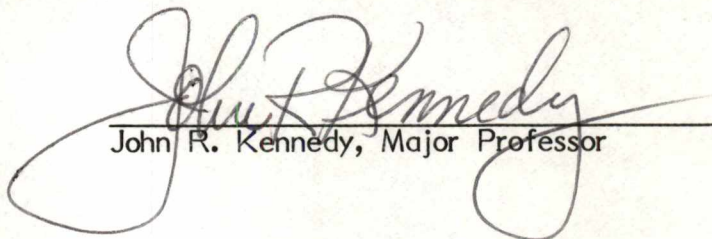
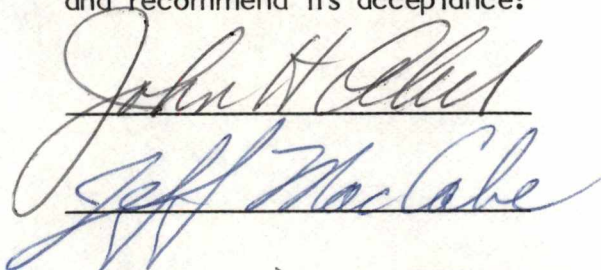


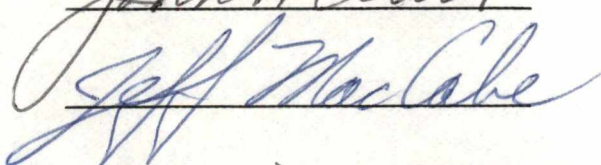
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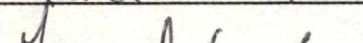
I am submitting herewith a dissertation written by Peggy R. Girard entitled "Ciliary Activity in Rabbit Tracheal Epithelium and its Regulation by Calcium, Sodium, and Cyclic Adenosine 3',5'-Monophosphate." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

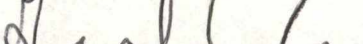

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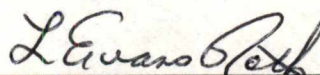

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CILIARY ACTIVITY IN RABBIT TRACHEAL EPITHELIUM AND ITS
REGULATION BY CALCIUM, SODIUM, AND CYCLIC
ADENOSINE 3',5'-MONOPHOSPHATE

A Dissertation
Presented for the
Doctor of Philosophy
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Peggy R. Girard

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ABSTRACT

Both calcium and cyclic adenosine 3',5'-monophosphate appear to play a role in the regulation of ciliary activity in rabbit tracheal epithelium. Elevation of cellular calcium levels using calcium ionophore A23187 stimulates ciliary motility in both explant and outgrowth cells. Prolonged exposure of the cells to A23187 leads to a reduction in ciliary frequency and ultimately to cessation. Reactivation studies utilizing demembranated ciliated cortices suggest that high levels of Ca^{2+} may act directly on the axoneme to exert an inhibitory effect on motility.

Depletion of cellular calcium by exposure to A23187 plus EGTA caused an immediate reduction in ciliary activity in explant and outgrowth cells. This may be due to a detrimental effect on mitochondria since the studies using demembranated models suggest that calcium is not necessary for reactivation of cilia.

Exposure of intact ciliated cells to theophylline and cholera toxin, agents which have been reported to elevate cellular cAMP levels, and to 8-Br cAMP, a cAMP analog, results in an increase in ciliary frequency. Direct exposure of the ciliary axoneme to cAMP using demembranated ciliary preparations causes enhanced ciliary amplitude and increased duration of reactivation.

Neither calcium nor cAMP are necessary for initiation or maintenance of ciliary motility but probably act to modulate ciliary activity. The interaction between calcium and cAMP in the control of ciliary motility is unknown but the present investigation suggests that increased intracellular calcium may stimulate cellular cAMP production and in turn increase ciliary frequency.

Tracheal epithelium is a sodium-transporting tissue and the movements of sodium and calcium in this tissue appear to be interrelated. In the presence of veratridine, an alkaloid which has been shown to elevate sodium levels in some cells, ^{45}Ca uptake is increased and coincides with a stimulation of ciliary frequency. Ouabain, which causes an increase in cellular sodium levels through an inhibition of $\text{Na}^+\text{K}^+\text{ATPase}$, reduces ciliary activity but calcium fluxes are unchanged. Unlike ouabain, veratridine causes increased intracellular sodium levels in the presence of functioning $\text{Na}^+\text{K}^+\text{ATPase}$. Therefore cellular K^+ levels should be unaltered by veratridine and the difference between the effects of these two drugs on ciliary motility and cellular calcium levels may be related to intracellular K^+ levels. The stimulation of ciliary frequency by veratridine may be the result of increased intracellular calcium as for cilia exposed to A23187. Exposure of the ciliated epithelium to depolarizing concentrations of extracellular K^+ or to Na^+ -free medium, does not affect ciliary frequency.

