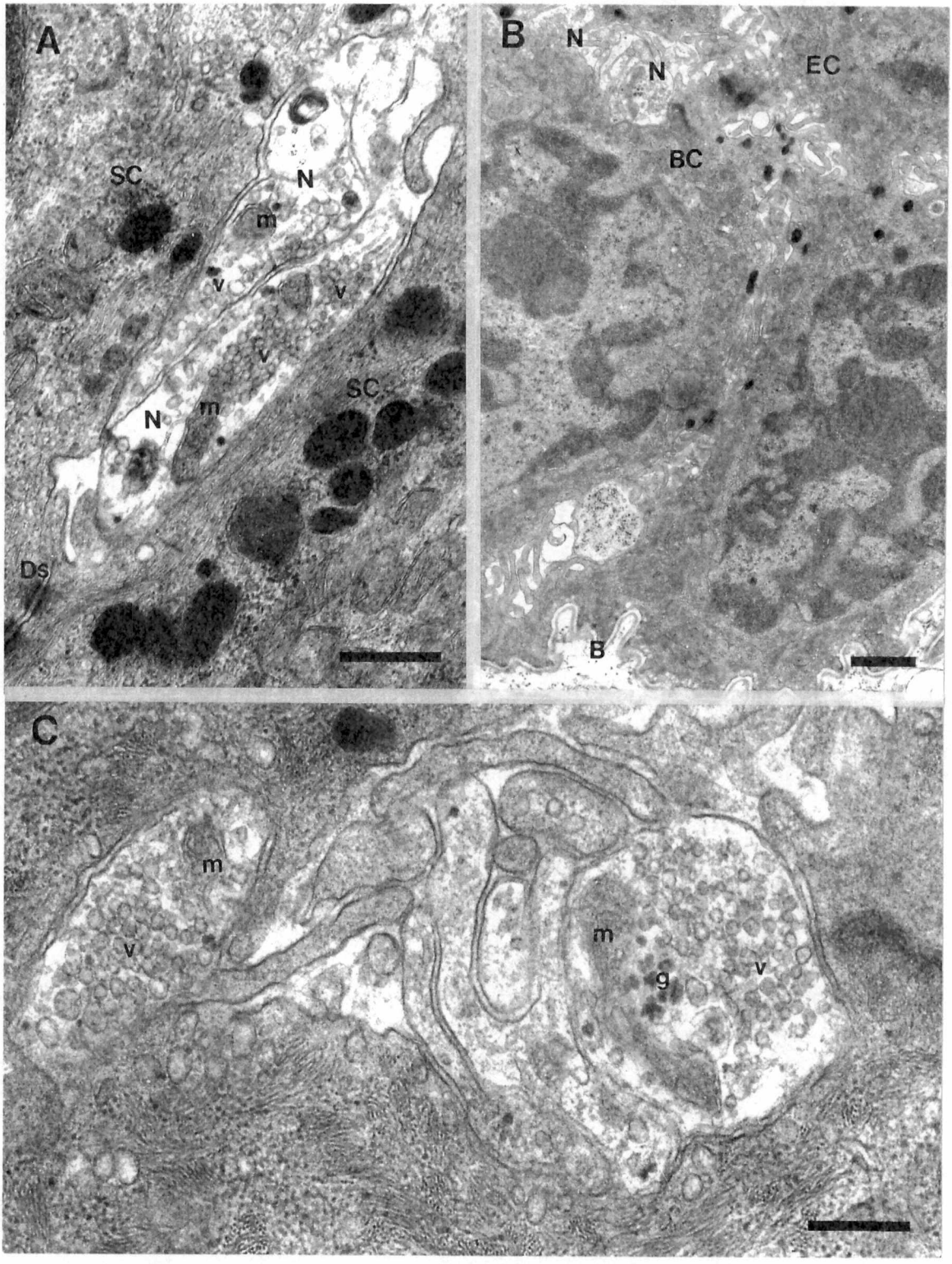

A
CC
SC
B
B
CC
C
v
m
v

nerve endings on its basal surface. Many small neurotransmitter vesicles and also a mitochondrion are seen in the two nerve endings (Fig. 2C). Ultrastructural features of the nerve endings (Fig. 3) are comparable to the established structural characteristics of cholinergic nerve endings (Richardson, 1966). The nerve endings contain a number of neurotransmitter vesicles which are clear small vesicles, glycogen particles and mitochondria (Fig. 3A,B & C)). Nerve endings were found to terminate on secretory basal cells (Fig. 3A,B) as well as on ciliated epithelial cells (Fig. 2B,C). These nerve endings are located between basal and columnar surface cells in regions with numerous microvillous projections (Figs. 2-3) but not along the basal lamina. No synaptic structures were found in our studies. There were no sensory cells (Puchelle, 1984) in the region where nerve endings were seen, which suggests that these nerve endings were not from sensory nerves. Considerable effort was made to find nerve endings in outgrowths, but no nerve fibers or endings were found.

Effect of electrical stimulation of palatine nerve on explant and outgrowth ciliary beat frequency

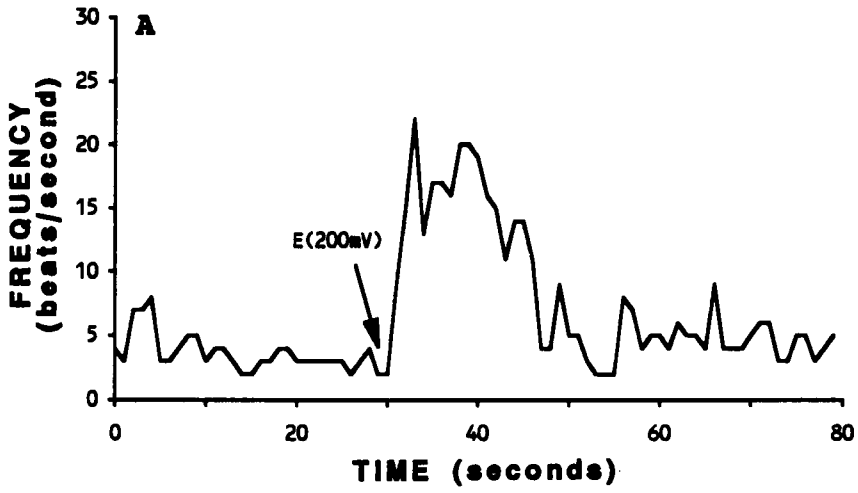
Although it has been shown that electrical stimulation of the palatine nerve increases mucociliary transport rate both *in vivo* and *in vitro* (Slaughter and Aiello, 1982; Hernandez and Aiello, 1992), no studies of the effect of nerve stimulation on ciliary beat frequency at the single cell level

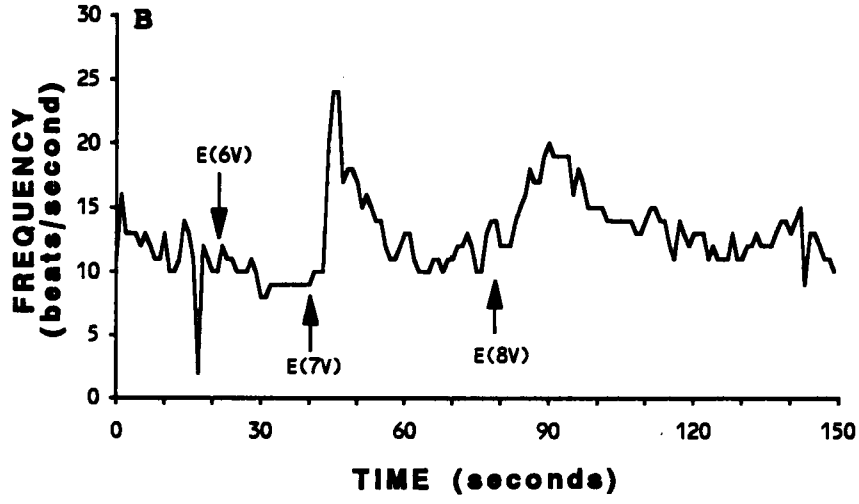
Fig. 3. Fine structures of the intra-epithelial nerve endings. (A). Two nerve endings (N) between basal secretory cells (SC). Numerus small neurotransmitter vesicles (v) and mitochondria (m) are shown in nerve endings. Desmosome (Ds) located between the two cells. Bar = 0.5 μm. (B). A group of nerve endings (N) between basal cells (BC) and superficial epithelial cells (EC). Basal lamina (B). Bar = 1 μm. (C). Enlargement of the nerve endings shown in (B). Numerus small neurotransmitter vesicles (v), glycogen particles (g) and several mitochondria (m) are shown in the nerve endings. Bar = 0.5 μm.

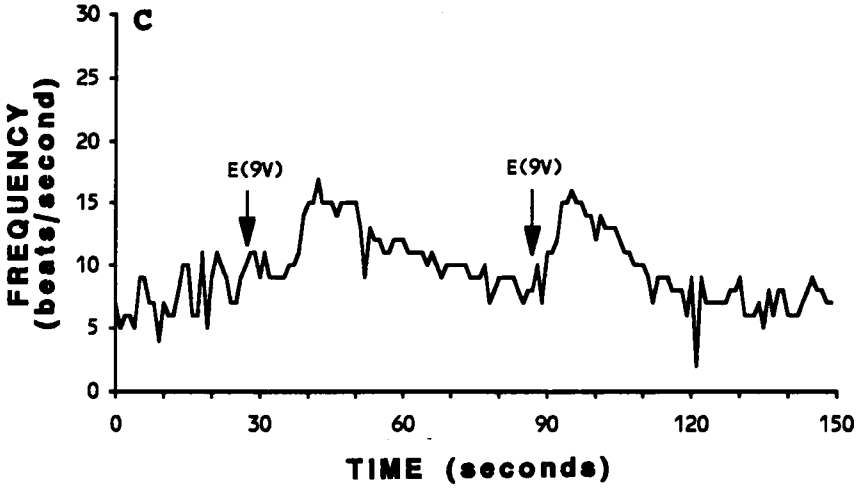

A
SC
N
m
v
v
v
SC
N
m
Ds
B
N
N
EC
BC
B
C
m
v
m
g
v

have been conducted. In this study, we investigated the effect of electrical stimulation of the palatine nerve on ciliary beat frequency directly at the single cell level. Electrical stimulation of the palatine nerves was performed on fresh palate-palatine nerve preparations of three frogs and on cultured palate-palatine nerve preparations of two frogs. Under this stimulus, resting cilia on the explant showed instant response with rapid beating which lasted for about 5-20 seconds. The results showed that the threshold of stimulus on fresh tissue preparations was about 80 mV (10 ms, single stimulus). Since fresh tissue was always moving in the plastic culture dish, it was impossible to measure single cell ciliary response over time. Individual frequency values, however, could be obtained from the edge of the palate. With two day cultured palate-palatine nerve preparations, the palates attached to culture dishes, and outgrowth appeared. Thus a ciliary beat frequency time curve of a single ciliated cell in the outgrowth was easily recorded and plotted. The response of the outgrowth cell (Fig. 4A) was a rapid increase in beat frequency immediately after stimulation of the nerve, in this case reaching a level of 22 beats/second and returning to baseline of 4 beats/second after about 20 seconds. The threshold of stimulation of the palatine nerve for a 2 day culture was about 200 mV (10 ms, single stimulus).

Fig. 4. Ciliary response to electrical stimulation. (A). Ciliary frequency of an outgrowth cell in response to a single pulse (10 ms) of 200 mV electrical stimulation to the palatine nerve of a 2 day explant culture. (B). Ciliary frequency increase of an explant cell in response to electrical stimulation of the culture. Threshold stimulus is 7 V. (C). Ciliary frequency increase of an outgrowth ciliated cell in response to the electrical stimulation of the culture.


A
FREQUENCY (beats/second)
E(200mV)
TIME (seconds)


B
FREQUENCY (beats/second)
E(6V)
E(7V)
E(8V)
TIME (seconds)


C
FREQUENCY (beats/second)
E(9V)
E(9V)
TIME (seconds)

Effect of electrical stimulation on outgrowth cell ciliary beat frequency of the culture with explant intact

After it was established that ciliary beat frequency could be increased by electrical stimulation of the palatine nerve *in vitro* cultured outgrowth without the palatine nerve attached but with the explant present were stimulated to determine whether they would also show increase in ciliary beat frequency. Stimulation of the explant and outgrowth culture directly resulted in an increase in ciliary beat frequency. In the example presented (Fig. 4B) an increase of 10-12 beats/second over a baseline frequency of about 12 beats/second was observed. In this sample a second stimulus following initial response by about 30 secondsinduced a second ciliary response with a frequency peak of 20 beats/second. The results confirmed that ciliary beat frequency was able to be increased by electrical stimulation, but the threshold of the stimulation was increased to 6-8 V (Fig. 4B) which was about a 100 magnitude higher than the threshold (80 mV) for palatine nerve stimulation. Outgrowth cells which appear to lack innervation, also respond to electrical stimulation in a manner comparable to explant cells. Figure 4C shows an outgrowth cell which received a 9 V stimulus at 30 second and 90 second intervals. After both stimuli, ciliary beat frequency increased to between 16-18 beats/second from a baseline frequency of about 8 beats/second. Thus, it is apparent that ciliated cells both on the explant and outgrowth could be electrically stimulated.

The fact that ciliated cells respond to electrical stimulation *in vitro* raised the question of how palate ciliated cell response to electrical stimulation is controlled. There are at least two possibilities, (1) electrical stimulation causes nerve endings to release neurotransmitters which mediate ciliated cell response, and (2) ciliated cells respond to the electrical field stimulation directly by increasing ciliary activity. In order to determine whether the response to electrical stimulation was attributed to release acetylcholine from nerve endings, explant cultures from 6 frogs were incubated in 10^{-4}M atropine in frog Ringer's solution. Atropine treatment inhibited the explant and outgrowth response to electrical stimulation. Results (Fig. 5) showed that, before the atropine treatment, an outgrowth cell responded to a 10 V electrical stimulation with rapid increase in ciliary frequency (Fig. 5A); within 5 minutes of atropine treatment the response of the outgrowth cell was abolished (Fig. 5B). When atropine was removed from the culture dish and the culture was rinsed with frog ringer's, the ciliated cells again responded to the 10 V stimulation (Fig. 5C). In some experiments atropine treatment did not abolish ciliated cell response to electrical stimulation but did significantly reduce the duration of the response. Figure 6A shows such an example where an electrical stimulus (10 V) induced a ciliary response prior to atropine treatment. Frequency increased from 7-8 beats/second to a

Fig. 5. Atropine blockage of ciliary response of outgrowth ciliated cells to electrical stimulation. (A). Before atropine treatment an outgrowth ciliated cell responds to a 10 V electrical stimulus with frequency increase. (B). Five minutes of 10^{-4}M atropine treatment blocks ciliary response of the cell to the electrical stimulus. (C). Removing the atropine from the culture dish results in the recovery of ciliary response of the cell to electrical stimulation.

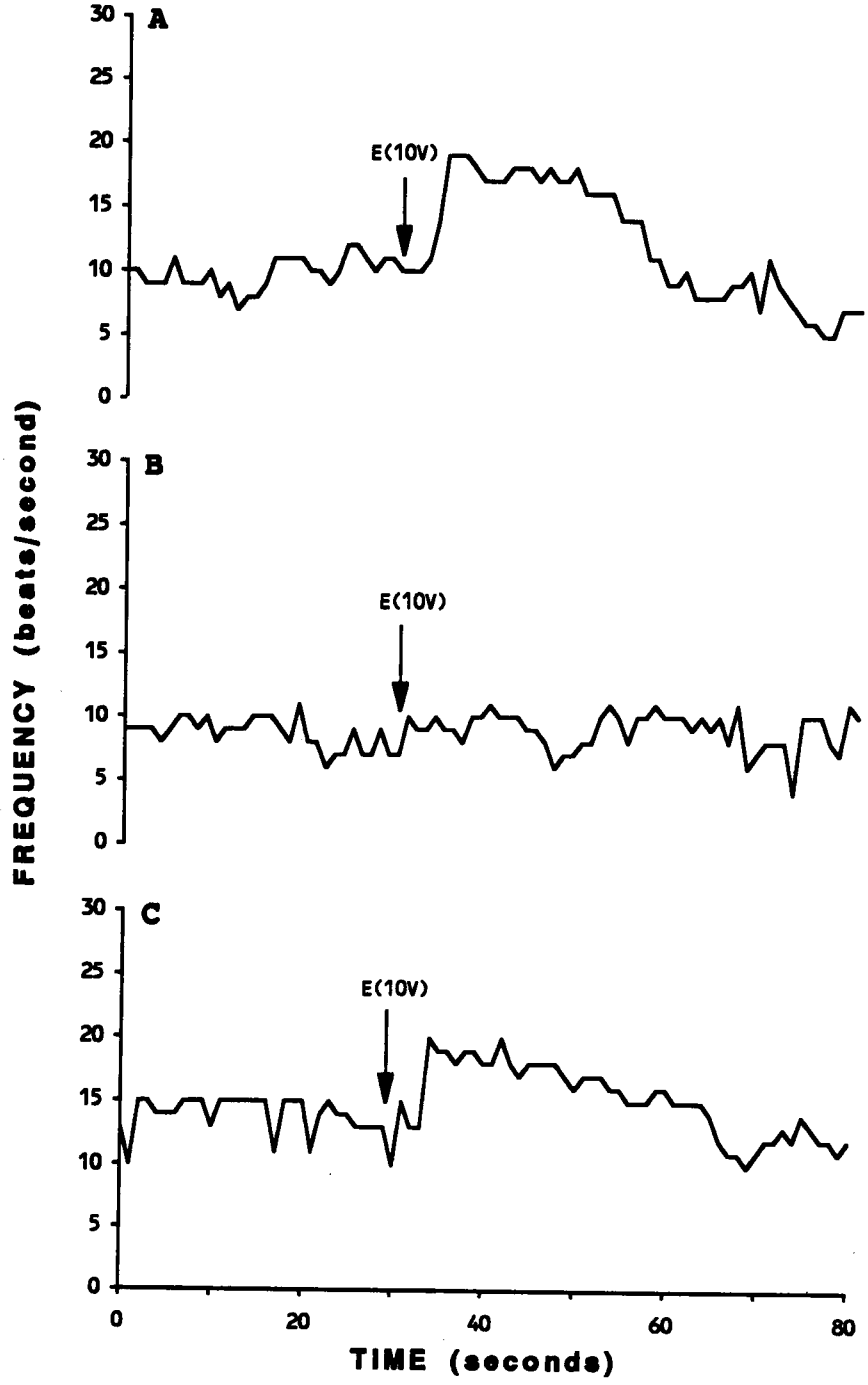
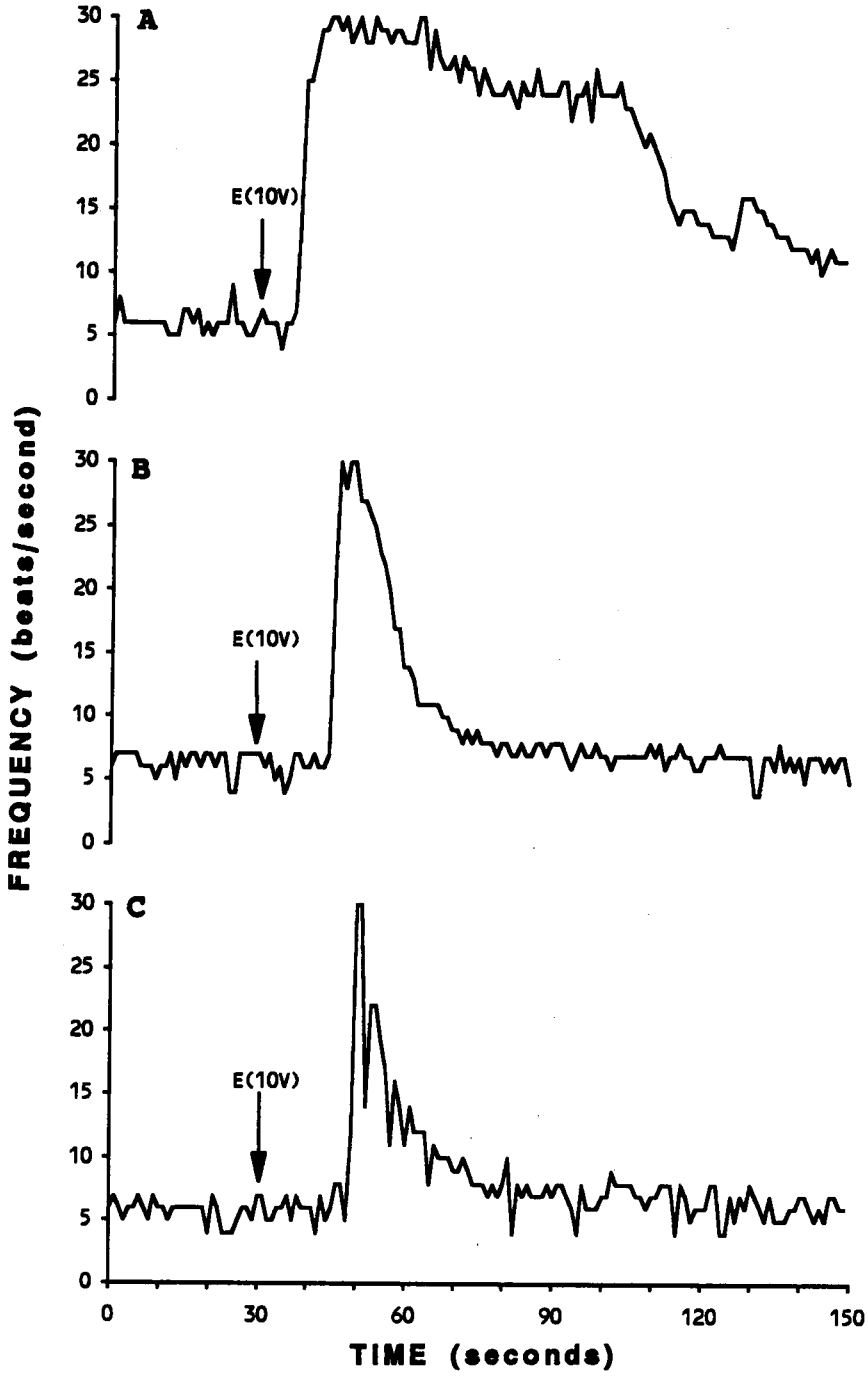

A
B
C
E(10V)
E(10V)
E(10V)
30
25
20
15
10
5
0
FREQUENCY (beats/second)
0
20
40
60
80
TIME (seconds)

Fig. 6. Atropine limits the duration of frequency increase of ciliated cell in response to electrical stimulation. (A). Before atropine treatment an outgrowth ciliated responds to a 10 V electrical stimulus with frequency increase. The increased frequency persists for about 90 seconds. (B). Two minutes of 10^{-4}M atropine treatments results in the declining of the duration of frequency increase to about 30 seconds. (C). Twenty minutes of the atropine treatment limits the duration of frequency increase to about 20 seconds.