TABLE OF CONTENTS

SECTION		PAGE
	PART I. CALCIUM REGULATION OF CILIARY ACTIVITY IN RABBIT TRACHEAL EPITHELIAL EXPLANTS, OUTGROWTHS, AND DEMEMBRANATED MODELS	
I.	INTRODUCTION	2
II.	MATERIALS AND METHODS	5
	Tissue Preparation	5
	Quantitation of Ciliary Frequency	6
	⁴⁵ Ca Uptake	6
	⁴⁵ Ca Efflux	7
	Calculations	8
	Isolation of Ciliated Cortices	8
	Reactivation of Ciliated Cortices	9
	Electron Microscopy	10
III.	RESULTS	11
	Effects of Calcium on Ciliary Frequency	11
	⁴⁵ Ca Uptake and Efflux in the Presence of A23187	27
	Reactivation Studies	42
IV.	DISCUSSION	44
	LIST OF REFERENCES	52
	PART II. THE MODULATION OF CILIARY ACTIVITY BY CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE IN RABBIT TRACHEAL EPITHELIAL EXPLANTS, OUTGROWTHS, AND DEMEMBRANATED MODELS	
I.	INTRODUCTION	58
II.	MATERIALS AND METHODS	61
	Tissue Preparation	61
	Quantitation of Ciliary Frequency	61
	Reactivation Studies	61
III.	RESULTS	63
	Changes in Ciliary Frequency	63
	Reactivation Studies	67

SECTION	PAGE
IV. DISCUSSION	69
LIST OF REFERENCES	73

PART III. CILIARY ACTIVITY AND THE INTERRELATIONSHIP OF SODIUM AND CALCIUM MOVEMENTS IN THE TRACHEAL EPITHELIUM OF RABBITS

I. INTRODUCTION	79
II. MATERIALS AND METHODS.	82
Tissue Preparation.	82
Quantitation of Ciliary Frequency.	82
$^{45}\text{Ca}^{2+}$ Uptake and Efflux	83
III. RESULTS	84
IV. DISCUSSION	93
LIST OF REFERENCES.	97
VITA	101

LIST OF TABLES

TABLE		PAGE
	PART I. CALCIUM REGULATION OF CILIARY ACTIVITY IN RABBIT TRACHEAL EPITHELIAL EXPLANTS, OUTGROWTHS, AND DEMEMBRANATED MODELS	
1.	Kinetic Analysis of $^{45}\text{Ca}^{2+}$ Uptake Curves	29
2.	$^{45}\text{Ca}^{2+}$ Efflux Curves: Kinetic Analysis	33
3.	Kinetic Analysis of $^{45}\text{Ca}^{2+}$ Uptake Curves	38
4.	$^{45}\text{Ca}^{2+}$ Efflux: Kinetic Analysis	40
	PART III. CILIARY ACTIVITY AND THE INTERRELATIONSHIP OF SODIUM AND CALCIUM MOVEMENTS IN THE TRACHEAL EPITHELIUM OF RABBITS	
1.	Kinetic Analysis of $^{45}\text{Ca}^{2+}$ Uptake Curves	87
2.	$^{45}\text{Ca}^{2+}$ Efflux: Kinetic Analysis	90