A
B
C
E(10V)
E(10V)
E(10V)
FREQUENCY (beats/second)
TIME (seconds)
0
5
10
15
20
25
30
0
30
60
90
120
150

peak frequency of 30 beats/second. The duration of increase was about 90 seconds. Two minutes after 10^{-4} M atropine treatment, the same cell still showed a rapid increase of frequency to the peak of 30 beats/second but the duration of the response was only about 30 seconds (Fig. 6B), about 60 seconds less than that of the control in frog Ringer's solution. Twenty minutes after the atropine treatment the ciliated cell still responded to the electrical stimulus (10 V) with a peak ciliary frequency of 30 beats/second but the duration of frequency increase was only about 20 seconds (Fig. 6C). These results suggest that atropine only partially inhibits electrically stimulated ciliary response.

Effect of electrical stimulation on outgrowth cell ciliary beat frequency in the absence of explants

The purpose of this experiment was to determine whether ciliated outgrowth cells devoid of nerves or nerve endings could respond to electrical stimulation. Since no nerve structures were found in the outgrowths, a denervated ciliated cell layer could be obtained by removing the explant from the primary cultures. In every experiment, the same ciliated cells in the outgrowths were subject to measurement before and after explant removal. A typical cell (Fig. 7A) was stimulated with 9 V at 30 and 90 seconds. In both cases, frequency increased from 4-6 beats/sec to 16-18 beats/sec with the explant present. When the explant was removed (Fig. 7B), no increase in frequency was observed with stimuli of either

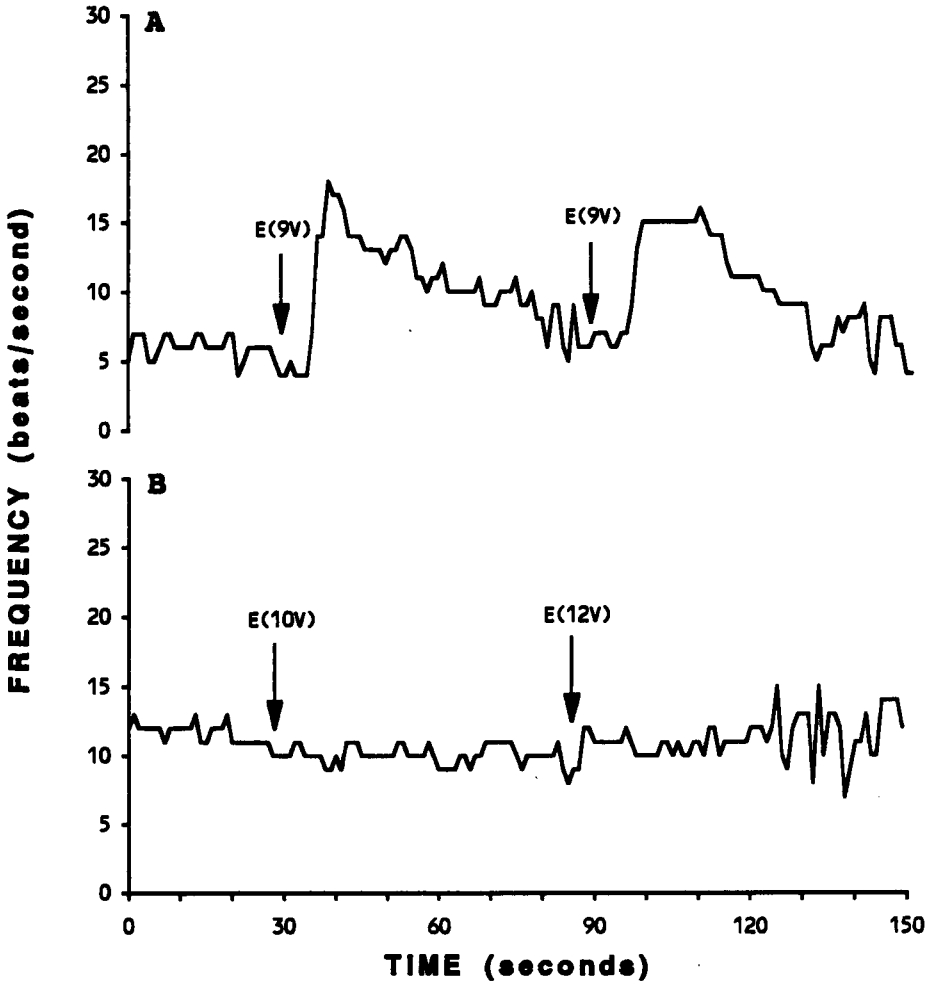


Fig. 7. Ciliary response of an outgrowth cell to electrical stimulation is explant dependent. (A). With intact explant an outgrowth ciliated cell responds to 9 V electrical stimulus with frequency increase. (B). After removal of the explant, the same outgrowth cell does not respond to even stronger electrical stimuli.

10 V or 12 V. The results showed that all the ciliated cells responded to electrical stimuli before removal of the explant, but the same cells did not respond to electrical stimuli after the explant was removed.

IV. DISCUSSION

This study presents evidence that palatine nerve endings are in direct contact with some ciliated epithelial cells, and stimulation of the palatine nerve *in vitro* excites ciliary beat. To our knowledge, this is the first study to demonstrate direct contact between the palatine nerve and palate ciliated cells. It is also the first *in vitro* study to examine the ciliary response of individual cells over time following electrical stimulation of the palatine nerve to explant cultures with intact nerve endings. As early as the 1930's, nerve influenced ciliary activity on frog palate had been observed. Studying the Japanese bull frog, Seo (1931) found that electrical stimulation of the glossopharyngeal nerve from the tongue activated a reflex that resulted in a increase in mucociliary transport on the palate. It was thought that the efferent arm of the reflex was the palatine nerve. Following experiments involving transection of the cranial and sympathetic nerves, Lucas (1935) reported the palatine nerve was from the 7th cranial nerve, a parasympathetic nerve and was responsible only for activation of ciliary movement. Further investigations (Slaughter and Aiello, 1982; Hernandez and Aiello,1992) confirmed the reflex and found that mucociliary transport and thus ciliary beat were increased by electrical stimulation of the glossopharyngeal nerve or the palatine nerve *in vivo*. The nerve stimulation induced ciliary activity was partially

inhibited by atropine (Slaughter and Aiello, 1982; Hernandez and Aiello, 1992). Mucus transport rate on the frog palate was also increased by administration of acetylcholine, and the effect could be blocked by atropine (Slaughter and Aiello, 1982; Maruyama et al., 1983). Direct measurement of ciliary frequency revealed that acetylcholine increased ciliary frequency and the effect was blocked by atropine (Aiello *et al.*, 1991). Dopaminergic inhibitory innervation to ciliated cells has also been suggested in the frog palate (Maruyarma et al., 1983) and the mussel *Mytilus edulis* (Paparo and Aiello, 1970; Catapane et al., 1978). Cilioinhibitory innervations have been observed in some organisms such as the mussel (Takahashi and Murakami, 1968), the scallop (Stommel and Stephens, 1985), and Velliger larve of a gastropod (Mackie et al., 1976). However, our *in vitro* results were similar to those of the *in vivo* study by Slaughter and Aiello (1982) and Hernandez and Aiello (1992) which favored only the cilioexcitatory function of the palatine nerve. The finding of intra-epithelial palatine nerve endings, the direct innervation of ciliated cells, completes the efferent arm of the reflex arch to ciliated cells of frog palate. The observations of increased ciliary activity by electrical stimulation of the nerve directly verifies the stimulatory effect of the palatine nerve on the ciliated cells.

The intra-epithelial nerve endings have typical axon terminal morphology with many neurotransmitter vesicles,

mitochondria and glycogen particles. These transmitter vesicles resemble the 'synaptic' vesicles first described by De Robertis *et al.*, (1955) and Palay (1956), and classified as cholinergic by Richardson (1966). No synapse structure was found in these nerve endings, which were comparable to other intra-epithelial nerve endings described by Jeffery *et al.*, (1973) in normal rat airways, by Addicks *et al.*, (1982) in the mucosa of rabbit maxillary sinus, by Cook *et al.*, (1987) in the saccopleural membrane of the domestic fowl (*Gallus gallus*) and by Mackie *et al.*, (1976) in gastropod larvae. These authors have suggested that neurotransmitter released from nerve endings were able to diffuse in the intercellular space and bind to their receptors causing cellular response.

Ciliated cells have been thought to be excitable cells because they are sensitive to mechanical, electrical, chemical, and hormonal stimuli (Slaughter and Aiello, 1982; Sanderson *et al.*, 1988, 1990; Spungin and Silberberg, 1984; Weiss *et al.*, 1992). However, ciliated cell response to electrical field stimulation has only been well studied on protozoan ciliates, such as *Paramecium,* which responds to depolarization stimuli by changing direction and increasing frequency of ciliary beat (Eckert, 1972; Brehm and Eckert, 1978; de Peyer and Machemer, 1982). The present experiments show that the ciliated cells of the frog palate respond to electrical stimulation by ciliary beat frequency increase, but the response is dependent on the continuity of the ciliated

cells with the explant which contains palatine nerve endings. As soon as the continuity is destroyed, *e.g.*, by removing the explant from the culture or making a cut between the explant and the outgrowth, the ciliated cells themselves do not respond to electrical stimulation by increasing ciliary beat frequency. Our results suggest (1) that the ciliary activity of ciliated cells in the frog palate are not directly electrically excitable, but dependent rather (2) on the intra-epithelial nerve endings which are still functional in the primary culture. These suggestions are further supported by the evidence that atropine inhibits ciliated cells response to electrical stimulation in the outgrowth culture. Therefore, we conclude that the ciliary response of frog explant/outgrowth culture cells to electrical stimulation is mediated by cholinergic innervations and muscarinic receptors of the tissue, but not to a direct response of ciliated cells to electrical stimulation such as with *Paramecium*.

In the present study, we looked extensively for intra-epithelial nerve endings in the explant and also for nerve migration into the outgrowth. The results showed that the intra-epithelial nerve endings, although relatively abundant, did not make contact with most epithelial cells in the palate. Also, no nerve fibers or endings were found in outgrowths. However, the ciliary response involved ciliated cells not only in explants , but also in outgrowths. These results suggest that an intercellular communication mechanism might be

involved in the progression of the ciliary response from explants to outgrowths. The possible intercellular communication could be mechanical or hydrodynamic (ciliary beat of one cell activating cilia of the adjacent cell causing a stimulated beat) or chemical (intracellular signal molecules passed through gap junctions from one cell into the next cell). The mechanical intercellular communication has been suggested earlier (Iravani, 1969; Aiello and Sleigh, 1977; Sanderson and Sleigh, 1981). Since ciliated cells in outgrowths are flattened, cilia of neighboring cells are not in close continuity in outgrowths. Thus it seems unlikely that mechanical/hydrodynamic intercellular communication could be the main mechanism for the propagation of the ciliary response. Therefore, intercellular signal transduction through gap junctions may be the most likely mechanism for propagation of the ciliary response throughout the outgrowths. Studies on rabbit trachea ciliated epithelial cultures by Sanderson *et al.*, (1988) showed that the ciliated cells were indeed electrically coupled. They also found that gap junctions existed between cells in both epithelial explant and outgrowth. Ciliary response from a mechanically stimulated cell could gradually be transmitted to adjacent cells. The intercellular signal molecule was calcium or inositol trisphosphate (Sanderson et al., 1990). A more recent study showed that intercellular propagation of calcium waves through the rabbit tracheal ciliated epithelium was mediated by

inositol trisphosphate (Boitano *et al.*, 1992). Comparing these results, we believe that a similar mechanism may be applicable to the frog palate epithelium once the key cell has been stimulated to increase frequency by acetylcholine. Future study will concentrate on intercellular signal transduction and the signal molecule(s).

In summary, the frog palate ciliated epithelium is an innervated tissue. The ciliary activity is under the direct influence of the cholinergic palatine nerve, and excitation of this nerve to increase ciliary beat frequency is the dominant effect on ciliary activity. Ciliated cells are not excitable by direct electrical stimulation in the absence of the palatine nerve, yet the nervous signals may be transduced from innervated cell to cell without innervation through intercellular communication which causes all ciliated cells to respond to nervous stimulation. Finally, the explant culture system can be useful as both an innervated ciliated epithelial model (complete culture) *in vitro* and a denervated ciliated cell culture model (formed by removing the explant from the culture). With this *in vitro* model and electrical stimulation, the study of nerve controlled ciliary activity may be facilitated.

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PART III

CHARACTERIZATION OF MORPHOLOGY AND CILIARY ACTIVITY OF FROG PALATE EXPLANT CULTURE

I. INTRODUCTION

Among the ciliary systems, the frog palate is a particularly interesting tissue for studying ciliary activity and mucus transport (Wilson *et al.*, 1975; Aiello and Sleigh, 1977; Puchelle *at al.*, 1982; Spungin and Silberberg, 1984). The tissue is easy to obtain, very tolerant to experiment conditions and can be maintained in an explant culture for *in vitro* study (Eshel *et al.*, 1985; Aiello *et al.*,1991). In addition, the ciliated epithelium of the frog palate has been found to be similar in structure and ciliary function to the mammalian tracheo-bronchial ciliated epithelia (Puchelle *et al.*, 1984; Zahm *et al.*, 1986). It has been used to study the effects of various neurotransmitters, hormones and drugs on ciliary activity (Giordano *et al.*, 1977; Slaughter and Aiello, 1982; Maruyama *et al.*, 1983, 1984, 1985; ; Aiello *et al.*, 1988, 1991; Ovadyahu *et al.*, 1988; Hernandez and Aiello, 1992; Weiss *et al.*, 1992).

While explant cultures of frog palate ciliated epithelium have proven to be a valuable method in the study of ciliary activity and its regulation (Eshel *et al.*, 1985, 1987; Hernandez *et al.*, 1992; Weiss *et al.*, 1992), only few studies have examined palate outgrowth cell ciliary frequency (Eshel *et al.*, 1985; Aiello *et al.*, 1991). That study did not consider the possibility of time related changes in structure or ciliary frequency. We have observed that ciliary activity of the frog palate outgrowth cells in culture is not constant

either in beat frequency or time related pattern of beat. However, little information has been available on the relationship between ciliary activity and culture age. In addition, it has been known that frog palate is an innervated tissue (Seo, 1931; Lucas, 1935; Slaughter and Aiello, 1982). We have found intra-epithelial nerve endings and direct innervation of ciliated cells in the palate (Part II). It was also verified that the ciliary response of the frog palate explant culture to electrical stimulation was mediated by the intra-epithelial cholinergic nerve endings (Part II). If ciliated cells of the explant respond to electrical stimulation with increase of ciliary frequency, it would be concluded that there were functional nerve endings surviving in the explant. Finally, the morphology of the frog palate explant and outgrowth culture has not been characterized. This study was undertaken to characterize the changes in morphology, ciliary beat frequency, ciliary beat pattern, ciliary response to electrical stimulation and acetylcholine of palate outgrowth cells over three weeks in primary culture.

II. MATERIALS AND METHODS

Tissue culture

Frogs (*Rana pipiens*) were sacrificed by pithing the brain and the spinal cord. The palate tissue culture was prepared according to the methods of Aiello *et al.*, (1991). Briefly, the palate mucosa was excised, rinsed with frog Ringer's solution (133 mM NaCl, 2.4 mM $NaHCO_3$, 1.1 mM $CaCl_2$, 1.9 mM KCl, 11 mM Glucose and 10 mM HEPE, pH 7.2) to remove mucus and blood, and placed in amphibian culture medium (Life Technologies Inc. Grand Island, NY) supplemented with 10% Nu-serum, 50 units/ml of penicillin and 50 μg/ml of gentamicin. The palate was pinned onto a piece of dental wax and cut into 1 mm^2 pieces. One or two pieces of the palate explant were placed, connective tissue side down, in a 35 mm plastic culture dish (Falcon) with 0.8 ml of the culture medium (Wolf amphibian culture medium, GIBCO). The tissues were cultured in humidified air at 25^0C. Epithelial outgrowths appeared in 24 hours. After secure attachment, the culture medium was kept at 1.5 ml and changed every 48 hours. The cultures were maintained for up to 3 weeks.

Light and electron microscopy

The fresh palate and explant cultures (day 3, 7, 14 and 20) were washed 2 times in ice cold phosphate buffer (0.1 M, pH 7.4) for 5 minutes. The tissues were fixed in a mixed fixative containing 1 part 2.5% glutaraldehyde in phosphate

buffer (pH 7.4) and 1 part 1.66% OsO_4 in phosphate buffer (pH 7.4) for 1 hour on ice. Dehydration was through a graded ethanol series, and the tissue was embedded in EM bed-812 resin (Electron Microscopy Sciences). For light microscopy, 1 μm sections were prepared and stained with 1% toluidine blue in 1% borax. For electron microscopy, 70-80 nm sections were stained with uranyl acetate and lead citrate, and examined with an Hitachi H-600 electron microscope at 75 kV. For scanning electron microscopy, the isolated frog palate and explant cultures were rinsed with the frog Ringer's solution to remove mucus. The fixation and dehydration were the same as methods mentioned above. The tissues were then dried with the Ladd critical point dryer (Ladd Research Industries, Inc.), and coated with gold/palladium using a Denton vacuum DV-515 automatic evaporator (Cherry Hill Industrial Center, Cherry Hill N.J.). Samples were viewed with an Hitachi S-800 scanning electron microscop operated at 20 kV.

Optical spectrum analysis of single cell ciliary beat frequency

Outgrowth cells from 3, 5, 8, 10, 13, 15 and 20 days in culture were subjected to ciliary beat frequency measurement with the optical spectrum analyzer system described by Kennedy and Duckett (1981). Culture dishes were placed on the stage of an inverted microscope (Nikon, DIAPHOT-TMD) with a television camera (RCA, TC1005) positioned in parallel with the viewing eyepieces. The image of beating cilia was

recorded with a video tape recorder (Panasonic, AG-1950) and observed on a television monitor. A photomultiplier detector (Oriel model 2870) with a limiting aperture was positioned over the image on the monitor and the signal fed to a frequency spectrum analyzer (Hewlett-Packard model 358A) which displayed the spectrum on an oscilloscope screen. The major peak of this spectrum corresponds to the dominant ciliary beat frequency, and is updated every half second. Measurements of ciliary frequency were made in real time on individual cells. Every cell was moniyored for 10 seconds and the highest frequency was recorded as the fundamental ciliary frequency of the cell. Thirty to fifty cells from each culture period (3-20 days) were measured randomly in rapid succession and the values averaged.

Time related analysis of ciliary beat pattern

To observe the ciliary beat pattern of individual cells over time, an IBM PC (IBM, PS/2, model 50Z) computer, with data acquisition and ciliary beat frequency analysis programs installed, was linked with the optical spectrum analyzer system. With this system, the ciliary beat frequency of a single cell was monitored for 300 seconds, and a spectrum corresponding to the ciliary beat was recorded by the computer each second. The fundamental frequency of the spectrum was recorded as the ciliary frequency of the cell during that second. In this manner, second by second, at the end of the

300 seconds the computer produced a curve representing the ciliary beat pattern over the 300 seconds. Outgrowths from the explant cultures of day 3, 7, 14 and 20 underwent this measurement.

Determination of the relationship between cessation of ciliary beat and culture age

The objective of this experiment was to determine what percentage of ciliated cells stop beating at least once in a 5 minute observation period as a function of culture age. Outgrowths of day 3, 7, 14 and 20 explant cultures were studied; the same 5 cultures were used for one entire series of experiments. For every outgrowth, 20 cells near the explant (in a 5 cell range from the edge of the explant) and 20 cells distant from the explant (at least 10 cells away from the edge of the explant) which were actively beating at the beginning of the experiment were chosen to be continually observed on the video monitor. Cells which stopped beating completely were marked on the monitor. At the end of the 5 minutes, the percentage of 20 cells which stopped beating at least once were calculated.

Determination of relationship between ciliary response to electrical stimulation or acetylcholine and culture age

Pieces of fresh palate tissues and explant cultures were subjected to electrical stimulation. Ciliary frequency was measured on single cells to determine the cellular responses

to the stimuli. A pair of electrodes (platinum wire of 0.008 inch in diameter) was connected to a Grass stimulater (S6, Grass Instrument). Stimuli used in these experiments were single pulses of monophasic rectangular wave, 10 ms in duration with a voltage range between 2 V -- 20 V. To stimulate fresh palate tissue, a piece of palate (connective tissue side down) was pinned to the bottom of a 35 mm plastic culture dish and covered with 1 ml of frog ringer's. The culture dish was attached on the stage of the inverted phase contrast microscope. The pair of electrodes was submerged in the frog ringer's with a piece of palate between, but without making contact with the tissue. To stimulate the explant culture, the culture medium was replaced with frog ringer's. The culture dish and electrodes were mounted on the microscope as mentioned above for the fresh palate. Ciliary responses to the electrical stimuli were observed in real time and recorded on video tapes. Frequencies were recorded from the edge of fresh palates and from ciliated cells of the outgrowth in the range of 2-5 cells from the edge of the explant. Ciliary beat pattern changes in response to the electrical stimulation were measured in real time. With this procedure, thresholds of electrical stimuli were determined for explant cultures over the three week culture period. Ciliary response to acetylcholine was also determined on the explant cultures of different age in a three week culture period with the methods of Aiello *et al.*, (1991).

III. RESULTS

Morphological observations

The frog palate ciliated epithelium is a stratified epithelium which is composed of two to four layers of cells (Fig. 1A-insert). The superficial layer of the epithelium contains primarily ciliated cells and mucus cells (Fig. 1A). Plaques of sensory cells are also present in some regions of the palate . Cilia are very tightly packed on the epithelial surface. Both actively beating cilia (Fig. 1B) and resting cilia (Fig. 1C) can be observed with the scanning electron microscope. Actively beating cilia are seen in different positions of the beat cycle (Fig. 1B). In comparison, resting cilia (Fig. 1C) are all flattened in one direction on the surface of the epithelium. Mucus cells are not readily identifiable in surface morphology, but regions called sensory plaques can be observed. The organization of sensory plaques is not considered here. Below the superficial layer are one to three layers of basal cells. Some of the basal cells appear to have non-distinct characteristics but some contain many small electron dense granules in the cytoplasm. The distribution of these granules is mostly in the apical half of the cytoplasm and have been suggested by Maruyama *et al.*, (1983) to contain dopamine. Their proximity to ciliated and mucus cells suggests some role in regulation of surface cell function. The characteristics of a basal secretory cell are also shown in Fig. 3A.

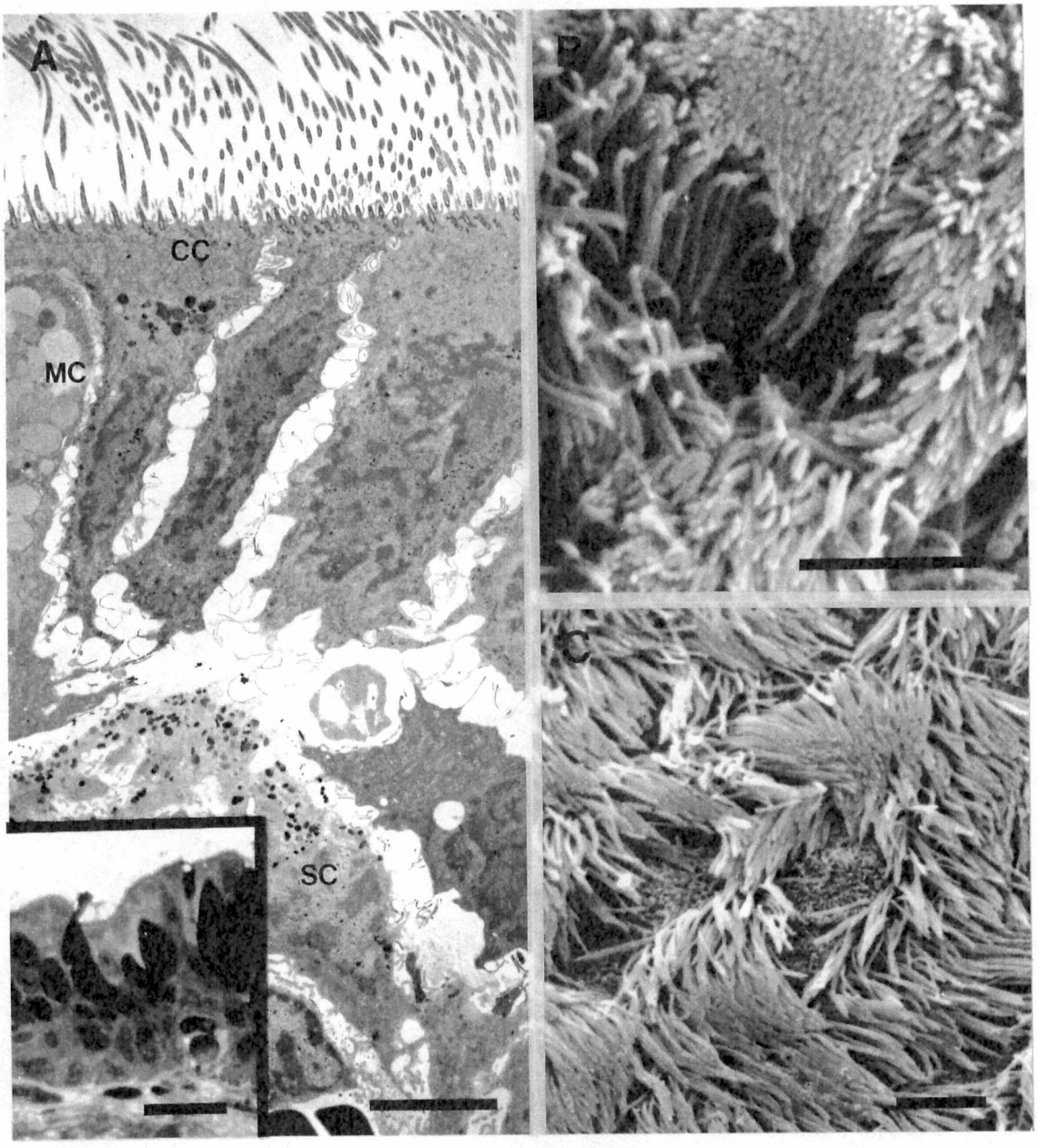
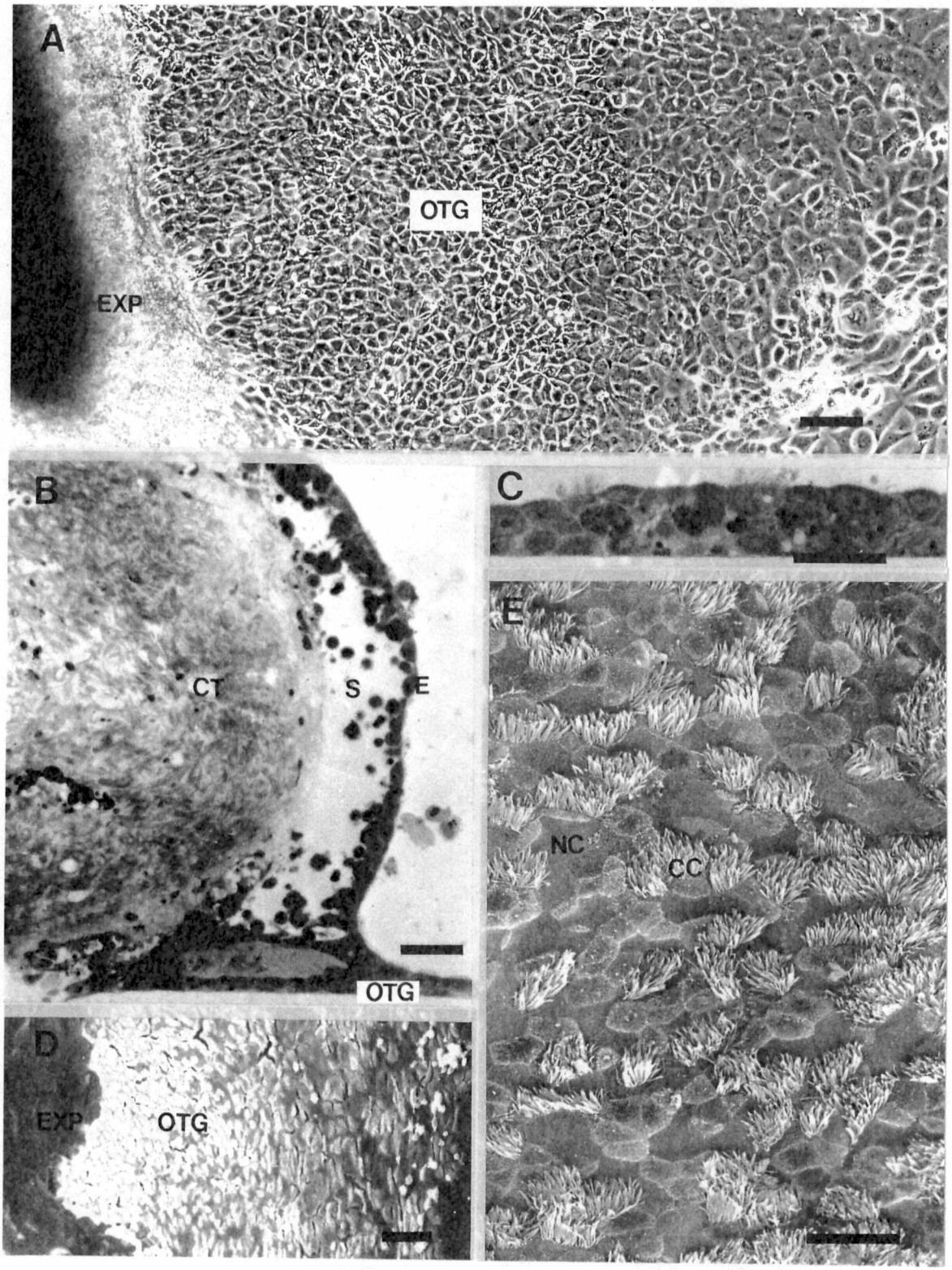


Fig. 1. Micrographs of frog palate explant. (A). A transmission electron micrograph shows ciliated cells (CC), mucus cell (MC) and basal secretory cells (SC) of the frog palate. Bar = 5 μm. Insert: a light micrograph of the stratified ciliated epithelium of the palate. Bar = 40 μm. (B). a scanning micrograph of cilia in different stages of the beat cycle of the palate. Bar = 5 μm. (C). A scanning micrograph of resting cilia of the palate. Bar = 5 μm.

Pieces of palate called explants provide the cells for the outgrowth. Formation of the outgrowth occurs by migration of cells from the palate explant. Phase contrast microscopy reveals that the outgrowth appears around the explant within 24 hours. Expansion of the outgrowth (Fig. 2A) progresses until day 9 or 10. During the remaining culture time, cells are gradually lost from the edge of the outgrowth. In days 14 to 20 of cultures, some ciliated cells migrate away from the outgrowth, losing contact with other cells, but their cilia are still able to beat actively. The cells closest to the explant have a smaller apical surface than the more distant cells, suggesting that as cells migrate from the explant they become more squamous in shape. Examination of the explant culture in cross section shows that the epithelial layer migrates outward to become the outgrowth (Fig. 2B). By 14-20 days in culture the underlying connective tissue separates from the epithelium (Fig. 2B). However, the superficial layer of the epithelium is still intact. Cross-sections of outgrowth show that it is composed of more than one layer, mostly two or three layers, of cells (Fig. 2C). Cilia still appear on the surface of the outgrowth, but they are not as tightly packed as in the palate. With scanning electron microscopy, the ciliated cells are clearly shown in both low and high magnification (Fig. 2D,E). At low magnification, it is evident that close to the explant the ciliated cells are more tightly packed and the apical surface of each cell is

Fig. 2. Micrographs of the frog palate outgrowth cultures. (A). A phase contrast micrograph shows a day 5 outgrowth culture. Explant (EXP), outgrowth (OTG). Bar = 100 μm. (B). A light micrograph of a cross section of a day 20 outgrowth culture. A large space (S) is shown between the epithelium (E) and the connective tissue (CT), which is caused by degradation of the tissue. Outgrowth (OTG). Bar = 100 μm. (C). A light micrograph of the cross section of a outgrowth shows the characteristic stratified ciliated epithelium. Bar = 20 μm. (D). A low magnification scanning micrograph of a palate explant (EXP) with outgrowth (OTG) culture. Bar = 100 μm. (E). Scanning micrograph of a outgrowth shows ciliated cells (CC) and non-ciliated cells. Bar = 20 μm.


A
OTG
EXP
B
CT
S
E
OTG
C
E
NC
CC
D
EXP
OTG

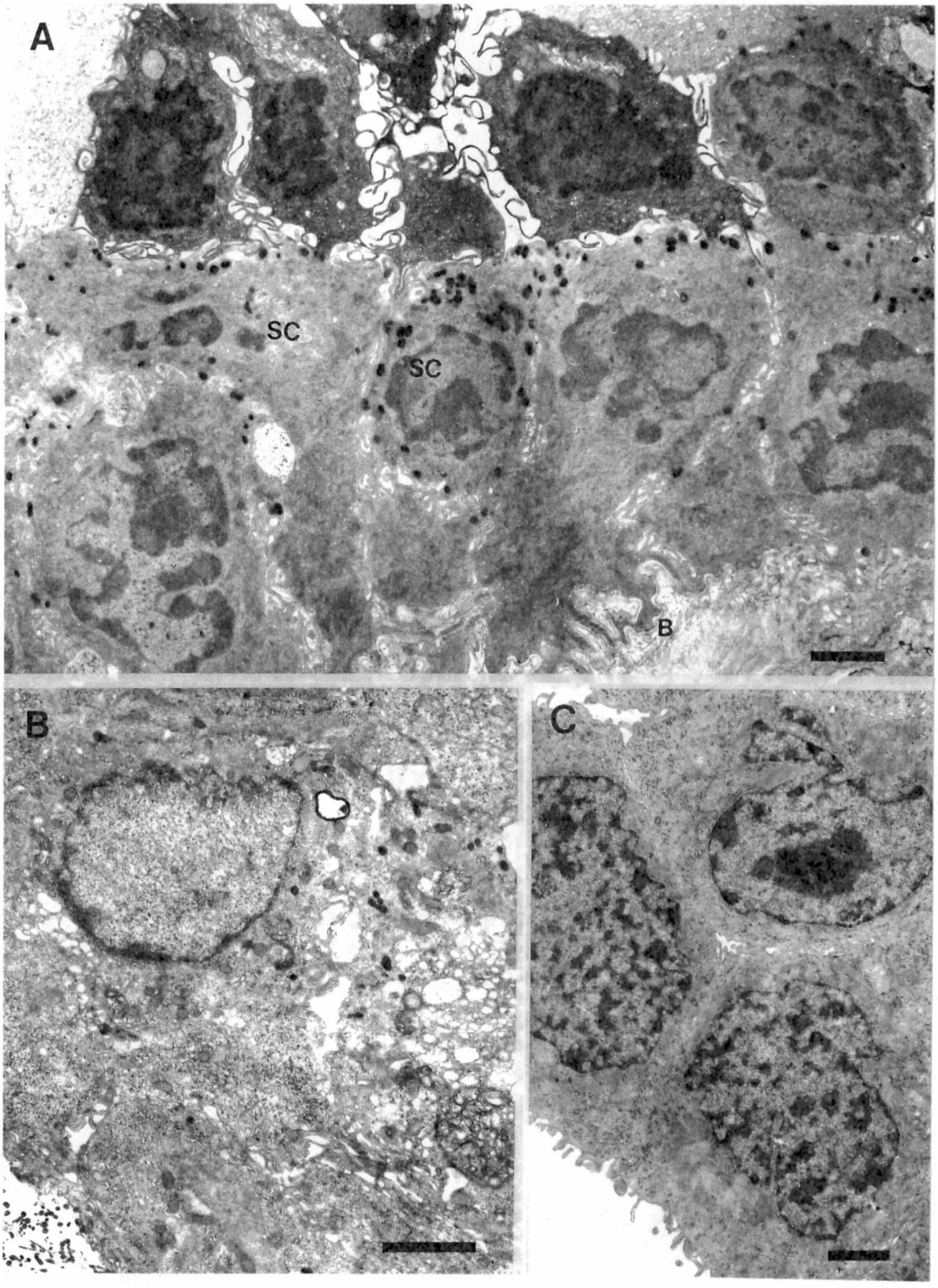
smaller (Fig. 2D). At high magnification, not only ciliated cells, but also non-ciliated mucus cells could be clearly differentiated from each other (Fig. 2E). However, examination of sections of this tissue suggests that mucus cells lack secretions in 14-20 day cultures.

Basal secretory cells are common components of fresh frog palate as mentioned above. They are characteristically located on the bottom layer of the epithelium. They contain numerous electron opaque secretory granules which are concentrated above the nucleus, towards the plasma membrane which contacts the bottom of ciliated or mucus cells (Fig. 3A). The number of granules in the secretory cells decrease rapidly over time in culture. Loss of granules occurs as early as day 3 of culture (Fig. 3B). Although the cells still have some secretory granules, the granules are only sparsely distributed in the cytoplasm. The basal secretory cells of day 7 cultures are similar to that of day 3 cultures, and still contain a few granules in the cytoplasm. The secretory granules disappear from the basal cells by day 14 to 20 of culturing (Fig. 3C).

Change in ciliary beat frequency as a function of culture age

The purpose of this experiment was to determine whether or not there is any change in ciliary beat frequency as a function of culture age. To accomplish this ciliary beat frequency of 50 ciliated outgrowth cells were measured

Fig. 3. Fine structures of basal secretory cells. (A). In fresh palate, basal secretory cells (SC) contain many electron dense granules in the cytoplasm. Basal lamina (B). Bar = 2 μm. (B). In 3 day cultured explant, the basal secretory cells contain only a few secretory granules. Bar = 2 μm. (C). In day 20 cultured explant, the basal secretory cells do not have secretory granules in the cytoplasm. Bar = 2 μm.


A
SC
SC
B
B
C

repeatedly on different days (3-21 days) over a three week culture period. The ciliary beat frequency increased gradually (Fig. 4) in the first two weeks (10.89±0.48 beats/second on day 3, 14.51±0.58 beats/second on day 5, 15.07±0.41 beats/second on day 8, 16.71±0.40 beats/second on day 10 and 19.05±0.42 beats/second on day 13). The frequency of cultured cells plateaued at the high frequency on day 13 and remained there throughout the third week in culture (19.39±0.45 beats/second on day 15 and 20.10±0.36 beats/second on day 20). Thus it appeared that the older the culture, the higher the ciliary beat frequency in the three week culture period. It was often observed in cultures older than 14 days that some ciliated cells, which migrated away from and lost contact with the outgrowth, showed a high ciliary beat frequency (about 18-20 beats/second).