LIST OF FIGURES

FIGURE		PAGE
PART I. CALCIUM REGULATION OF CILIARY ACTIVITY IN RABBIT TRACHEAL EPITHELIAL EXPLANTS, OUTGROWTHS, AND DEMEMBRANATED MODELS		
1.	Transmission Electron Micrograph of Tracheal Epithelium With Ciliated Columnar Cell, and Goblet Cell	12
2.	Scanning Electron Micrograph of Tracheal Epithelial Explant Showing Extensive Covering of Cilia on the Surface of the Cells	12
3.	View of the Edge of the Trachea Epithelial Explant With Adjacent Outgrowth	14
4.	Higher Magnification Scanning Electron Micrograph of Outgrowth Cells Showing the Group of Cilia on Each Cell .	14
5.	Thin Section Through a Ciliated Outgrowth Cell Showing the Squamous Nature of the Cells and a Cluster of Cilia	14
6.	Higher Magnification of a Ciliated Outgrowth Cell Showing Microfilamentous Network Present Among the Basal Bodies and Ciliary Rootlets	16
7.	The Lower Curves of Each Graph Show the Effect on Ciliary Frequency of Various Concentrations of Ca^{2+} on the Culture Medium.	19
8.	Comparison of the Effects of $1 \times 10^{-5}\text{M}$ A23187 on the Frequency on Cilia in Explant and Outgrowth Cells.	25
9.	^{45}Ca Uptake by Isolated Tracheal Epithelial Cells in the Presence and Absence of $1 \times 10^{-5}\text{M}$ A23187	28
10.	a) ^{45}Ca Efflux from an Untreated Tracheal Epithelial Cell Suspension. b) ^{45}Ca Efflux From A Tracheal Epithelial Cell Suspension in the Presence of $1 \times 10^{-5}\text{M}$ A23187	30
11.	The Effects of $1 \times 10^{-5}\text{M}$ Antimycin A on Ciliary Frequency in Outgrowth and Explant Cells	34

Figure

Page

12.	Effects of Ca^{2+} -Free Medium Containing 1mM EGTA on Explant Ciliary Frequency and Effects of Ca^{2+} -Free Medium Containing EGTA Plus $1 \times 10^{-5}\text{M}$ A23187	35
13.	^{45}Ca Uptake by a Suspension of Tracheal Epithelial Cells With and Without 1mM EGTA and $1 \times 10^{-5}\text{M}$ A23187 in the Medium	37
14.	^{45}Ca Efflux From Tracheal Epithelial Cells in the Presence of 1mM EGTA and $1 \times 10^{-5}\text{M}$ A23187 Showing a Fast, Intermediate, and Slow Component of Runout	39
15.	Effects of $1 \times 10^{-4}\text{M}$ Trifluoperazine on Ciliary Frequencies in Explants and Outgrowths	41
16.	Light Micrograph Showing Ciliated Apical Cortices from the Tracheal Epithelium	43

PART II. THE MODULATION OF CILIARY ACTIVITY BY CYCLIC ADENOSINE 3',5'-MONOPHOSPHATE IN RABBIT TRACHEAL EPITHELIAL EXPLANTS, OUTGROWTHS, AND DEMEMBRANATED MODELS.

1.	Effects of 10mM Theophylline on Ciliary Frequency in Explant and Outgrowth Cells	64
2.	Effects of 0.5 mM 8-Br cAMP on the Frequency of Cilia in Explant and Outgrowth Cells	65
3.	Effects of 50 ng/ml Cholera Toxin on Ciliary Frequency in Outgrowth Cells	66

PART III. CILIARY ACTIVITY AND THE INTERRELATIONSHIP OF SODIUM AND CALCIUM MOVEMENTS IN THE TRACHEAL EPITHELIUM OF RABBITS

1.	Effect of $1 \times 10^{-4}\text{M}$ Veratridine on Ciliary Frequency in Explant and Outgrowth Cells	85
2.	^{45}Ca Uptake by a Cell Suspension of Tracheal Epithelial Cells in the Presence and Absence of $1 \times 10^{-4}\text{M}$ Veratridine	86

3. a) ^{45}Ca Efflux From Tracheal Epithelial Cells. b) ^{45}Ca
Efflux from Tracheal Epithelial Cells in the Presence
of 10^{-4}M Veratridine 88
4. Effect of 1mM Ouabain on Ciliary Frequency in Explant and
Outgrowth Cells 92