Change in the pattern of ciliary beat in individual cells over time as a function of culture age

The objective of this experiment was to examine the ciliary beat pattern of individual cells over time as a function of culture age. The ciliary beat frequency, second by second for 5 minutes, was recorded at the single cell level from outgrowths of day 3, 7, 14 and 20 cultures (Fig. 5) to determine the pattern of ciliary beat over time. Ten cells from each day were measured. The results from day 3 and 7 cultures (Fig. 5A) showed big fluctuation in the pattern of ciliary beat with many cilia even stopping their beat for

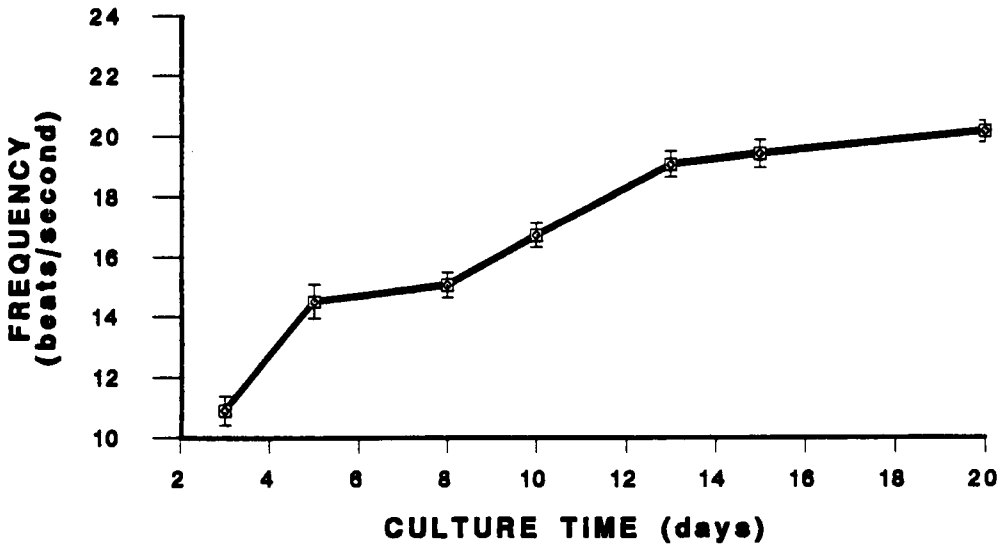
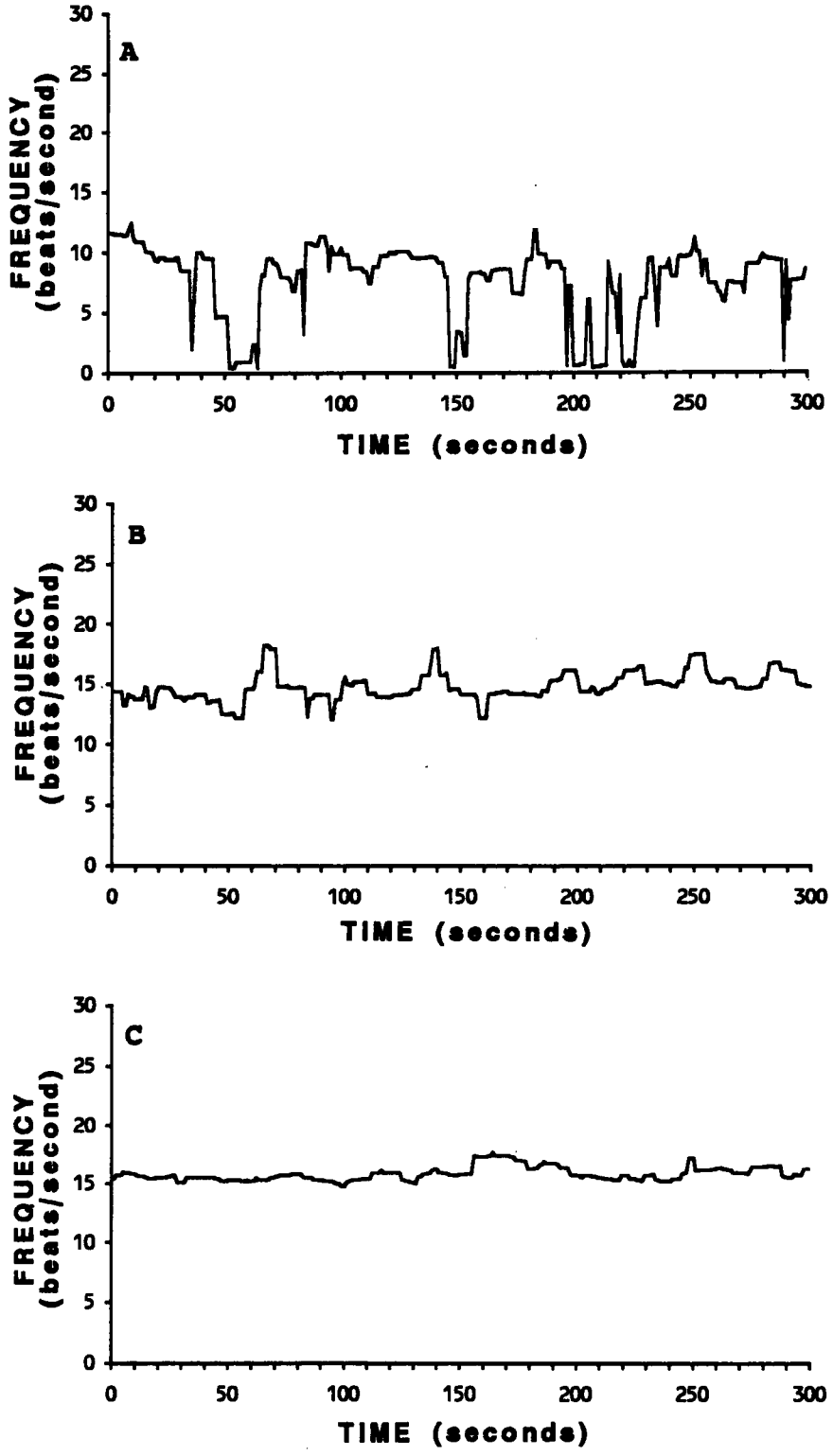


Fig. 4. Ciliary beat frequency change in outgrowth cells during 20 day culture period. The frequency gradually increases with culture time. Each point represents ciliary frequency (mean±SE) of 50 cells.

Fig. 5. Ciliary beat frequency patterns of individual outgrowth cells during 20 days in culture. (A). From a day 3 culture. (B). From a day 14 culture. (C). From a day 20 culture.


A
FREQUENCY (beats/second)
30
25
20
15
10
5
0
0
50
100
150
200
250
300
TIME (seconds)
B
FREQUENCY (beats/second)
30
25
20
15
10
5
0
0
50
100
150
200
250
300
TIME (seconds)
C
FREQUENCY (beats/second)
30
25
20
15
10
5
0
0
50
100
150
200
250
300
TIME (seconds)

short intervals (1-several seconds). With day 14 cultures (Fig. 5B), the fluctuation decreased significantly and little or no cessation of beat was evident. Most cells showed a periodic increase above a mean frequency with only occasional fluctuations below the mean. On day 20 of culture (Fig. 5C), the ciliary beat pattern became relatively constant with occasional fluctuations above a baseline frequency. These results revealed that the ciliary beat pattern changed from large fluctuation in frequency above and below a mean to fluctuations above a baseline but no fluctuations below the baseline. Theses changes were a function of the culture age.

Relationship between cessation of ciliary beat and culture age

Since it had been found that individual cells showed large fluctuation around a mean frequency, some stopped beating in the early outgrowth cultures, and some gave much less fluctuation and no cessation of beating in the older cultures, we examined cultures to determine the proportion of cells which stopped beating at different ages of culture. Cells from ten explant cultures were measured on day 3, 7, 14 and 20. A total of 20 cells near the explant (in the 5 cell range from the edge of the explant) and 20 cells distant from the explant (at least 10 cells away from the edge of the explant) were examined for ciliary activity for each culture. The percentage of cells which stopped beating at least once in 5 minutes was obtained and was expressed as mean±SE. The

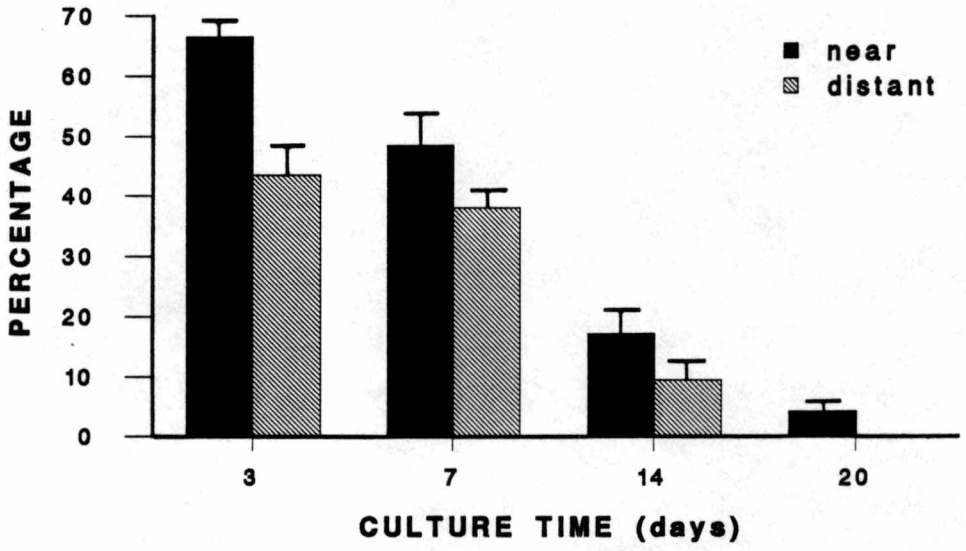


Fig. 6. Percentage of ciliated outgrowth cells which stopped beating at least once during a 5 minute measurement period from 3, 7, 14 and 20 day cultures. Blackened column represents cells near the explant. Stripped column represents cells distant from the explant. The differences of the percentage between outgrowths of different days and between near and distant ciliated cells are all significant ($p<0.01$, one way ANOVA). Each column represents 100 ciliated cells (mean±SE).

results showed (Fig. 6) that for the 3 day cultures, (66.5±2.7)% of measured cells in the group near the explant and (43.5±5.1)% in the distant group stopped beat at least once in the 5 minutes. For the 7 day cultures, the percentages were (48.5±5.1)% in the near group and (38±2.7)% in the distant group. The ciliated cells at day 14 showed (17.2±3.9)% and (9.44±3.3)% for the near and distant groups respectively. On day 20, many outgrowth cells had lost ciliation, so that all the cells were put into one group. Only (4.2±1.5)% of cells stopped beating. Statistical analysis (one way ANOVA) showed that the difference between the different day groups and between the near group and the distant group in the same day were all significant ($p<0.01$). These results suggest that in the early period of outgrowth formation, most ciliated outgrowth cells are affected by an inhibition mechanism so that their ciliary beat pattern showed frequent stops. Over extended culture time, the inhibitory effect declined in the culture condition so that the ciliary beat became more regular with little or no cessation of beat. The fact that cells in the distant group showed significant lower percentage of cessation of beat than that of cells in the group near the explant suggested the inhibitory effect was stronger closer to the explant than in the more distant part of the outgrowth.

Ciliary response to electrical stimulation and acetylcholine as a function of culture age

As indicated above, no morphological evidence was found for the existence of nerve endings in outgrowth epithelium. Furthermore, removal of the explant from 3 day cultures resulted in loss of the ability to electrically stimulate a ciliary beat frequency increase in outgrowth cells (Part II). To further examine changes in culture response to electrical stimulation over time, thresholds were established for 3, 5, 7, 9, 11, 14 and 21 day cultures. Threshold stimulus was defined as the stimulus with the lowest voltage which caused measurable ciliary beat frequency increase. For fresh palate, four of six experiments showed a threshold of 4 V stimulus (Part I). For 3 and 5 day cultures, five of seven experiments showed that 6 V was the threshold level. For 7, 9 and 11 day cultures, eight of ten experiments showed that the threshold increased to 8 V. On day 14 of culture, two of three experiments showed the threshold was 10 V, and on day 19 and 21 of culture, all of the seven experiments showed the threshold rose to 12 V. The gradual increase of the thresholds over culture time suggested that sensitivity of nerve endings which survived in the explant gradually declined.

To examine the possibility that the loss of sensitivity to electrical stimulation was also related to sensitivity loss of ciliated cells to acetylcholine during the culture, sensitivity of outgrowth cells to acetylcholine was examined.

A dose response of ciliated cells to the acetylcholine (10^{-9}M -- 10^{-3}M) was performed on a 21 day culture. Ciliary response to acetylcholine started at 10^{-7}M and the maximal response appeared at 10^{-5}M. The response was blocked by atropine, which was identical to the result obtained with outgrowth cells of 3-7 day cultures (Part I). This result indicated that the sensitivity of three week explant cultures to acetylcholine was not affected. The muscarinic receptors on the membrane of ciliated cells apparently were not lost in culture. It was concluded that frog palate explant culture which had gradually lost sensitivity to electrical stimulation during the culture was not related to the ability of ciliated cell to respond to acetylcholine.

IV. DISCUSSION

It has been suggested that the formation of the outgrowth of the frog palate explant culture is the result of migration of the epithelial layer from the explant (Aiello *et al.*, 1991). It has also been suggested that the outgrowth is a monolayer of ciliated epithelium (Eshel *et al.*, 1985; Weiss *et al.*, 1992). However, no morphological studies have described frog palate explant cultures. Our morphological observations verified that the epithelial layer migrates from the explant forming the outgrowth. In some experiments, we observed that removal of the explant from the culture results in disappearance of the outgrowth in 24 hours. This suggests that the survival of the outgrowth in culture is explant dependent. A similar situation has been found for rabbit tracheal epithelial outgrowth cultures (Kennedy and Ranyard, 1983). The migration of the epithelium into the outgrowth is found to progress for 9-10 days of culture, which is observed as an expansion of the outgrowth. After that, the migration stops and cells are gradually lost from the edge of the outgrowth, as observed by the gradual shrinking of the outgrowth.

Both light and electron microscopy show that frog palate ciliated epithelium is a stratified epithelium. The outgrowth is also a stratified epithelium. Only in the area near the outer edge is the outgrowth a monolayer. Thus it is evident that the outgrowth maintains some of the histological

characteristics of the palate epithelium. The stratified outgrowth of ciliated epithelial tissue in culture has also been reported for rat (Heckman *et al.*, 1978), Human (Stoner *et al.*, 1980) and rabbit (Kennedy and Ranyard, 1983) respiratory epithelium in culture.

Although the outgrowths maintain the layered histologic characteristics of the epithelium, the migration causes the epithelial cell shape to change from columnar and cuboidal into cuboidal and squamous. This cellular shape change increases the surface area of epithelial cells. Since cilia are still localized on the central area of cell surface in the outgrowth, the cellular shape change causes isolation of the cilia of adjacent cells facilitating the study of ciliary activity at the single cell level. Despite the cellular shape change, ciliated cells, mucus cells and non-ciliated cells still can be easily recognized in the outgrowth. Similar cellular shape change has been reported in mammalian ciliated epithelial cultures (Kennedy and Ranyard, 1983; Sanderson and Dirksen, 1985), and it appears to have no significant effect on ciliary function.

In our experiments, the explant cultures could be maintained for three weeks. However, only the cultures in the first two weeks do not show significant cell death. Kept too long in the culture, the outgrowth and connective tissue of the explant will degrad caused by cell death. The deterioration of outgrowths of frog palate cultures after the

20th day in culture has also been reported by Eshel *et al.*, (1985).

The ciliary beat frequencies of frog palate outgrowth cells have been studied on cultures from the 3rd through the 20th day (Eshel *et al.*, 1985). They focused on the second-by-second change of ciliary frequency, and an average ciliary frequency of 9 Hz was reported as the normal ciliary frequency for frog palate explant cultures. The ciliary beat frequency of frog palate outgrowths has also been studied by Aiello *et al.*, (1991) for cultures of day 3 through 7. It was reported that ciliated cells showed significant fluctuations of frequency with frequent stopping and starting and an average ciliary frequency of 9.51-15.17 beats/second. The ciliary frequency change as a function of culture time has not been addressed in any reported studies. Our study shows that the ciliary frequency of the outgrowths gradually increases in the first two weeks and remains relatively constant at a high frequency through the third week in culture. In this study the ciliary frequencies of cultures less than 10 days old show comparable values to the reported average ciliary frequencies, and are comparable to ciliary frequencies from fresh isolated frog palate (14.4±2.9 Hz, Puchelle *et al.*, 1982).

The pattern of ciliary beat also changed during the culture. On day 3 and 7, the pattern showed significant fluctuation and frequent stopping. After day 14, the

fluctuation greatly decreased, thus resulting in a more constant beat pattern. The percentage of outgrowth ciliated cells which stopped beating gradually declined during the three weeks in culture. Since the fluctuation of ciliary beat has been a known characteristic of frog palate (Eshel *et al.*, 1985; Zahm *et al.*, 1986; Aiello *et al.*, 1991), our current results suggest that frog palate explant cultures in the first week have similar ciliary activity as the ciliary activity of the palate *in vivo*, while older culture will gradually lose the characteristic ciliary beat pattern.

The mechanism responsible for inducing cessation of ciliary beat in the frog palate is not well understood. Maruyama *et al.*, (1983) found dopamine containing cells in the palate epithelium of Japanese bull frog, and reported that 10^{-6}M dopamine significantly inhibited ciliary movement *in vivo*. Morphological evidence indicates that the secretory basal cells in *Rana pipiens* may be comparable to the dopamine cells in the Japanese bull frog and thus may be inhibitory. The granules are localized in the superficial half of cytoplasm close to the ciliated cells. Second, the ciliary frequency of cultured cells gradually increased during the three week culture period while the granules in the secretory cells gradually decreased to zero. Third, the ciliary activity gradually changed from a pattern of large fluctuations and frequent stops into a pattern with little fluctuation and no cessation. Fourth, the percentage of cells

which stopped beating gradually declined in culture while the secretory cells gradually degranulated. All these observations suggest that the inhibition of ciliary activity may be from secretory granules of these basal secretory cells.

However, we have failed to inhibit ciliary frequency of outgrowth cells with dopamine *in vitro*. The possibilities may be that the dopamine receptors are located on the basal lateral side of the ciliated cell, and in the culture condition exogenous dopamine can not penetrate the outgrowth to bind to the receptor. Similar results have been reported by Aiello *et al.*, (1991) who suggest that species difference may be the reason for failure of dopamine inhibition of ciliary activity in *Rana pipiens*.

Various studies have shown that the palatine nerve, a parasympathetic nerve in the frog palate, can stimulate the mucus transport rate on the palate (Seo, 1931; Lucas, 1935; Slaughter *et al.*, 1982; Aiello *et al.*, 1991). Recently, we found intra-epithelial nerve endings which contact the palate ciliated cells directly, and electrical stimulation of both the palatine nerve and the explant culture verified that innervation is cilioexcitory and is mediated by muscarinic receptors on the ciliated cells (Part II). We also found that outgrowth ciliated cell response to electrical stimulation is mediated by nerve endings in the explant since removal of the explant abolishes outgrowth cell response to electrical stimulation and atropine inhibits the response (Part II).

Intra-epithelial nerve endings (Part II) may still function for up to two weeks in the explant culture since electrical stimulation induced significant increase in ciliary beat in these cultures. Explant cultures older than two weeks usually did not respond well to electrical stimulation. This may be caused by degradation of nerve endings in long term culture. The threshold of electrical stimulation necessary to induce ciliary response gradually increases from 4 V (for fresh palate) to 12 V (for day 21 cultures). The threshold change suggests that the number of functional nerve endings gradually decreases in culture. On the other hand, the outgrowth ciliated cells maintain the ability to respond to acetylcholine through the three week culture period. This further supports the interpretation that older cultures showed lower sensitivity to electrical stimulation due to degradation of nerve endings but not to the inability to respond to acetylcholine.

In summary, we observed that (1) ciliary frequency increased gradually until 14 days, after which time it was maintained at a relative constant level (18-20 beats/sec). (2) The ciliary beat pattern also changed during culture. On day 3 and 7, the pattern showed significant fluctuations above and below a mean, with frequent cessation of activity. After day 14, the fluctuation greatly decreased, resulting in a more constant baseline frequency beat pattern. (3) The percentage of ciliated cells which stopped beating gradually declined

during the three weeks in culture. (4) The threshold of electrical stimulation necessary to increase ciliary frequency gradually increased from 4 V (for fresh palate) to 12 V (for day 21 cultures), suggesting that the number of functional nerve endings were gradually decreased in the culture. (5) Ciliated cells were able to respond to acetylcholine treatment after three weeks in culture, indicating ciliated cells maintained muscarinic receptors to acetylcholine throughout the culture period. These results suggest that outgrowths less than 10 days old more closely represent the physiological and morphological condition of intact palate epithelium than do older cultures.

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PART IV

GAP JUNCTIONAL INTERCELLULAR COMMUNICATION MEDIATES PROPAGATION OF CILIARY ACTIVITY ON FROG PALATE EPITHELIUM

I. INTRODUCTION

Effective mucociliary transport is dependent on the coordinated ciliary beat of numerous ciliated cells. Cilia are packed closely together on ciliated epithelia, and they will interact with their neighbors when they beat. This interaction creates metachronal coordination of ciliary activity (Sanderson and Sleigh, 1981; Marino and Aiello, 1982). *In vitro* studies on rabbit tracheal ciliated cells in culture (Sanderson *et al.*, 1986, 1988) found that mechanical stimulation of a single ciliated cell propagated the ciliary response to many neighboring ciliated cells. With electric coupling technique and freeze fracture electron microscopy, Sanderson *et al.*, (1988) also reported gap junctional intercellular communication between the cells of the epithelium. It is believed that intercellular communication by way of gap junctions is an important mechanism to coordinate ciliary activity for mucociliary transport (Sanderson *et al.*, 1988). Except for the studies on the rabbit tracheal ciliated cells in culture mentioned above, the function of gap junctional intercellular communication in coordination of ciliary activity has not been reported for other ciliated epithelia.

Previous studies (Part I) have revealed that both mechanical stimulation of single ciliated cells and electrical stimulation of frog palate explant cultures can induce rapid and transient increase of ciliary beat frequency and regular

progression of the ciliary response to many neighboring cells. Outgrowths are formed by migration of cells from the explants. Ciliated cells of outgrowths are flat in shape with cilia in the center of the apical surface so that cilia of neighboring cells can not interact directly (Part III). Since ciliary interaction can not account for the propagation of stimulated ciliary activity in our experimental system, we believe the gap junctional intercellular communication may be the mechanism.