PART I

CALCIUM REGULATION OF CILIARY ACTIVITY IN RABBIT
TRACHEAL EPITHELIAL EXPLANTS, OUTGROWTHS,
AND DEMEMBRANATED MODELS

I. INTRODUCTION

Calcium appears to play a key role in the control of ciliary activity. However its affect varies with the type of cell or tissue examined. In Paramecium, Ca^{2+} regulates both the direction of effective stroke and the frequency of ciliary beat through voltage-sensitive calcium channels (Eckert, 1972 and Machemer, 1974). In response to a number of environmental stimuli the cell membrane depolarizes, opening these calcium channels. The subsequent increase in intracellular Ca^{2+} concentration has been demonstrated to trigger ciliary reversal (Eckert, 1972; Naitoh and Eckert, 1974). Demembranated models reactivated with MgATP^{2-} have also been shown to undergo ciliary reversal in response to Ca^{2+} , swimming forward in the presence of Ca^{2+} at concentrations below 10^{-6}M and backward at $5 \times 10^{-5}\text{M}$ (Naitoh and Kaneko, 1972). Increasing the calcium concentration from 10^{-4}M to 10^{-3}M causes a slowing and then arrest of cilia (Naitoh and Kaneko, 1972).

Murakami and Takahashi (1975), on the other hand, demonstrated that mussel gill cilia activated to beat with 10^{-7}M 5-hydroxytryptamine showed irregularly repeated calcium-dependent ciliary arrests (usually less than 1 sec duration) upon addition of 10^{-6}M calcium ionophore A23187. Satir (1975) also observed ciliary arrest in mussel gill cilia in the presence of 10^{-5}M A23187 and 6.25 to 12.5 mM Ca^{2+} . He reported partial inhibition with a ten-fold dilution of either Ca^{2+} or ionophore. Studies by Walter and Satir (1978) using demembranated cilia from three regions of mussel gill (lateral, laterofrontal, and frontal) showed that all cilia beat normally at Ca^{2+} concentrations less than 10^{-7}M . Lateral cilia were arrested differentially at 10^{-6}M Ca^{2+} . At

Ca^{2+} concentrations greater than 10^{-4}M , most cilia of all three regions were arrested.

Several studies indicate that the control of ciliary activity by calcium in ciliated epithelia of vertebrates differs from that of protozoa and mussel gills. In ciliated cells of salamander oviduct, iontophoretic injection of Ca^{2+} led to an increased ciliary frequency (Eckert and Murakami, 1972). On the other hand, Lee and Verdugo (1976) showed that a decrease in extracellular Ca^{2+} using EDTA inhibited ciliary beat in rabbit oviduct. Verdugo (1980) reported evidence that indicates a calcium-dependent coupling of hormonal stimulus to ciliary activity in rabbit oviduct. He suggested that prostaglandin E_2 increased ciliary frequency through a release of intracellularly sequestered calcium into the cytosol. However, in none of these studies have actual measurements of Ca^{2+} changes in response to experimental manipulation of ciliary frequencies been made.

While the reversal response of Paramecium, the arrest response of gill cilia and the frequency response of vertebrate cilia are all calcium dependent, it is unclear whether similar mechanisms may occur in the axonemes to initiate these different responses. Two steps are likely to be involved in the mediation of these motility responses by Ca^{2+} : (a) an influx of Ca^{2+} across the cell membrane or a release of Ca^{2+} to the cytosol from intracellular stores followed by (b) modulation of ciliary activity through an interaction of Ca^{2+} with components associated with the axoneme. However, little is known regarding either step in mammalian ciliated cells.

The present study was undertaken to examine the relationship between Ca^{2+} induced frequency changes and cellular calcium levels in rabbit ciliated

tracheal explants and outgrowths. Demembranated models were also employed to examine the direct effects of calcium on axonemal function.

II. MATERIALS AND METHODS

Tissue Preparation

Tracheas were removed from young adult rabbits of mixed breed and sex from the cricoid cartilage to the bifurcation to the lungs. The cartilagenous portion of the tracheas were cut longitudinally into four segments and the epithelium was dissected from the underlying cartilagenous rings. The membranous region of each trachea was discarded since this region showed the greatest degree of variation of cell types. The epithelial strips were cut into 2 mm segments and these explants were cultured in Waymouth's MB 752/1 tissue culture medium (Gibco Laboratories, Grand Island N.Y.) to obtain outgrowths of ciliated cells.

For studies using cell suspensions, the epithelial cell layer was separated from underlying connective tissue using a modification of the technique of Tandler, et al. (1981). Strips of epithelial tissue were incubated in a Ca^{2+} - Mg^{2+} -free Earles balanced salt solution containing 20 mM EDTA at 37°C in a 5% CO_2 -95% air atmosphere. After one hour, the epithelium was removed from the underlying connective tissue with a scalpel. The resulting sheet of epithelium was rinsed free of EDTA by centrifugation and washing the pellet several times with Waymouth's medium. Cells were gently drawn into and out of a pipette in order to obtain a uniform cell suspension, and then equilibrated in Waymouth's medium for 1.5 hours. Ciliated cells in these cell suspensions retain their normal ciliary frequencies. They also show the same responses to the test substances used in this study on both tracheal explants and outgrowths.