In this study, we have examined the role of gap junctions in intercellular communication between epithelial cells of frog palate with dye coupling using Lucifer Yellow CH and freeze fracture electron microscopy. Microinjection of Lucifer Yellow into cells and observation of the dye transfer with fluorescent microscopy has been an established method to study the gap junctional intercellular communication in different tissues (Stewart, 1978, 1981; Burt and Spray, 1989; Mehta *et al.*, 1989; Saez *et al.*, 1989; Bouille *et al.*, 1991). Lucifer Yellow CH is a negative charged fluorescent chemical with a molecular weight of 457.2 daltons. It can not pass through the cell membrane, but can diffused from cell to cell through gap junctions. The progression of mechanical stimulated ciliary activity has also been tested with the stimulation of either ciliated cells, mucus cells or non-ciliated cells in culture. We found that both dye transfer and the progression of the ciliary response between outgrowth

cells were blocked by halothane. Halothane, a volatile anesthetic, is known to be an effective blocker of the gap junctional intercellular communication in various tissues (Hauswirth, 1968, 1969; Wojtczak, 1985; Terrar and Victory, 1988; Burt and Spray, 1989; Sanderson *et al.*, 1990). The evidence presented supports the interpretation that gap junctional intercellular communication is an important mechanism in coordination of ciliary activity for mucociliary transport in the frog palate.

II. MATERIALS AND METHODS

Tissue culture

Frogs (*Rana pipiens*) were sacrificed by pithing the brain and the spinal cord. The palate mucosa was excised and prepared for tissue culture as previously described (Part I). The tissues were cultured in humidified air at 25°C. Epithelial outgrowth appeared in 24 hours. Tissue culture outgrowths between day 2 and day 7 were used in this study.

Optical spectrum analysis of single cell ciliary beat frequency change with time

Measurement of ciliary beat frequency change, second by second, of the frog palate outgrowth cells was performed with an optical spectrum analyzer system described by Kennedy and Duckett (1981) following the procedure described in Part I and II.

Test ciliary response to mechanical and electrical stimulation

Mechanical and electrical stimuli on ciliated cells in outgrowth cultures were performed with a micropipette and a Grass electrical stimulator respectively as described in Part I. Electrical stimuli used in these experiments were monophasic rectangular wave single pulses 10 ms in duration and with voltages from 8 V to 10 V. The ciliary activity of the ciliated cells before, during and after the stimulation were recorded on video tape which was used for optical spectrum analysis of ciliary frequency changes in response to

the stimulation.

Iontophoretic microinjection of fluorescent dye

Dye transfer between ciliated cells, and between ciliated cells and mucus cells was observed under fluorescent microscopy with the method of Stewart (1978, 1981). A 5% (w/v) solution of Lucifer Yellow CH (Sigma) prepared in 150 mM lithium chloride (Merck) was used to fill the glass micropipette (tip diameter -- 1 μm) by inserting the basal end of the micropipette into the dye solution. The dye solution was absorbed into the tip of the micropipette by capillary action. The remainder of the micropipette was filled with 150 mM lithium chloride by injection. The micropipette was mounted in a micromanipulator (Narishihe, Japan) on an inverted microscope (Nikon DIAPHOT-TMD). An iontophoretic injecting current was generated with an electrical stimulator (S6, Grass Instrument). The current was introduced to the micropipette through a pair of platinum wire electrodes (0.008 inch in diameter) with the negative pole in the micropipette and the ground pole in the medium. The resistance of the micropipette filled with 5% Lucifer Yellow CH in 150 mM lithium chloride was 29-30 MΩ. The electrical stimulator was set to generate a -20 mV output of 10 ms duration and 10 Hz in frequency. With that setting, current was about 30 nA. The iontophoretic injection continued until the impaled cell was brightly fluorescent (10-20 seconds were required in most

experiments). After injection, the cells were observed for 2 minutes to determine whether dye transfer occurred. The dye transfer was observed with a Nikon microscope with a 100 W Hg lamp illumination using a Nikon B-2A filter block (BA 520/DM 510/Ex 450-490).

Exposure of explant cultures to halothane

A saturated solution of halothane (Sigma) in frog ringer's was prepared by mixing 15 ml of frog ringer's with 5 ml halothane in a capped bottle for at least 3 hours at room temperature (Sanderson *et al.*, 1990). The solution was stored overnight at 4°C. According to Burt and Spray (1989), a solution prepared in this way is 16-17 mM halothane as measured by chromatography. Experimental solutions were made by diluting the saturated stock solution with frog ringer's in capped tubes immediately before using.

Freeze fracture electron microscopy

Fresh frog palate explant and 4 day explant cultures were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 hours at 4°C. The fixed tissue was soaked in 25% glycerol in cacodylate buffer overnight at 4°C. Freezing of the tissue was done on gold specimen discs by immersion into liquid ethane cooled by liquid nitrogen, after which specimen discs were stored in liquid nitrogen. Freeze fracture was performed with a Balzers BAF 400 freeze-etching system.

Fracture was done at -115°C under a vacuum of at least 1 x 10^{-6} torr. The fractured surface was coated with platinum and carbon. Replicas were cleaned in 40% chromic anhydride overnight, rinsed with distilled water and picked up on 200-mesh copper grids. Replicas were observed and photographed with a Hitachi H-600 transmission electron microscope.

III. RESULTS

Propagation of ciliary response and fluorescent dye transfer between outgrowth cells

Under phase contrast light microscopy, three kinds of cells were recognized in outgrowths of the frog palate explant cultures, ciliated cells and non-ciliated cells with and without mucus. The question of whether stimulation of any these cell types in the outgrowth resulted in the transmission of a signal to other ciliated cells was tested. Mechanical stimulation was used to stimulate a single cell without disturbing adjacent cells. Stimulation of non-ciliated cells without mucus or mucus cells resulted in the transmission of a signal to adjacent cells in the outgrowths. This transmission was viewed as a frequency increase in adjacent ciliated cells (The same situation had been observed from mechanical stimulation of ciliated cells in Part I). When a non-ciliated cell (without mucus) was stimulated, ciliated cells at a distance of several cells responded to the stimulus with increased ciliary frequency. The induced ciliary response was transmitted to more distance cells with a lag time and a gradually decreasing ciliary frequency (Fig. 1A). When a mucus cell (also lacking cilia) was stimulated, a similar ciliary response was induced, and a similar transmitting pattern was evident (Fig. 1B). In one previous study (Part I), we had observed that a ciliated cell responded to a mechanical stimulus immediately by increasing ciliary frequency and the ciliary response was transmitted to adjacent

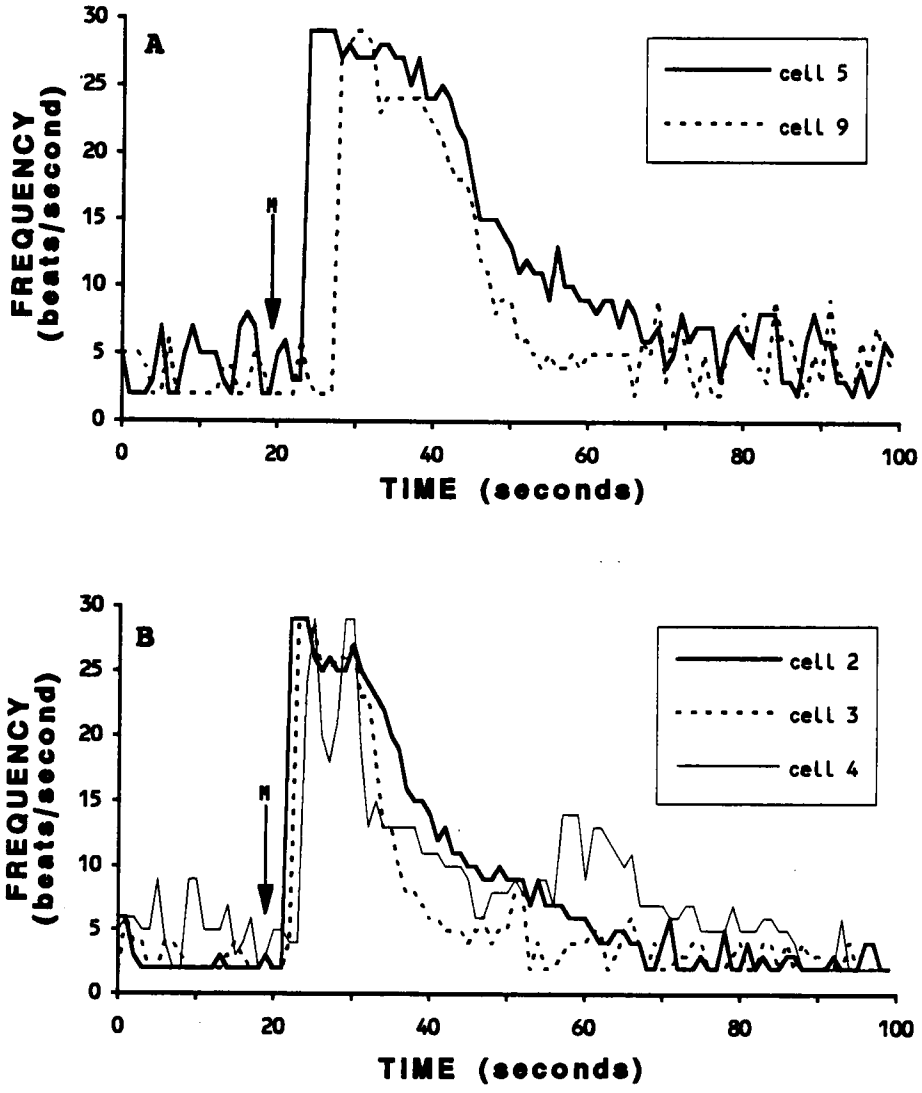


Fig. 1. Ciliary response to mechanical stimulation of outgrowth cells. (A) Stimulation of a non-ciliated cell induces ciliary response to a distance of 5 and 9 cells respectively. (B) Stimulation of a mucus cell induces ciliary response propagation to a distance of 2, 3 and 4 cells respectively.

ciliated cells. All experiments were repeated at least 10 times and similar results obtained.

Lucifer Yellow CH was iontophoretically injected into both ciliated cells and mucus cells of the outgrowth respectively. Two minutes after the injection, the Lucifer Yellow CH was always transferred to adjacent cells. An example of the dye coupling by microinjection of Lucifer Yellow CH into a ciliated cell was shown in Fig. 2A & B, and a similar result was shown in Fig. 2C & D from the same experiment on a mucus cell. In both examples, Lucifer Yellow CH was successfully transferred to more than ten adjacent cells from the originally microinjected cell. This indicated that gap junctions were present between all outgrowth cells.

Halothane blocks propagation of ciliary response and dye transfer between outgrowth cells

Mechanical stimulation of a ciliated cell always induced ciliary response from not only the stimulated cell but also adjacent ciliated cells (Fig. 3A). Within one minute after the frog ringer's was replaced by 3-5 mM halothane in frog ringer's, mechanical stimulus of the same ciliated cell could only induce a ciliary response from the stimulated cell. The progression of the ciliary response to adjacent cells was blocked (Fig. 3B). These results verified that halothane did not abolish the ability of ciliated cell to respond to mechanical stimulation, but blocked the progression of ciliary response between ciliated cells.

Fig. 2. Fluorescent dye transfer between outgrowth cells. (A) A phase contrast micrograph shows microinjection of Lucifer Yellow CH into a ciliated cell (+). Micropipette (arrow). (B) Fluorescent micrograph of the same area in (A) shows the dye transfer from microinjected cell (+) to many adjacent cells two minutes after the injection. (C) A phase contrast micrograph shows microinjection of Lucifer Yellow CH into a mucus cell (+). Micropipette (arrow). (D) Fluorescent micrograph of the same area in (C) shows the dye transfer from the microinjected cell (+) to adjacent cells two minutes after the injection. Bars = 100 μm.

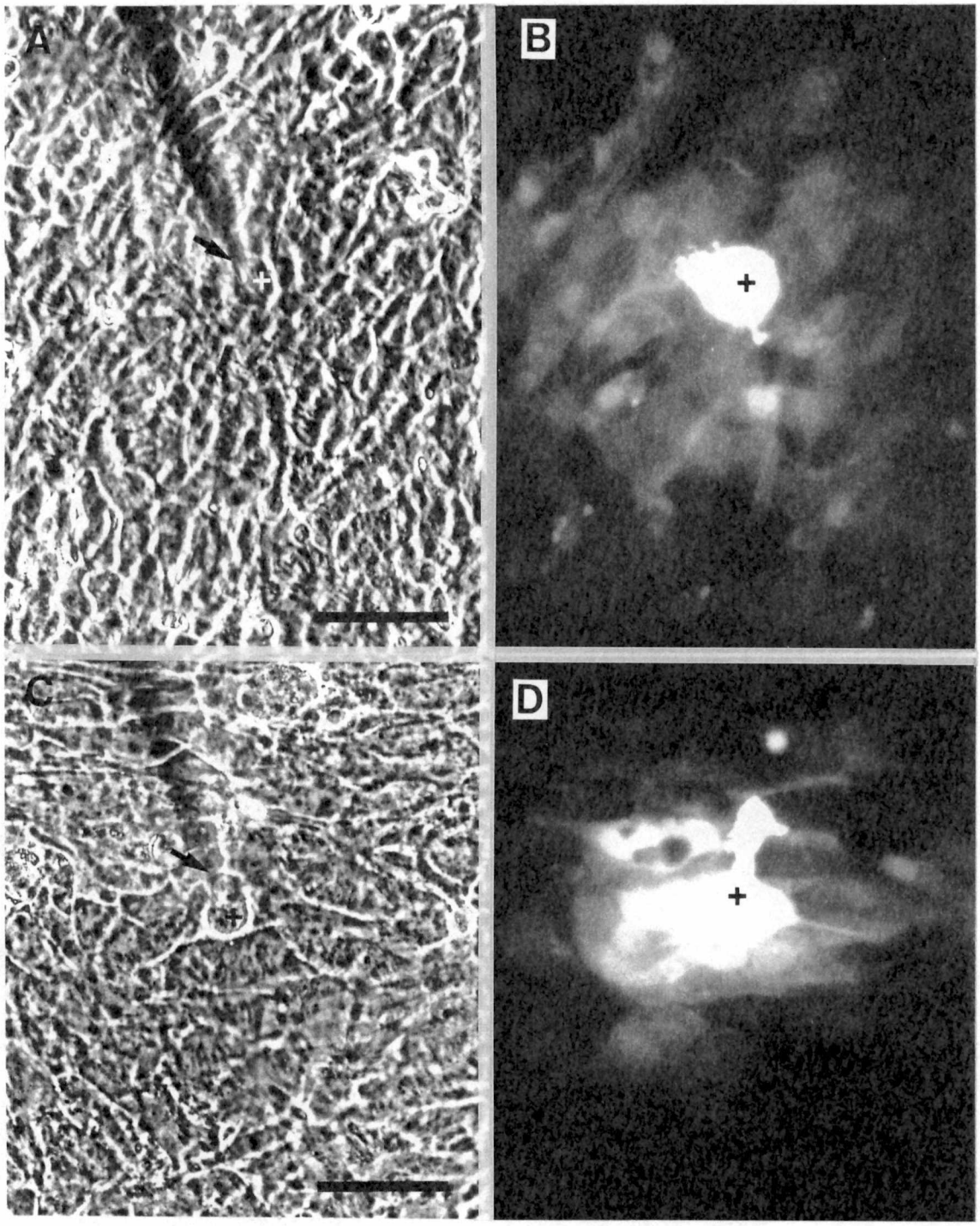
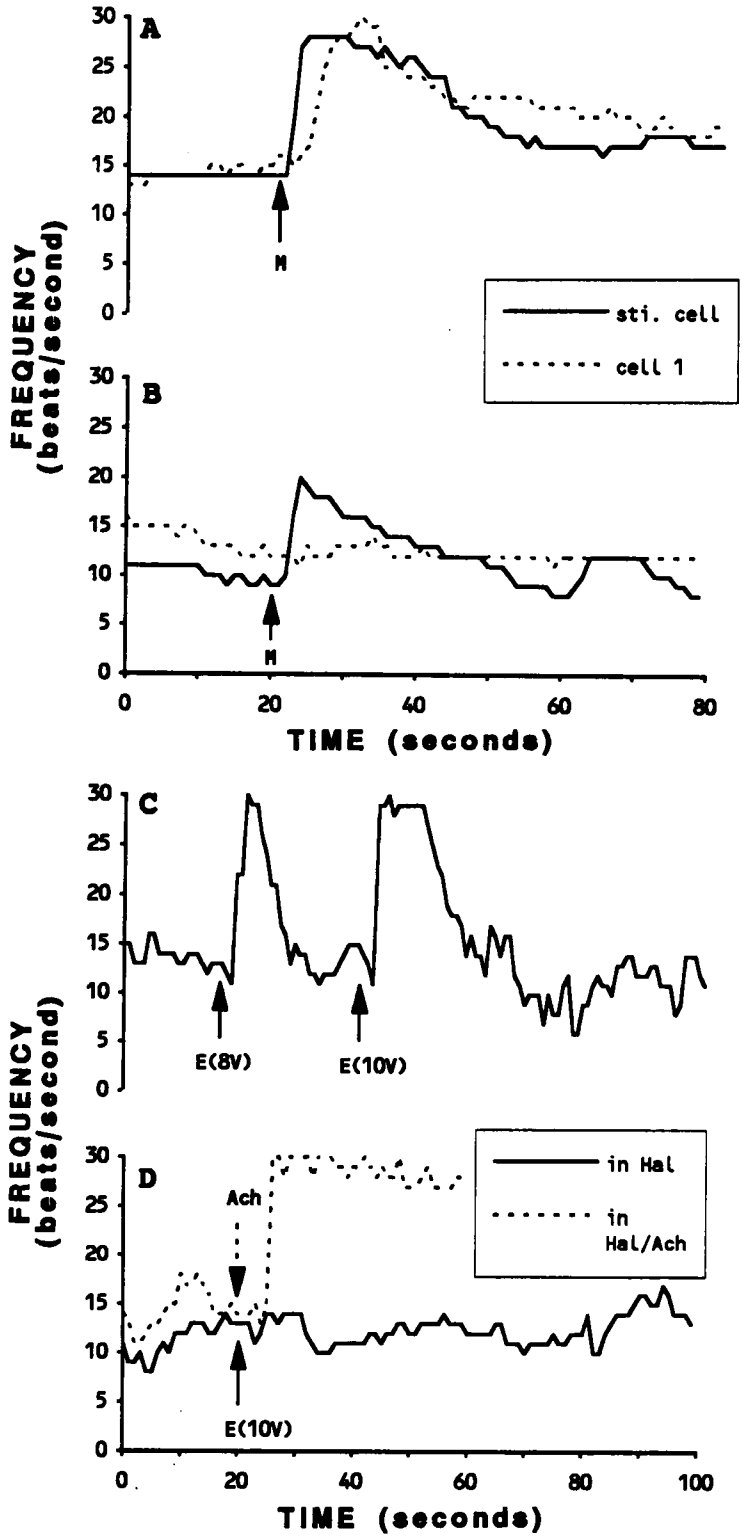

A
B
C
D

Fig. 3. Halothane blockage of the propagation of ciliary response between outgrowth cells. (A) In frog ringer's a ciliated cell (sti. cell) responds to a stimulus by increase in frequency. The response spreads to adjacent cell (cell 1). (B) After one minute in 4 mM halothane, the same ciliated cell as (A) is stimulated again. The stimulated cells can still respond, but the adjacent cells does not respond. (C) An outgrowth cell in frog ringer's responds to 8 V and 10 V stimuli by increasing frequency. (D) After one minute in 4 mM halothane (Hal) the same cell as in (C) does not respond to 10 V stimulus, but still can respond to 10^{-5}M acetylcholine (Ach).


A
B
C
D
FREQUENCY (beats/second)
TIME (seconds)
M
sti. cell
cell 1
E(8V)
E(10V)
Ach
in Hal
in Hal/Ach

Electrical stimulation of frog palate explant cultures induced ciliary frequency increase in the explant and the response was transmitted to ciliated cells in outgrowths. Stimulations of 8 V and 10 V both caused increase in the ciliary frequency of outgrowth cells (Fig. 3C). The ciliary response of the outgrowth cell could be blocked by halothane (3-5 mM) treatment in less than one minute (Fig. 3D). The ciliated cell, however, could still respond to acetylcholine in the presence of halothane (Fig. 3D). These data indicate that electrical stimulation results in frequency increase in outgrowth cells by a signal transmitted through gap junctions rather than by diffusion of acetylcholine released from nerve endings in the explant.

Dye transfer between the epithelial cells in culture was also blocked by the 3-5 mM halothane. When cells were treated with halothane, Lucifer Yellow CH microinjected into the outgrowth ciliated cell did not spread to adjacent cells even 5 minutes after the microinjection (Fig. 4A,B). After extensive rinsing (5 times) with frog ringer's the dye did spread to adjacent cells (Fig. 4C).

Freeze fracture observation of gap junctions

Gap junctions were found in both fresh palate epithelium (Fig. 5A,B) and the epithelium of 4 day outgrowth cultures (Fig. 5C,D). Figure 5A&B show examples of gap junctions in the palate epithelium. Cilia are evident on the cell surface

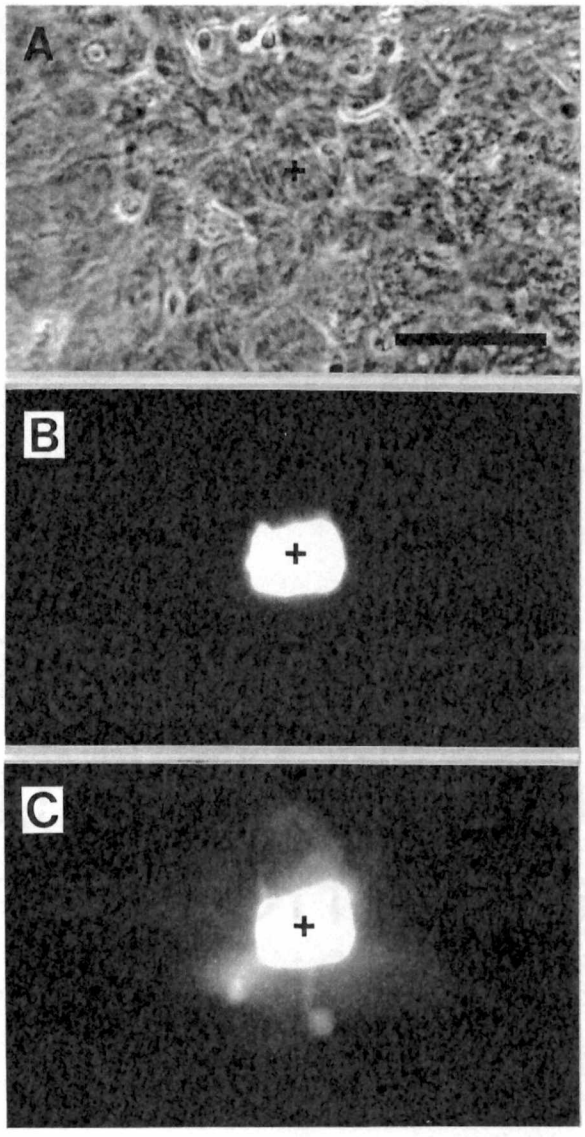
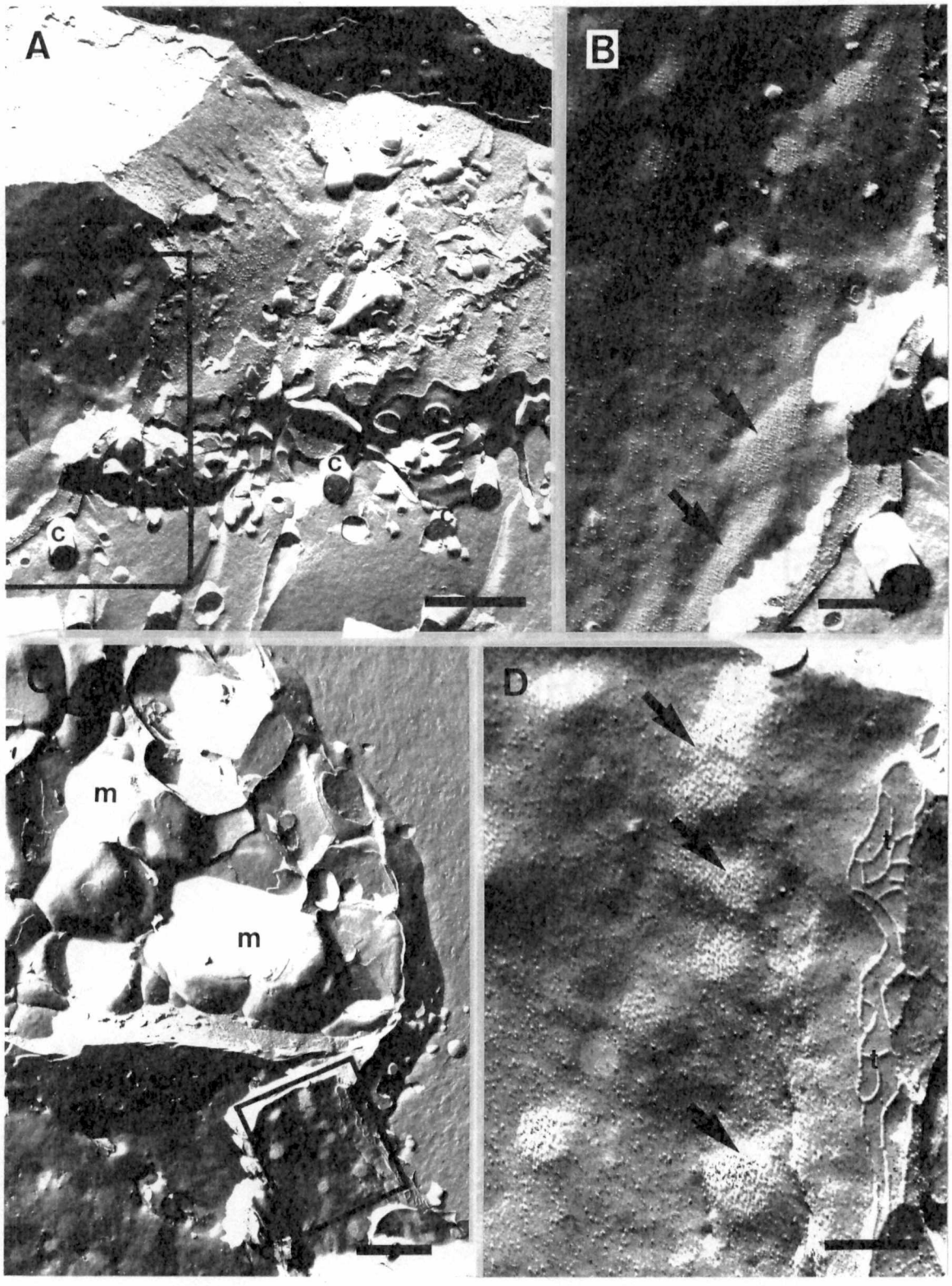


Fig. 4. Halothane blockage of fluorescent dye transfer between outgrowth cells. (A) Phase contrast micrograph shows a ciliated cell (+) microinjected with Lucifer Yellow CH in the presence of 4 mM halothane. (B) Five minutes after the injection, the dye is still limited to the cell(+). (C) After extensive rinsing with frog ringer's (5 times), the dye transfers into adjacent cells from the injected cell (+). Bar = 100 μm.

Fig. 5. Freeze fracture electron micrographs of gap junctions. (A) Gap junctions(arrows) on the lateral surface of a explant ciliated cell. Cilia (c). Bar = 1 μm. (B) Enlargement of the black lined area of (A) shows gap junction structure (arrows). Bar = 0.5 μm. (C) Gap junctions and tight junctions (in the square) on a lateral surface of a outgrowth mucus cell. Mucus granules (m). Bar = 1 μm. (D) Enlargement of the square area of (C) shows gap junctions (arrows) and tight junctions (t). Bar = 0.25 μm.


A
c
c
c
B
C
m
m
D
t
t

(lower half of Fig. 5A). Gap junctions are exposed on one lateral surface of the cell. Gap junctions are of different sizes but all are composed of aggregations of intramembrane protein particles, connexons (Fig. 5B). Gap junctions were also found in the outgrowths of 4 day palate outgrowth cultures. Figure 5C&D shows an example of gap junctions on the lateral surface of an outgrowth cell. The tight junction above the gap junction area was also evident (Fig. 5C,D). Morphologically, the gap junctions were the same as the gap junctions found in fresh palate epithelium. Thus there is morphological evidence to support the physiological data which indicates that gap junctions are present between both explant and ciliated and non-ciliated outgrowth cells.

IV. DISCUSSION

Mucociliary transport is the major function of ciliated epithelium whether it is movement of materials into the esophagus as with the frog palate or movement of materials away from the lungs as in mammalian tracheal epithelium. This function can only be performed by cooperative activity of many ciliated cells. Local mechanical stimulation of the frog palate has been found to accelerate mucociliary transport and ciliary beat frequency both *in vivo* (Slaughter and Aiello, 1982) and *in vitro* (Spungin and Silberberg, 1984). Using frog palate explant cultures, the computer aided optical spectrum analyzer and video microscopy, we have been able to study the propagation of ciliary response between cells induced by mechanical stimulation. Our previous results have shown that mechanical stimulation of a single ciliated cell or electrical stimulation of frog palate cultures always induced a ciliary frequency increase. Furthermore the ciliary response was propagated cell by cell from the point of initiation of the response to many adjacent cells (Part I). Gap junctional intercellular communication between epithelial cells of the palate may be one of the mechanisms mediating cooperative activity of ciliated cells. In this study, the presence of gap junctions between frog palate epithelial cells and their function in propagation of ciliary response between the epithelial cells were confirmed. Similar results have been reported on rabbit tracheal ciliated epithelium in culture by

Sanderson *et al.*, (1988).

Frog palate epithelium is composed of ciliated cells, mucus and serous cells, intermediate cells, basal cells, and patches of vomero-nasal epithelium with sensory receptors and supporting cells (Puchell *et al.*, 1984). The intercellular communication between not only ciliated cells but also different type of cells should be important in mediating the cooperative ciliary activity of the epithelium for mucociliary transport. In previous studies (Part I), we have observed that upon stimulation of single ciliated cells the ciliary response is propagated to adjacent ciliated cells. In the present study, we further tested whether mechanical stimulation of non-ciliated cells and mucus cells could induce ciliary response of the explant culture and whether gap junctions were present between different types of cells in the epithelium. We found that (1) mechanical stimulation of either ciliated cells, mucus cells or non-ciliated cells in culture resulted in the propagation of a ciliary response between cells; (2) microinjection of Lucifer Yellow CH into either ciliated cells or mucus cells revealed significant dye coupling between injected cells and adjacent cells; (3) freeze fracture electron microscopy revealed gap junctions between the epithelial cells. Morphologically, these gap junctions are similar to the gap junctions observed in rabbit tracheal ciliated epithelium (Sanderson *et al.*, 1988). They are also comparable to the gap junctions found in lens epithelium and

fiber cells of frog, rabbit, rat and human (Lo W.K. and Harding, C.V. 1986) and other tissues (Friend and Gilula, 1972; Raviola *et al.*, 1980).

Further evidence indicating the presence of gap junctional intercellular communication in frog palate epithelium was that 3-5 mM halothane blocked both the progression of stimulated ciliary response and dye transfer between ciliated cells. Halothane (3-5 mM) uncoupling of ciliated cells by blocking gap junctions has also been reported from a study on rabbit tracheal epithelial cells in culture (Sanderson *et al.*, 1990). The specificity of 3-5 mM halothane on the gap junctional intercellular communication has been tested in the present study. It was observed that halothane treatment only blocked progression of mechanical stimulated ciliary responses but not the ability of ciliated cell to respond to direct mechanical stimulation. Also, the dye transfer inhibited by halothane treatment could be recovered by washing with frog ringer's. These facts suggest that intercellular communication by gap junctions is a common mechanism to mediate cooperative ciliary activity among frog palate epithelial cells. The presence of such a mechanism may explain how ciliated epithelium can respond to local stimulation of just a few cells by small particles. That stimulation results in an increase in the ciliary frequency of many ciliated cells which in turn increases the mucociliary transport. The present study also suggests that gap junctions

are involved in mediating the transfer of palatine nerve signal to the palate epithelium. Slaughter and Aiello (1982) found that electrical stimulation of the palatine nerve both *in vivo* and *in vitro* stimulated mucus transport of the frog palate. Direct electrical stimulation of the frog palate explant culture can stimulate ciliary activity (Part I) as can electrical stimulation of the palatine nerve (Part II). But the ciliary response always starts at the explant and is gradually passed to ciliated cells of the outgrowth (Part II). Upon removing the explant from the culture, the ciliary response of outgrowth ciliated cells to electrical stimulation is abolished (Part II). Atropine also inhibits the ciliary response of explant cultures to electrical stimulation. This suggests that the ciliary response to electrical stimulation is mediated by the palatine nerve endings in the explant. Electron microscopic study (Part II) revealed that the palatine nerve endings are very close to some ciliated cells in the epithelium, but not every epithelial cell in the palate is innervated. These observations suggest that gap junctional intercellular communication is involved in coordination of palatine nerve stimulated ciliary activity. Specifically, when a few innervated cells are stimulated by acetylcholine from the palatine nerve, the signal can pass through gap junctions between innervated cells to cells without innervation resulting in the spread of the response over a large number of ciliated cells. This hypothesis is further

supported by the fact that the progression of ciliary responses induced by electrical stimulation can be blocked by a one minute treatment of 3-5 mM halothane. While halothane inhibits the passage of the electrically stimulated ciliary responses, the ciliated cells can still respond to a stimulation such as acetylcholine treatment.

In summary, the present study revealed that there is gap junctional intercellular communication between different types of cells in the frog palate epithelium. Intercellular communication by gap junction provides an integrated control mechanism for regulation of the ciliary activity of large numbers of cells in the epithelium. Stimulation of a single cell or a few cells induces ciliary activity of many cells. Mechanical stimulation and gap junction communication may represent a mechanism of local activation of mucociliary transport in response to exogenous particles. Electrical stimulation and gap junction communication provides a mechanism of more general activation of mucociliary transport mediated by the palatine nerve.

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PART V

CALCIUM MOBILIZATION FROM INTRACELLULAR STORES STIMULATES CILIARY RESPONSE OF FROG PALATE CILIATED CELLS

I. INTRODUCTION

As an intracellular signal molecule, Ca^{2+} has an important role in the regulation of ciliary activity in ciliated cells. High concentrations of intracellular Ca^{2+} stimulate the ciliary beat frequency of mucociliary epithelium in the oviduct of the salamander Necturus (Eckert and Murakami, 1972), rabbit tracheal epithelium (Verdugo *et al.*, 1983; Girard and Kennedy, 1986; Sanderson *et al.*, 1988, 1989, 1990), and dog tracheal epithelium (Kakuta *et al.*, 1985). In response to mechanical stimulation, an increase of intracellular Ca^{2+} always preceded increase in ciliary beat frequency of rabbit tracheal epithelium (Sanderson *et al.*, 1992).

Sources responsible for intracellular Ca^{2+} increase are known to be either extracellular or intracellular stores (Rasmussen and Barrett, 1984; Satir and Sleigh, 1990; Nicotera *et al.*, 1992). Both mechanical stimulation and Ca^{2+}-ionophore A23187 are believed to induce ciliary beat frequency increase through the same mechanism, Ca^{2+} entering the cell from the exterior resulting in an increase of intracellular Ca^{2+} (Girard and Kennedy, 1986; Sanderson and Dirksen, 1986, 1989). It is believed that stretch-activated channels on the cell membrane of rabbit tracheal epithelium (Kourie *et al.*, 1990) are responsible for Ca^{2+} influx in the cell after mechanical stimulation. On the other hand, mobilization of Ca^{2+} from intracellular stores has been suggested in the transduction of

mechanical stimulation of ciliated epithelium. Stimulation induced increase of intracellular Ca^{2+} concentration could spread through adjacent ciliated cells in the absence of extracellular Ca^{2+} (Sanderson *et al.*, 1992), increasing the cytoplasmic Ca^{2+} concentration in the process. Mobilization of Ca^{2+} from intracellular stores has also been found to be important in hormonal induced ciliary response such as prostaglandin E_2 stimulation of ciliary activity in the rabbit oviduct (Verdugo, 1980).

Our previous studies have characterized the ciliary responses of frog palate outgrowth cultures to acetylcholine, mechanical and electrical stimulation (Part I). With this experimental system and computer aided optical spectrum analysis of ciliary beat frequency (Kennedy and Duckett, 1981; Aiello *et al.*, 1991), we can analyze ciliary activity change in real time. Comparison of ciliary activity after stimulation in the presence and absence of extracellular Ca^{2+} may give some indication about the roles of extracellular and intracellular Ca^{2+} sources in mediation of ciliary responses. The precise localization of intracellular Ca^{2+} stores in ciliated cells has not been investigated. Ultrastructural localization of intracellular Ca^{2+} with the oxalate/pyroantimonate technique (Borgers *et al.*, 1977; Griffiths *et al.*, 1983) may reveal the location of intracellular Ca^{2+} stores and its' mobilization in stimulated ciliated cells.

II. MATERIALS AND METHODS

Tissue culture

Frogs (*Rana pipiens*) were sacrificed by pithing the brain and the spinal cord. The palate tissue culture was prepared according to Aiello *et al.*, (1991). Briefly, the palate mucosa was excised, rinsed with frog Ringer's solution and cut into 1 mm^2 pieces and cultured as described previously (Part I). The tissues were grown in humidified air at 25°C. Epithelial outgrowths appeared in 24 hours. Tissue culture outgrowths between day 2 and day 7 were used in this study.

Optical spectrum analysis of single cell ciliary beat frequency change with time

Measurement of ciliary beat frequency on frog palate and explant cultures was performed with the optical spectrum analyzer system described by Kennedy and Duckett (1981) as described in Part I. Measurements of ciliary beat frequencies were made in real time on single cells. To observe change over time, an IBM PC (PS/2) computer with a data acquisition and analysis program was linked with the optical spectrum analyzer system. With this system, a single cell could be measured, second by second, for up to 400 seconds.

With this system, ciliary responses of frog palate explant cultures to acetylcholine (10^{-5}M) and electrical stimulation were tested under following conditions: (1) in frog Ringer's solution; (2) in frog Ringer's solution and 0.1 mM verapamil and (3) in calcium free frog Ringer's solution containing 0.1

mM verapamil and 1 mM EGTA. By using verapamil, EGTA and calcium free frog Ringer's solution, we could determine whether extracellular calcium was necessary for initiation of ciliary frequency increase in frog palate epithelial cells.

Ultrastructural localization of calcium in ciliated cells

To compare the location of intracellular calcium in normal beating ciliated cells with cells stimulated to increase frequencies, frog palate outgrowth cultures were treated with different stimuli. (1) The control group was normal beating ciliated cells without stimulation. (2) Electrically stimulated ciliated cells were subjected to 10 V (10 Hz, 10 ms pulse) for 10 seconds. (3) Mechanically stimulated ciliated cells were induced to respond with a fine needle touched to the explant twice in 10 seconds. (4) Acetylcholine stimulated ciliated cells were treated by 10^{-5} M acetylcholine for one minute. At the ends of the above defined stimuli, all ciliated cells were observed to responded with increased ciliary frequency. The cultures were fixed immediately with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for about 1 minutes before transfer into following fixative. As soon as the fixative was introduced into the culture dish, ciliary beating stopped.

Ca^{2+} localization was performed with the oxalate/pyroantimonate method developed by Borgers *et al.*, (1977). The cultures were further fixed in 2.5%

glutaraldehyde in 90 mM potassium oxalate (pH 7.4, adjusted with 0.1 M potassium hydroxide) overnight at 4°C. The cultures were rinsed with 7.5% sucrose in the 90 mM potassium oxalate (pH 7.4) for 5 minutes. Postfixation was performed in a solution of 1% osmium tetroxide and 2% potassium pyroantimonate in 0.01 N acetic acid (adjusted to pH 7.4 with 0.1 M potassium hydroxide) for 2 hours at 4°C, and rinsed with cold distilled water (pH 10 adjusted with 0.1 M potassium hydroxide). Dehydration was with a graded ethanol series, and the tissue was embedded in EM bed-812 (Electron Microscopy Sciences). For electron microscopy, 70-80 nm sections were stained with lead citrate only, and examined with an Hitachi H-600 electron microscope operated at 75 or 100 kV. The experiments were performed on two groups of eight outgrowth cultures each from two frogs.

The specificity of the calcium localization was examined by both energy-dispersive X-ray microanalysis techniques (Griffiths, *et al.*, 1983) and ethylene glycol bis(ß-aminoethyl ether) N,N'-tetra-acetic acid (EGTA) treatment on thin sections (Diculescu, *et al.*, 1971; Ravazzola, 1976; Wick and Hepler, 1980; Griffiths, *et al.*, 1983). Unstained thin sections were subjected to the X-ray microanalysis with a Link Pentafet detector and QX 2000 analysis system, interfaced with an Hitachi H-800 transmission electron microscope operated at 200 kV. The microanalysis was focused on the electron opaque precipitates in cells. The element content of the precipitate

was determined by the element specific X-ray energy spectrum which was collected through a Si(Li) detector over a time of 100 seconds. In EGTA treatment experiments, serial thin sections were cut from embedded tissue blocks. For each block, some of the sections were treated with 5 mM EGTA for 1 hour at 60°C. The rest of the sections were put in distilled water for 1 hour at 60°C as a control. After treatments, sections were stained with lead citrate, and observed with the H-600 electron microscope operated at 75 or 100 kV.

III. RESULTS

Initiation of ciliary response to stimulation is not extracellular calcium dependent

Using the Ca^{2+} channel blocker verapamil, we examined whether extracellular Ca^{2+} was necessary for ciliated cells to respond to various stimuli. Verapamil (0.1 mM) was used to block Ca^{2+} channels of ciliated cells of frog palate cultures in frog ringer's containing 1.1 mM Ca^{2+}. In controls without verapamil treatment, ciliated cells responded to 10^{-5}M acetylcholine with a rapid increase in ciliary beat frequency. The frequency remained at an elevated level (Fig. 1A) for at least 15 minutes. In comparison, after treatment with 0.1 mM verapamil for 5 minutes, ciliated cells could still respond to 10^{-5}M acetylcholine by increasing frequency of beat, but the elevated frequency remained for less than 1 minute. It gradually dropped to basal frequency (Fig. 1B).

When outgrowth cells in frog Ringer's solution were electrically stimulated with 10 V, ciliated cells responded with a rapid and transient increase of ciliary beat frequency (Fig. 2A). The same ciliated cell in the culture still responded to electrical stimulation 5 minutes after 0.1 mM verapamil treatment, but the ciliary frequency response was weaker than before verapamil treatment both in terms of speed of response and duration of stimulated ciliary beat (Fig. 2B). Five experiments were performed for both acetylcholine and electrical stimulation of the ciliated epithelial cultures with 0.1 mM verapamil, and comparable results were obtained.