Quantitation of Ciliary Frequency

Fresh explants, ciliated outgrowths, and cell suspensions were exposed to various extracellular concentration of Ca^{2+} (0, 0.8, 2.75, 5.4, 12.5 mM) and to Ca ionophore A23187 (Calbiochem-Behring Corp., LaJolla, CA) and EGTA. Explants were placed on a slide with a supported coverslip and sealed with parafin. Ciliary frequency of individual cells was measured employing a Leitz ortholux phase contrast microscope, an Oriel photomultiplier system and spectrum analyzer as previously reported (Kennedy and Duckett, 1981). Frequency data was obtained from the analog signal utilizing a spectrum analyzer and Fast Fourier Transform analysis. Ciliated outgrowths were examined using a Nikon Diaphot-TMD inverted microscope. The area of the outgrowth to be monitored was imaged on a Hitachi television monitor using a Hitachi TV camera. The photomultiplier system and spectrum analyzer described above were then employed to obtain frequency readings of individual cells from the TV monitor.

^{45}Ca Uptake

Cell suspensions of tracheal epithelial cells were preincubated in Waymouth's medium for 1.5 hrs. at 37°C in a 5% CO_2 -95% air atmosphere. Following preincubation, 5 $\mu\text{Ci/ml}$ ^{45}Ca (supplied by New England Nuclear, Boston, MA) and 10 $\mu\text{Ci/ml}$ ^3H -inulin (supplied by ICN Chemical and Radioisotope Div., Irvine, CA), to be used to measure extracellular space, were added to the medium with or without the agent to be tested. 100 μl tissue samples were removed at varying intervals from 2.5 to 90 min and layered over a 70% silicone oil - 30% dinonyl phthalate mixture in 400 μl microcentrifuge tubes. The cells were separated from the incubation medium by centrifugation at

11,000 g in a Beckman microfuge for 30 sec. After centrifugation, the tip of the tube with the cell pellet was removed and transferred to a scintillation vial containing 200 μ l protosol. The pellet was dissolved and 3 ml of Scintiverse (Fisher Scientific, Atlanta, GA) was added to each vial. Radioactivity was measured in a Beckman LS 8100 liquid scintillation counter under double isotope settings. 75 μ l Aliquots of the cell suspension were also taken during the course of the uptake experiment for protein determination (Lowry, 1951).

^{45}Ca Efflux

For efflux studies, the cell suspensions were preincubated after isolation for 30 min in Waymouth's medium followed by incubation for 1.5 hrs. in Waymouth's medium containing 10 μCi $^{45}\text{Ca}^{2+}$ and 20 μCi ^3H -inulin as a measure of extracellular space. After equilibration, the cells were centrifuged at 11,000 g for 30 sec in a Beckman microfuge and resuspended in 800 μ l of Waymouths medium containing either A23187 or A23187 and EGTA but without isotopes. Parallel controls were employed without ionophore or EGTA. At varying intervals (from 2.5 - 60 min) the cell suspension was centrifuged and medium removed and placed in a scintillation vial. Fresh medium was added and the pellet resuspended after each interval. To the aliquots of efflux medium, 2.5 ml of scintiverse was added and the $^{45}\text{Ca}^{2+}$ and ^3H -inulin measured by liquid scintillation spectrometry as above. The total amount of $^{45}\text{Ca}^{2+}$ which ran out of the cells during each time interval was calculated (pmoles $^{45}\text{Ca}^{2+}$ /mg protein). The cells were sonicated and aliquots measured for $^{45}\text{Ca}^{2+}$ and ^3H -inulin to determine the amount of $^{45}\text{Ca}^{2+}$ remaining in the tissue. Efflux curves were constructed and rate constants and efflux rates were calculated from the graphs.

Calculations

The assumptions and equations for calculating kinetic parameters of $^{45}\text{Ca}^{2+}$ uptake and efflux were obtained from Borle (1975). For graphical analysis of the uptake curves, semilogarithmic plots of rate vs. time were constructed.