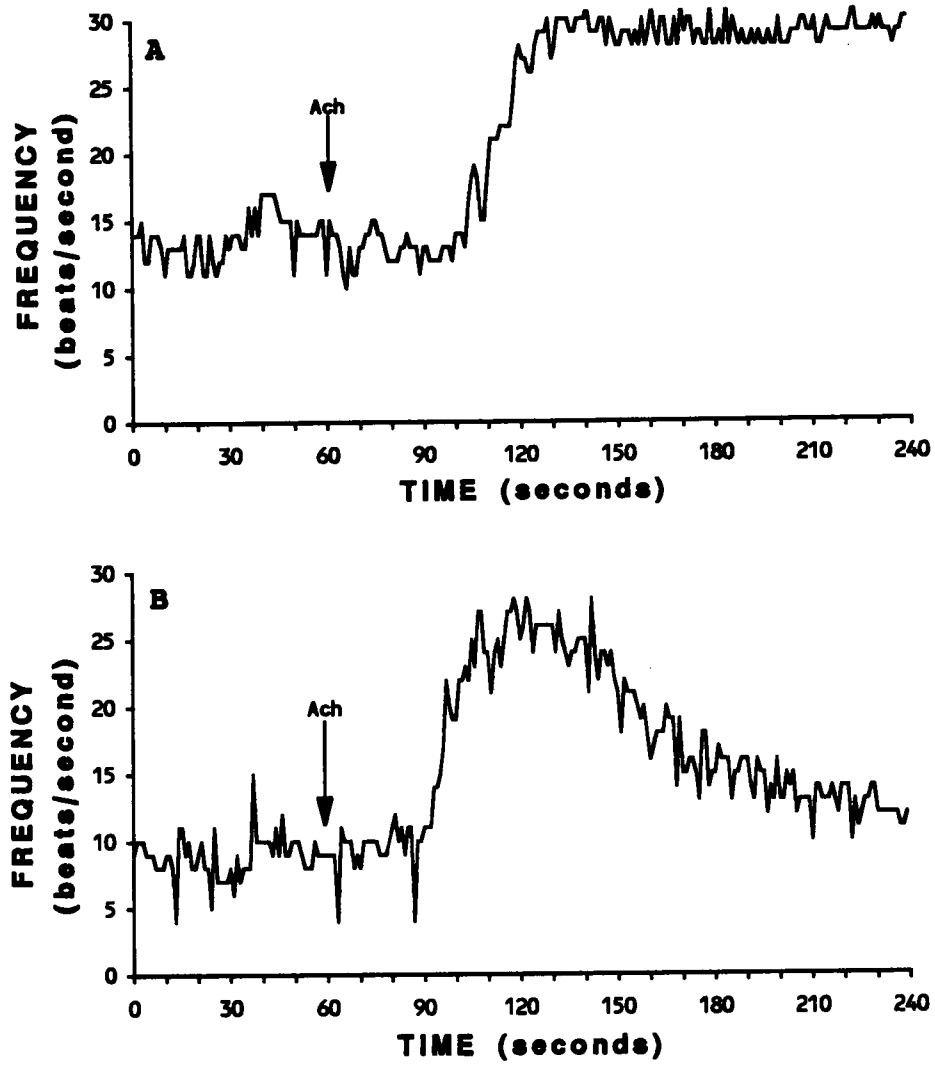


Fig. 1. Ciliary response to acetylcholine stimulation with and without verapamil. (A) Control in frog ringer's solution: a short time after 10^{-5}M acetylcholine treatment, ciliary frequency increases from basal level (about 15 beats/sec) to a plateau (about 30 beats/sec), and remains during the rest of measurement. (B) In frog ringer's solution containing 0.1 mM verapamil: five minutes after the verapamil treatment, the same amount of acetylcholine causes frequency increase. But the stimulated frequency remains for less than one minute, then gradually dropped to basal level in the presence of acetylcholine.

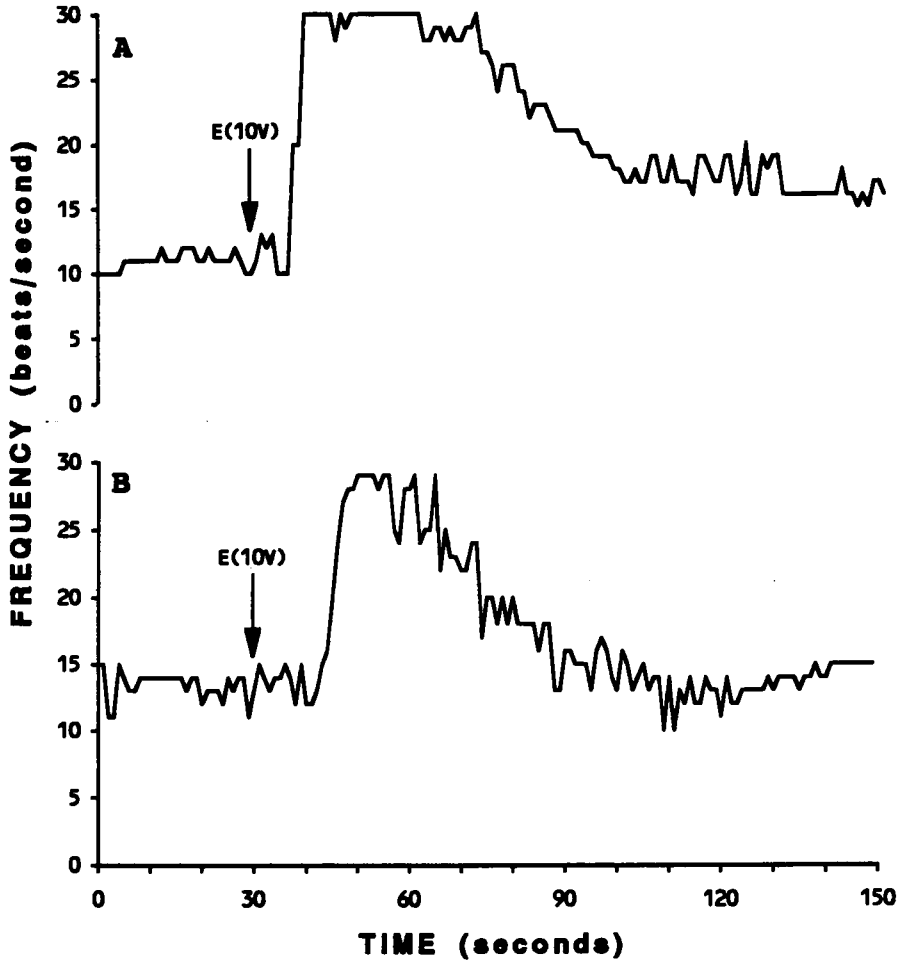


Fig. 2. Ciliary response to electrical stimulation with and without verapamil. (A) Before verapamil treatment: the ciliated cell rapidly responded to 10 V (10 ms) single stimulus with frequency increase, the stimulated frequency remains at the plateau for about 40 seconds. (B) Five minutes after 0.1 mM verapamil treatment: the ciliated cell still rapidly responds to the stimulation with frequency increase, but the stimulated ciliary frequency remains at the plateau for only about 20 seconds.

These results indicated that blocking cell membrane Ca^{2+} channels did not abolish ciliated cell response to various stimuli, but prolonging increased ciliary activity was greatly limited by the verapamil treatment. It appears that blockage of the Ca^{2+} influx did not affect the initiation of the ciliary response to stimulation, but it did affect the maintenance of increased ciliary beat frequency.

To test ciliary response in the absence of extracellular Ca^{2+}, frog palate outgrowth cultures were rinsed three times with Ca^{2+} free frog ringer's containing 1 mM EGTA and 0.1 mM verapamil and placed in the solution. Five minutes after the treatment, the cultures were subjected to either 10^{-5}M acetylcholine treatment or 10 V electrical stimulation. With acetylcholine treatment, ciliated cells still responded with a rapid increase in ciliary beat frequency, but the increased ciliary activity was not sustained at the high frequency. Instead, the frequency started to drop after a short time at the peak frequency (Fig. 3A). Ciliated cells did not respond to single 10 V stimulus, but responded to a 5 second repeated 10 V (10 Hz, 10 ms pulses) electrical stimulus (Fig. 3B). Although under stronger electrical stimulation, the ciliated cells in the Ca^{2+} free condition showed weaker response in terms of magnitude of increase and duration of increased ciliary activity (Fig. 3B). These results further supported the results from the verapamil experiments, and indicated that without extracellular Ca^{2+}, ciliated cells can still respond

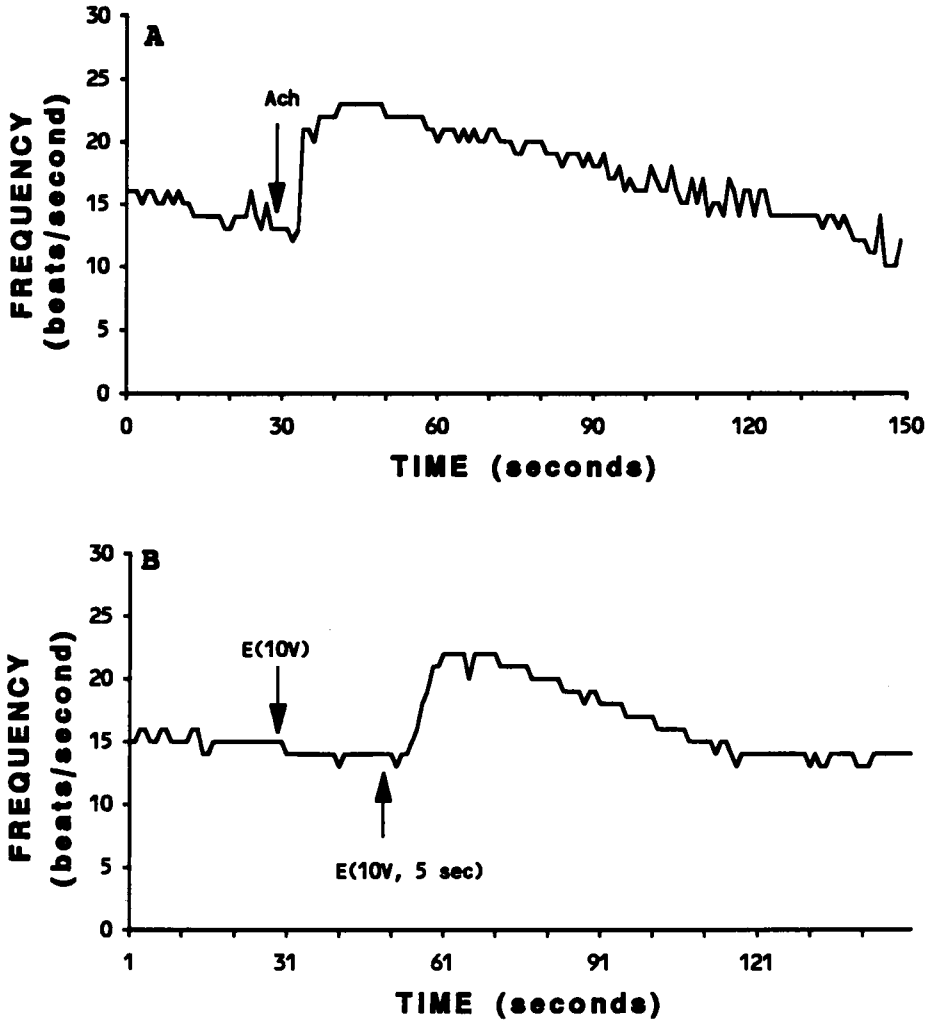


Fig. 3. Ciliary response to stimulation in Ca^{2+} free solution. The Ca^{2+} free frog ringer's solution contains 1 mM EGTA and 0.1 mM verapamil. (A) Five minutes after incubation in the Ca^{2+} free solution, ciliary response is still induced by 10^{-5}M acetylcholine, but the stimulated frequency remains at the plateau for less than 30 seconds. (B) Five minutes after in the Ca^{2+} free solution, 10 V single stimulus does not induce ciliary response, but the ciliary response appears right after 5 second repeated 10 V (10 Hz, 10 ms pulse) stimulation.

to stimulation. But maintenance of increased ciliary frequency is greatly limited. It appears that initiation of ciliary response to stimulation is not dependent on extracellular Ca^{2+}, but to maintain increased ciliary beat frequency at a high level requires extracellular Ca^{2+}.

Redistribution of intracellular calcium in response to various stimulation

Ultrastructural localization of Ca^{2+} in ciliated cells was studied with the oxalate/pyroantimonate method described above. Intracellular free Ca^{2+} appeared as electron opaque calcium pyroantimonate deposits when viewed with the electron microscope. Morphology of the deposits were various, from irregular shaped fine particles to large round droplets. In non-stimulated ciliated cells, the deposits were found mainly in the matrix of the mitochondria and euchromatin areas of the nuclei. Very few deposits were observed in the cilia or the cytoplasm (Fig. 4A & B). No significant deposits were found in the endoplasmic reticulum.

When ciliated cells were stimulated to beat actively, the location of intracellular Ca^{2+} was different from that of non-stimulated cells. The most significant change in most ciliated cells was that deposits in the mitochondria were significantly decreased. The deposits in the nuclei were also decreased. At the same time, deposits in cilia increased. The same situation was observed in ciliated cells under either electrical stimulation (Fig. 5A), mechanical stimulation

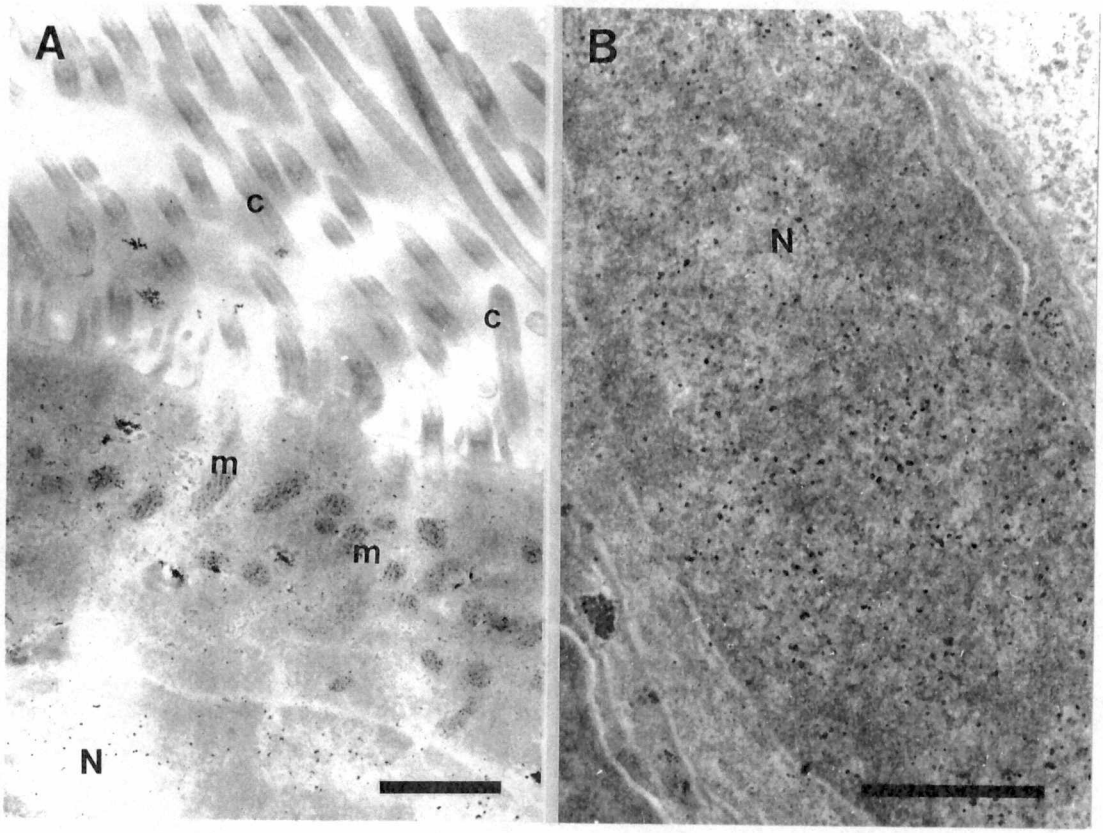
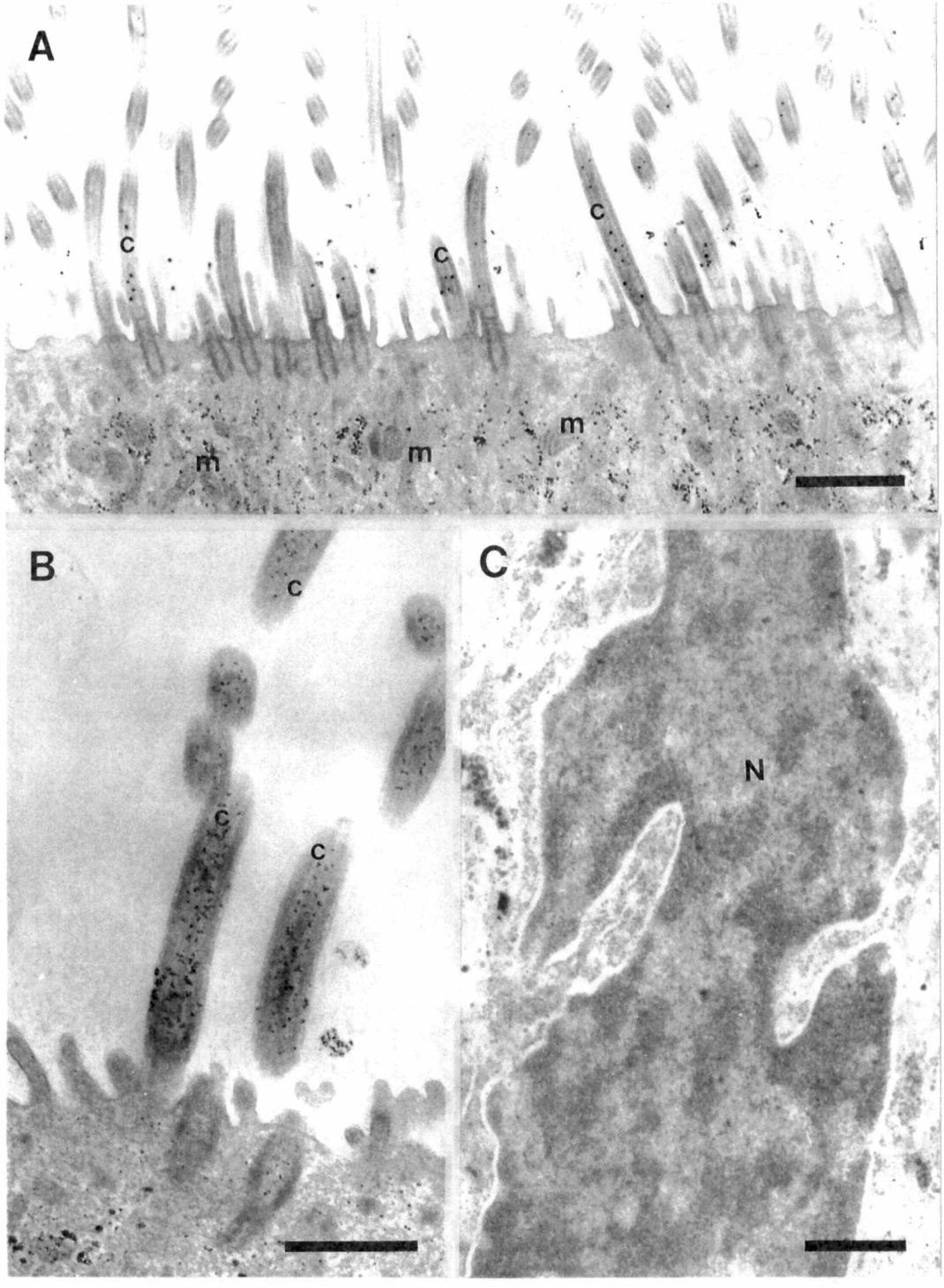


Fig. 4. Ultrastructural localization of Ca^{2+} in non-stimulated ciliated cell. (A) Calcium/pyroantimonate precipitates are present in the mitochondria (m) matrix and euchromatin area of the nucleus (N); little precipitate appeares in cilia (c). Bar = 1 μm. (B) Precipitates in the euchromatin area of a nucleus (N). Bar = 1 μm.