The uptake curves follow the general equation:

$$(dR/E)/dt = C_1 \exp(-k_1 t) + C_2 \exp(-k_2 t)$$

where R = radioactivity in the cells normalized on the basis of protein, E = specific activity of the medium, C = y -intercept of the asymptote of the component, and k = slope of the asymptote. The value for C is equivalent to kA , where A = pool size.

For $^{45}\text{Ca}^{2+}$ efflux, a semilogarithmic plot of R/E vs. time was constructed.

The efflux curves follow the general equation:

$$R/E = C_1 \exp(-k_1 t) + C_2 \exp(-k_2 t)$$

In this equation, C (the y -intercept of the asymptote of the component) is equivalent to pool size.

Isolation of Ciliated Cortices

Strips of ciliated tracheal epithelium were prepared as above. Isolated ciliated cortices were then obtained according to the method of Dirkson and Zeira (1981): Pieces of epithelial tissue were placed in test tubes containing approximately 3 ml of an isolation solution consisting of 0.25M sucrose, 0.02M HEPES, pH 7.5, 2 mM EDTA, 25 mM KCl, and 0.05% Triton X-100 (Calbiochem-Behring Corp., LaJolla, CA). The tissue was vortexed intermittently for 40 minutes. Pieces of the trachea were then removed and an equal volume of isolation solution containing 1% Triton X-100 was added to the suspension.

After a 60 second incubation, the suspension was vortexed for 2-3 seconds. The contents were then centrifuged for 10 min. at 500 g in a Sorvall SP/X centrifuge. The pellet, which consisted of cortices with demembrated cilia, some whole cells, and nuclei, was resuspended in the isolation solution without Triton X-100. The cortices were maintained at 4°C until ready for use that day.

Reactivation of Ciliated Cortices

A reactivation solution, consisting of 75 mM KCl, 2.5 mM MgSO₄, 0.1% β-mercaptoethanol, 20 mM Tris-HCl, and 0 to 10⁻²M Ca²⁺ (pH 8.0) was prepared fresh for each experiment. To obtain Ca²⁺ concentrations in the range of 10⁻⁸ - 10⁻⁶ M, Ca-EGTA buffers were prepared. Free Ca²⁺ concentrations were calculated following the method of Katz *et al.* (1970) using the stability constants of Ca²⁺ EGTA, Mg²⁺ ATP, and Ca²⁺ EGTA of Sillen and Martell (1964), Godt (1974) and O'Sullivan and Perrin (1963), respectively.

Fifteen μl of the ciliated cortices were mixed on a slide with 15 μl of reactivation solution with the Ca²⁺ concentration to be tested, and a coverslip placed over the mixture. Twenty-five μl of the reactivation solution was then added to the edge of the coverslip and drawn across the suspension of cortices by touching a piece of filter paper to the opposite edge of the coverslip in order to equilibrate them with the appropriate Ca²⁺ concentration. Twenty-five μl of reactivation solution containing 1 mM ATP was then added to the edge of the coverslip and drawn across the suspension of cortices. Reactivation was performed at room temperature. In some experiments either 25 μM or 100 μM trifluoperazine was added after reactivation.

Electron Microscopy

Outgrowths and explants were fixed in the plastic culture dishes in one part 2.5% glutaraldehyde and two parts 1% osmium tetroxide in 0.1M cacodylate buffer (pH 7.4) in ice for one hour. For transmission electron microscopy, samples were dehydrated in a graded ethanol series and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate and examined on a Hitachi H600 transmission electron microscope.

For scanning electron microscopy, tissue was dehydrated in a graded ethanol series and critical-point dried in a Bomar SPC-900/EX critical-point drier. Specimens were coated with gold:palladium (60:40) in a Denton DV-515 SM vacuum evaporator. Specimens were examined in an ETEC Autoscan scanning electron microscope.

III. RESULTS

Tracheal epithelial tissue of the rabbit (Fig. 1) is composed primarily of ciliated columnar cells, mucus-secreting goblet cells and basal cells. Goblet cells are relatively sparse in rabbit tracheal epithelium. These cells contain large secretory vesicles and are responsible for the production and secretion of mucus onto the epithelial surface. Basal cells are located on the basal lamina between the bases of the goblet and ciliated cells and have no contact with the lumen. Basal and ciliated cells are present in approximately equal proportions, each comprising about 40-45% of the epithelium. With SEM (Fig. 2), the tracheal surface appears to be extensively ciliated. Each ciliated cell has an apical width of approximately 10 μm and it has been estimated to contain 250 cilia (6 μm long, 0.2-0.3 μm in diameter).