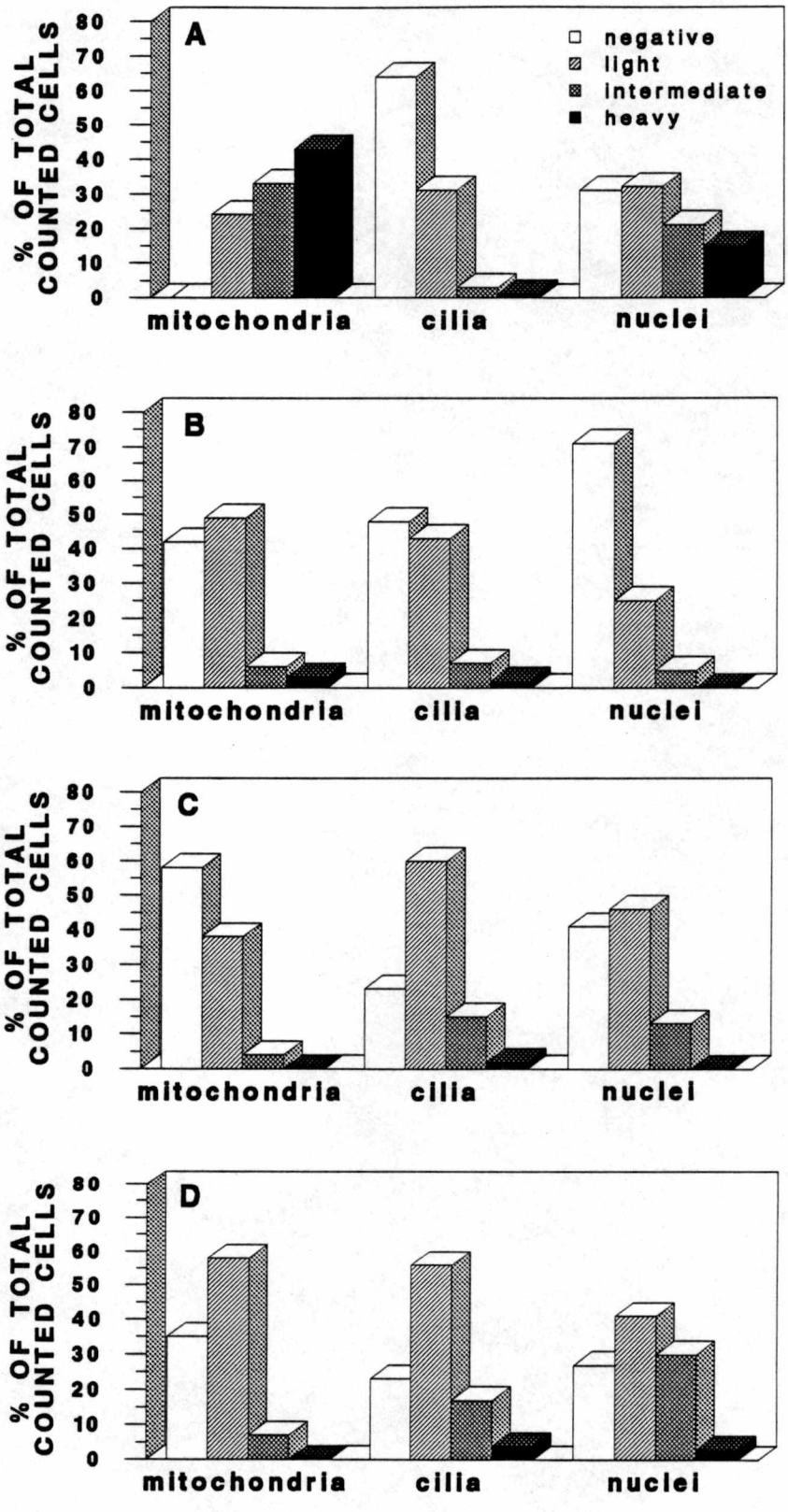
Fig. 5. Ultrastructural localization of Ca^{2+} in stimulated ciliated cell. (A) Considerable amount of the precipitate is shown in cilia (c) while there is very little precipitate in mitochondria (m) (from an electrically stimulated cell). Bar = 1 μm. (B) Many calcium/pyroantimonate precipitates are present in cilia (c) (from a mechanically stimulated cell). Bar = 0.5 μm. (C) There is no the precipitate in the nucleus (N) (from an acetylcholine treated cell). Bar = 0.5 μm.


A
c
c
c
m
m
m
B
c
c
c
C
N

(Fig. 5B) or acetylcholine treatment (Fig. 5C). Based on oxalate/pyroantimonate staining, a quantitative evaluation of the deposits in the mitochondria, cilia and nucleus were performed by scoring the deposits into four categories (negative, light, intermediate and heavy). At lest 100 different ciliated cells from each experimental group (non-stimulation, electrical stimulation, mechanical stimulation and acetylcholine stimulation) were observed with the electron microscope and scored.

The results showed that among non-stimulated ciliated cells (153 cells observed), 70-80% of them had heavy or intermediate deposits in mitochondria, a considerable number of cells had intermediate to heavy deposits in the nucleus while most of cells did not have deposits in their cilia or had only light deposits (Fig. 6A). In comparison, 70-90% of stimulated ciliated cells showed no or only light deposits in their mitochondria and nuclei while the number of cells with considerable deposits in cilia significantly increased (Fig. 6B, C & D). These results indicated that in response to stimulation, intracellular Ca^{2+} was released from mitochondria and nuclei and moved into cilia. This suggests that the mitochondria and nuclei are major sites for intracellular Ca^{2+} in normally beating non-stimulated ciliated cells. In response to stimulation, the Ca^{2+} is released from the storage sites into cytoplasm and the Ca^{2+} concentration in cilia increases, resulting in the initiation of increased ciliary frequency.

Fig. 6. Semi-quantitation of calcium/pyroantimonate precipitate in ciliated cells. (A) Control group: the plot was based on the observation of 153 different cells. (B) Electrical stimulation group: the plot was based on the observation of 102 different ciliated cells. (C) Mechanical stimulation group: the plot was based on the observation of 100 different cells. (D) Ach group: the plot was based on the observation of 100 different cells.


A
B
C
D
% OF TOTAL COUNTED CELLS
0
10
20
30
40
50
60
70
80
negative
light
intermediate
heavy
mitochondria
cilia
nuclei

Specificity of the calcium localization with the technique

The cell structures including mitochondria, cilia and nuclei were well preserved, and there were no electron opaque deposits in the untreated control tissues (Fig. 7A & B). The specificity of the technique was also verified by EGTA treatment of thin sections. The calcium/pyroantimonate deposits in mitochondria, cilia and nuclei in either non-stimulated or stimulated cells remained in distilled water treated sections (Fig. 7C). Sections from the same tissue treated by EGTA showed no deposits in these organelles (Fig. 7D). X-ray microanalysis on the deposits in cilia, mitochondria and nuclei revealed that the deposits were composed of calcium and antimony (Fig. 8). This evidence verified that the oxalate/pyroantimonate technique used in the present study identified intracellular Ca^{2+} specifically.

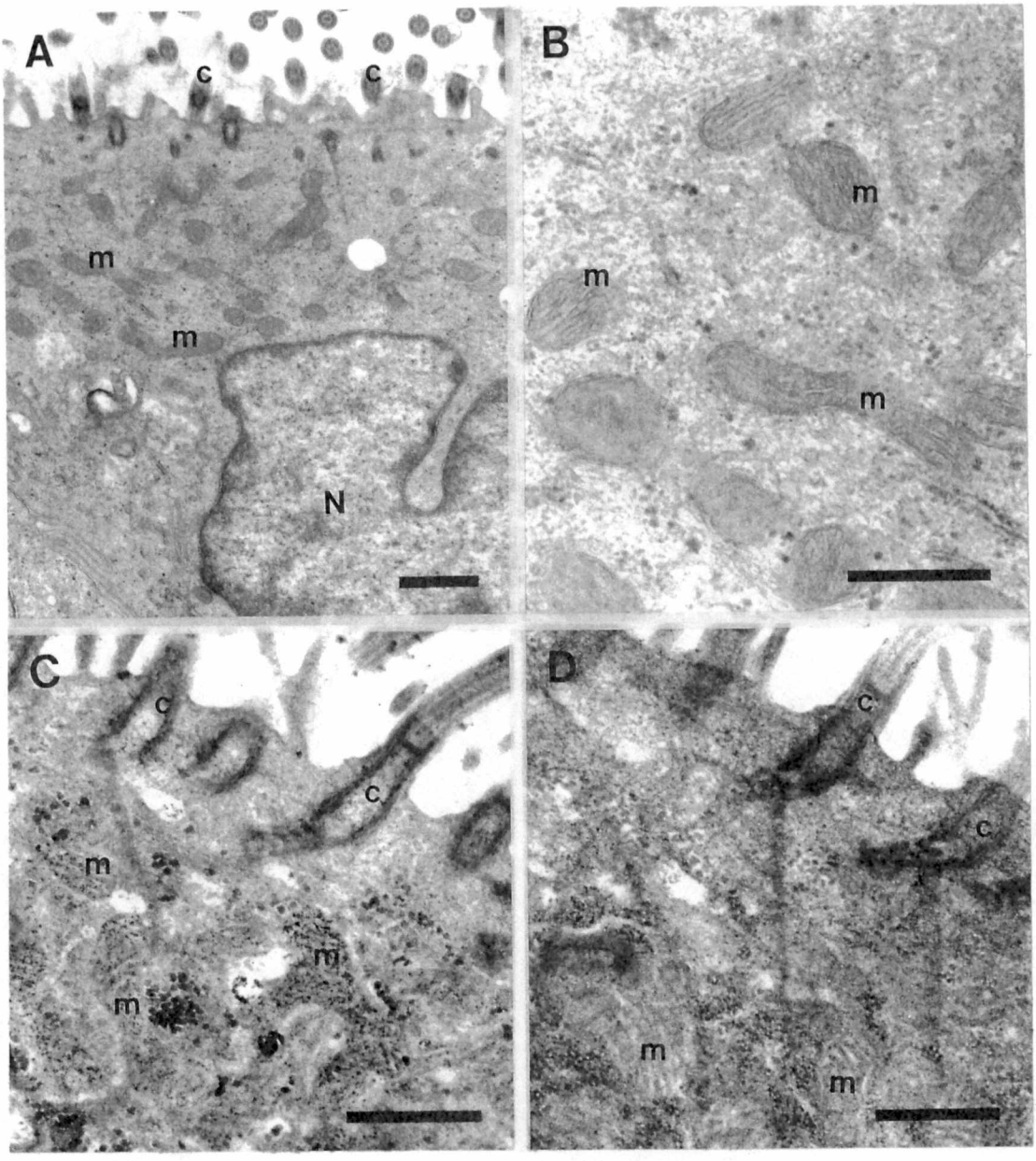


Fig. 7. Staining controls for the oxalate/pyroantimony method. (A) and (B) Negative control. The specific staining is omitted. There is no electron dense precipitate in the cell. Mitochondria (m), nucleus (N) and cilia (c). Bar = 1 μm (A), 0.5 μm (B). (C) The section washed in distilled water (60°C) for 1 hour. The precipitates are still in mitochondria (m). Bar = 0.5 μm. (D) The section treated with 5 mM EGTA at 60°C for 1 hour removes all calcium/pyroantimonate precipitate in mitochondria (m). Bar = 0.5 μm.

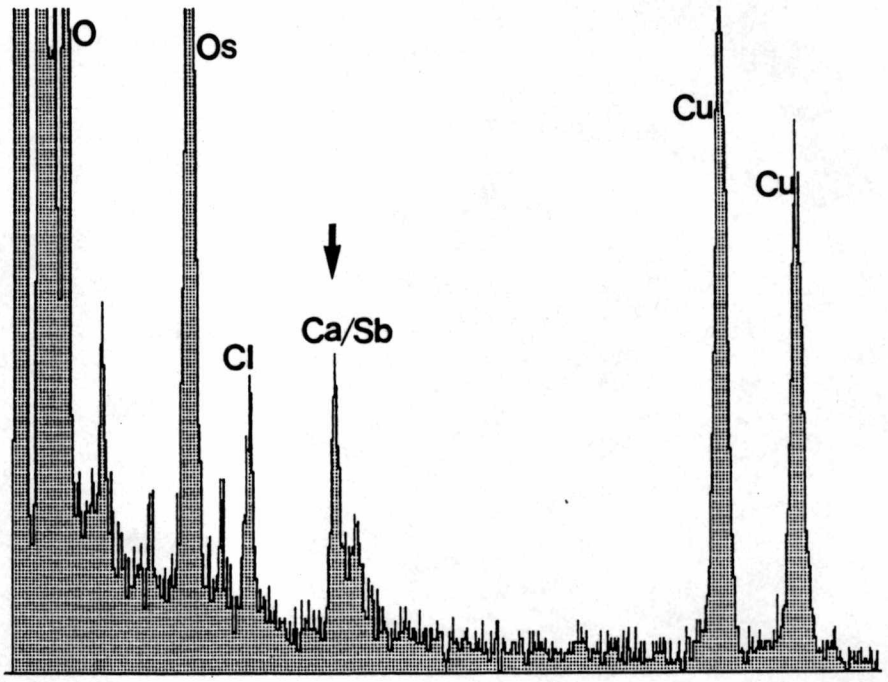


Fig. 8. A spectrum of X-ray microanalysis of electron opaque precipitate in a mitochondrion. One of the major peaks (arrow) of the spectrum indicates the presence of calcium/antimony (Ca/Sb). Oxygen (O), osmium (Os), chlorine (Cl) and copper (Cu) are also identified.

IV. DISCUSSION

Previous studies have shown that ciliary response of the frog palate culture to electrical stimulation is regulated by cholinergic nerve endings in the palate (Slaughter and Aiello, 1982; Hernandez and Aiello, 1992; Part II). In this study, ciliary response of frog palate cultures to extracellular acetylcholine or electrical stimulation in the presence and absence of extracellular Ca^{2+} was tested. The results in both cases showed that extracellular Ca^{2+} was not necessary for initiation of the ciliary response although the duration of the ciliary response was shorter after 0.1 mM verapamil treatment. Stronger stimulation was needed to induce ciliary frequency increase in Ca^{2+} free, 1 mM EGTA and 0.1 mM verapamil solution. Verapamil has been proven to be a specific Ca^{2+} channel blocker (Fleckenstein, 1977; Katz and Reuter, 1979; Triggle, 1980; Hille, 1984; Ruff, 1986; Hosey and Lazdunski, 1988). Micromolar verapamil can effectively block Ca^{2+} influx into a cell. It has been shown that 1 μM verapamil blocks Ca^{2+} channels of cardiac muscle cells in the presence of 1 mM extracellular Ca^{2+} (Lee and Tsien, 1983).

Although it has been reported that cytosolic Ca^{2+} concentration is elevated by mobilization of Ca^{2+} from intracellular stores in response to mechanical stimulation in mammalian ciliated epithelial cells (Sanderson *et al.*, 1990), the exact location of intracellular Ca^{2+} stores in ciliated epithelial cells is not known. In the present study, we found

that mitochondria and nuclei are the major Ca^{2+} stores in frog palate ciliated epithelial cells.

The oxalate pyroantimonate methods for the ultrastructural localization of intracellular Ca^{2+} was developed in a study of lung mast cells (Borgers *et al.*, 1977). It is based on the precipitation of Ca^{2+} with oxalate and the subsequent visualization of the precipitate by conversion to calcium pyroantimonate, a electron opaque particle. With this method, ultrastructural localization of Ca^{2+} in mitochondria, nuclei, intracellular vesicles, endoplasmic reticulum and cytoskeleton was successfully shown in various tissues, including lung mast cells (Borgers *et al.*, 1977), nervous tissues (Ohara *et al.*, 1979; Shaw and Morris, 1980; Griffiths *et al.*, 1983; Cornell-Bell and Finkbeiner, 1991), retina (Van Reempts *et al.*, 1982) and plant tissues (Wick and Hepler, 1980). The specificity of this technique for localization of Ca^{2+} was verified by EGTA treatment to remove the precipitates and X-ray microanalysis demonstration of calcium in the precipitates. Both of the validation processes were performed in the present study.

Since endoplasmic reticulum was another major Ca^{2+} storage site (Rasmussen and Barrett, 1984; Nicotera *et al.*, 1992) especially in liver cells and striated and cardiac muscles, we looked for the precipitate in the endoplasmic reticulum of ciliated cells, but no precipitate was found there. As the matter of fact, there is little endoplasmic reticulum in

ciliated cells, but mitochondria are abundant. These results suggested that the endoplasmic reticulum may not be a major Ca^{2+} storage site in ciliated cells of frog palate.

We found that mobilization of Ca^{2+} from mitochondria and nuclei to cilia occurred in frog palate ciliated cells under either electrical, acetylcholine or mechanical stimulation. Ca^{2+} mobilization from mitochondria appeared to be more significant than from the nucleus. In mitochondria of stimulated cells, calcium pyroantimonate precipitate was always greatly decreased compared to that of non-stimulated cells. On the other hand some nuclei of stimulated cells did not have the precipitate, but some still contained much of the precipitate. This observation suggested that mitochondria may be a more important Ca^{2+} source than the nuclei. Mitochondria have been identified as a major Ca^{2+} store in various tissues, but the role of mitochondria in intracellular Ca^{2+} signaling has been controversial (Rasmussen and Barrett, 1984). One view is that mitochondria serve the combined function of stabilizing cytosolic Ca^{2+} concentration and as a sink for Ca^{2+} in time of Ca^{2+} excess. Another is that mitochondria serve as a source of activator Ca^{2+}. Since Ca^{2+} in the mitochondria can be mobilized in so short a time (10 seconds after electrical or mechanical stimulation, and 1 minute after acetylcholine stimulation), we favor the view that mitochondria of frog palate ciliated cells serve as a source of activator Ca^{2+}. The mechanism of mobilization of Ca^{2+} from mitochondria in

response to stimulation is not clear.

Based on the present study, we propose that the ciliary response to continuous acetylcholine stimulation either by direct application or electrical stimulation of frog palate is a two step event which included (1) initiation of and (2) maintenance of increased ciliary frequency. In the first step, acetylcholine is bound to muscarinic receptors on the cell membrane causing the mobilization of Ca^{2+} from intracellular stores and elevation of cytosolic Ca^{2+}. Elevated cytosolic Ca^{2+} initiates the ciliary response. Since the muscarinic receptor has been found to be one of the G protein associated receptors (Berridge and Irvine, 1984; Kubo *et al.*, 1986; Matesic *et al.*, 1989; Birnbaumer *et al.*, 1990), the mechanism by which acetylcholine elevates cytosolic Ca^{2+} may be explained by the G protein mediated intracellular signal transduction pathway. It has been found in various cells (Berridge, 1987; Birnbaumer *et al.*, 1990; Catt *et al.*, 1991; Nicotera *et al.*, 1992) that upon binding of agonists to membrane receptors, activated G protein will stimulate a specific phospholipase C. This in turn causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the generation of two second messengers, inositol 1,4,5,-trisphosphat (IP_3) and diacylglycerol (DAG). The IP_3 can mobilize Ca^{2+} from intracellular stores while DAG activates protein kinase C resulting in phosphorylation of target proteins. In mammalian tracheal ciliated epithelium, it has been confirmed that IP_3

mediates Ca^{2+} release from intracellular stores (Sanderson *et al.*, 1990; Boitano *et al.*, 1992). Although there is no direct evidence that acetylcholine induces formation of IP_3 in frog palate epithelial cells, or that IP_3 mediates Ca^{2+} release from intracellular stores in this cell type, the similarity of function between the two tissues suggests that the same mechanism may function in frog palate epithelium.

In the second step, extracellular Ca^{2+} may influx into cells as a result of continuous acetylcholine stimulation so that high concentrations of intracellular free Ca^{2+} are available to increase ciliary frequency. It is not clear how acetylcholine can stimulate Ca^{2+} influx in ciliated cells. Acetylcholine may bind to muscarinic acetylcholine receptors and activate G protein sensitive potassium channels as found in cardiac muscle (Yatani *et al.*, 1987a,b; Birnbaumer, *et al.*, 1990). Potassium efflux through the channels driven by the potassium concentration gradient may cause hyperpolarization of the cell membrane. In turn, the hyperpolarization may open voltage operated channels (Rasmussen and Barrett, 1984; Nicotera *et al.*, 1992) on the cell membrane which facilitates the Ca^{2+} influx into the cell. The continuous effect of acetylcholine on the cells may cause continuous mobilization of Ca^{2+} from intracellular stores, and the external Ca^{2+} influx may prevent Ca^{2+} in the intracellular stores from being exhausted so that increased ciliary beat activity can be maintained. Since Ca^{2+} dependent potassium channels have been

found to be involved in extracellular ATP stimulation of ciliary activity of frog palate ciliated cells (Weiss *et al.*, 1992), we think the above explanation is an attractive possible mechanism.

In summary, this study suggests that extracellular Ca^{2+} is not necessary for initiation of ciliary beat frequency to increase in frog palate ciliated cells, but is responsible for maintaining the high frequency of beat after stimulation. The Ca^{2+} released from intracellular stores mediates the initiation of ciliary frequency increase, and mitochondria and nuclei are the major Ca^{2+} stores in the ciliated cells. In response to stimulation, extracellular Ca^{2+} moves into the cell to maintain an elevated level of ciliary frequency.

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VITA

Shaoyou Chu was born in Bengbu, Anhui Province, China on February 10, 1956. He graduated from Bengbu Second High School in 1974. After three years working in the countryside and then one year in the Bengbu Pencil Factory, he started his medical study in the spring of 1978. In the fall of 1982, he received his Bachelor of Medicine degree from Bengbu Medical College. He began his graduate study at Shanghai Second Medical University in the fall of 1982, and received his Master of Medicine degree in 1985. From 1985-1988, he was an assistant teacher and then promoted to a lecturer in the Department of Biophysics of Shanghai Second Medical University. In the spring of 1989 he began his graduate study at The University of Tennessee, Knoxville and was supported by a graduate teaching assistantship from the Department of Zoology. In May 1993, he was awarded the Doctor of Philosophy degree in Zoology and accepted a postdoctoral research position at Johns Hopkins University Medical school in Baltimore, Maryland.