After 5-6 days in culture, cells from small pieces of epithelial tissue (explants) migrate out onto the plastic petri dish forming a monolayer (Figs. 3 and 4). The cells in this outgrowth have changed from a columnar shape to a flattened appearance (Fig. 5). The cilia of each cell remain in a group on the cell surface. This appears to be due to a microfilamentous network among the ciliary rootlets and basal bodies (Fig. 6).

Effects of Calcium on Ciliary Frequency

Calcium concentration has been shown to be increased in the tracheobronchial secretions of patients with several types of respiratory disorders such as cystic fibrosis, bronchiectasis, and asthma (Nadel, et al., 1979). In order to determine whether changes in extracellular calcium concentration affects ciliary activity, the calcium concentration of the medium

Fig. 1. Transmission electron micrograph of tracheal epithelium with ciliated columnar cell (C), and goblet cell (G). Cilia (Ci) and microvilli cover the cell surface. x2800

Fig. 2. Scanning electron micrograph of tracheal epithelial explant showing extensive covering of cilia (C) on the surface of the cells. The cell without cilia is most likely a goblet cell (G). x6300

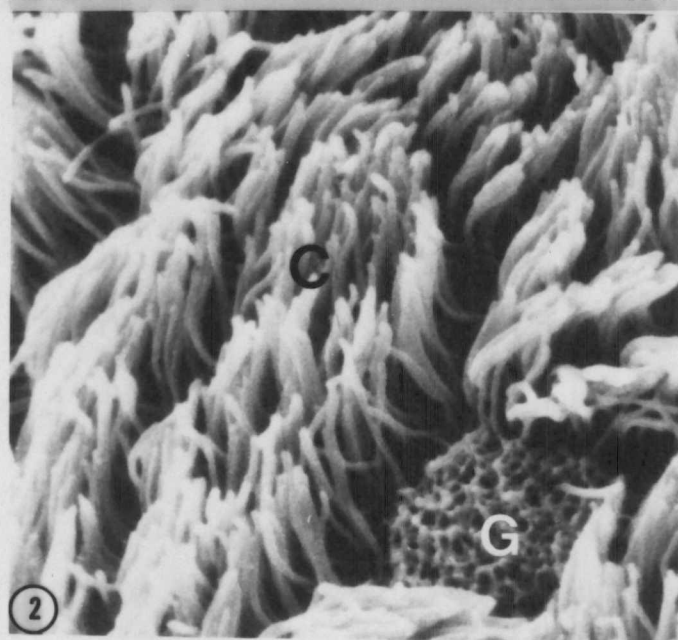
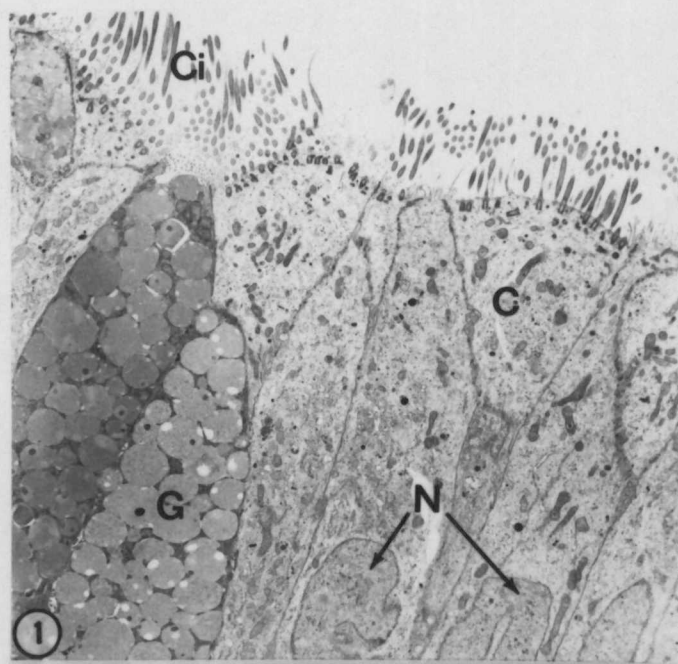


Fig. 3. View of the edge of the tracheal epithelial explant (E) with adjacent outgrowth. The spreading of the epithelial cells during formation of the outgrowth increases the apical membrane surface area and results in isolated groups of cilia (C). x260

Fig. 4. Higher magnification scanning electron micrograph of outgrowth cells showing the group of cilia on each cell. x1100

Fig. 5. Thin section through a ciliated outgrowth cell showing the squamous nature of the cells and a cluster of cilia (C). x4500

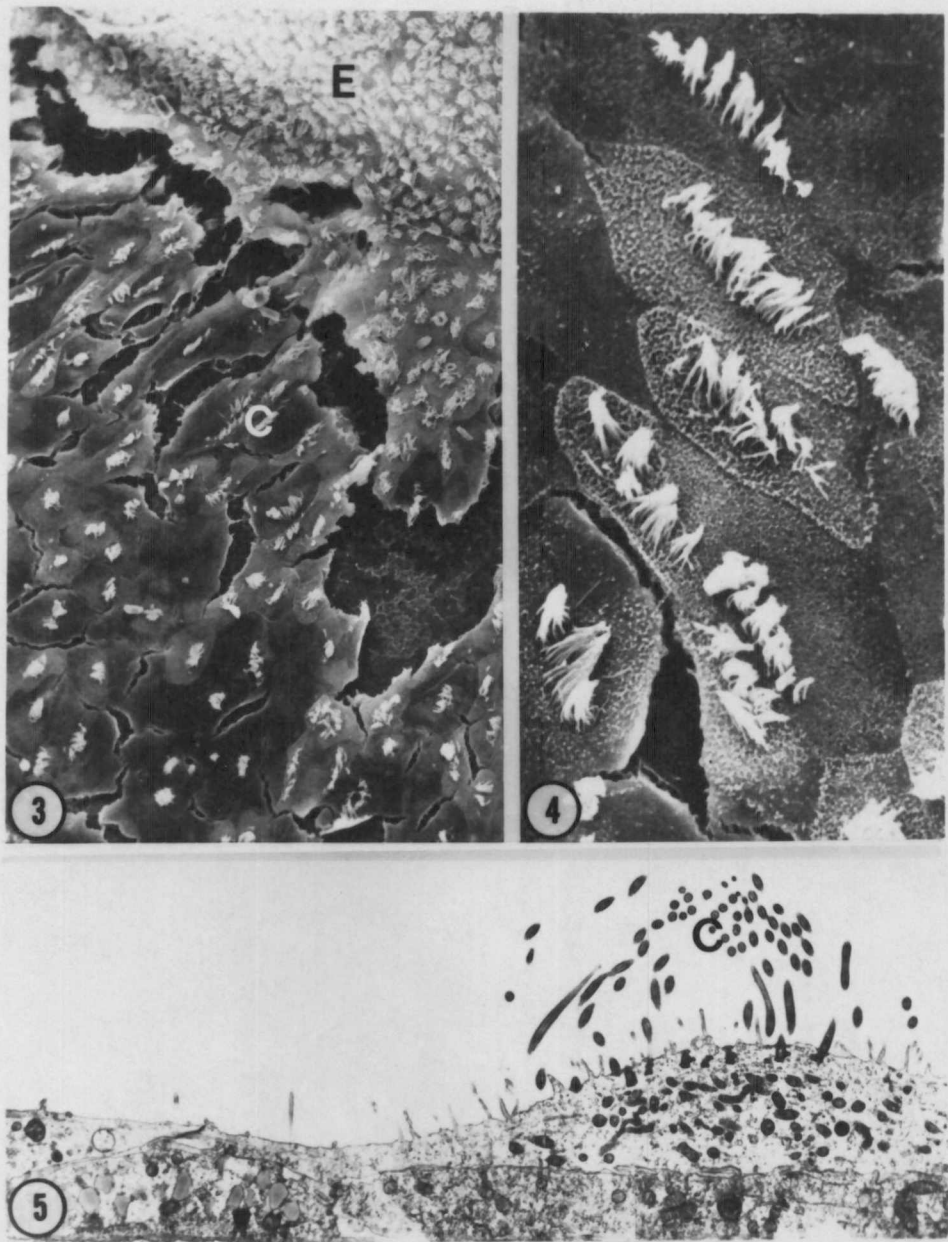
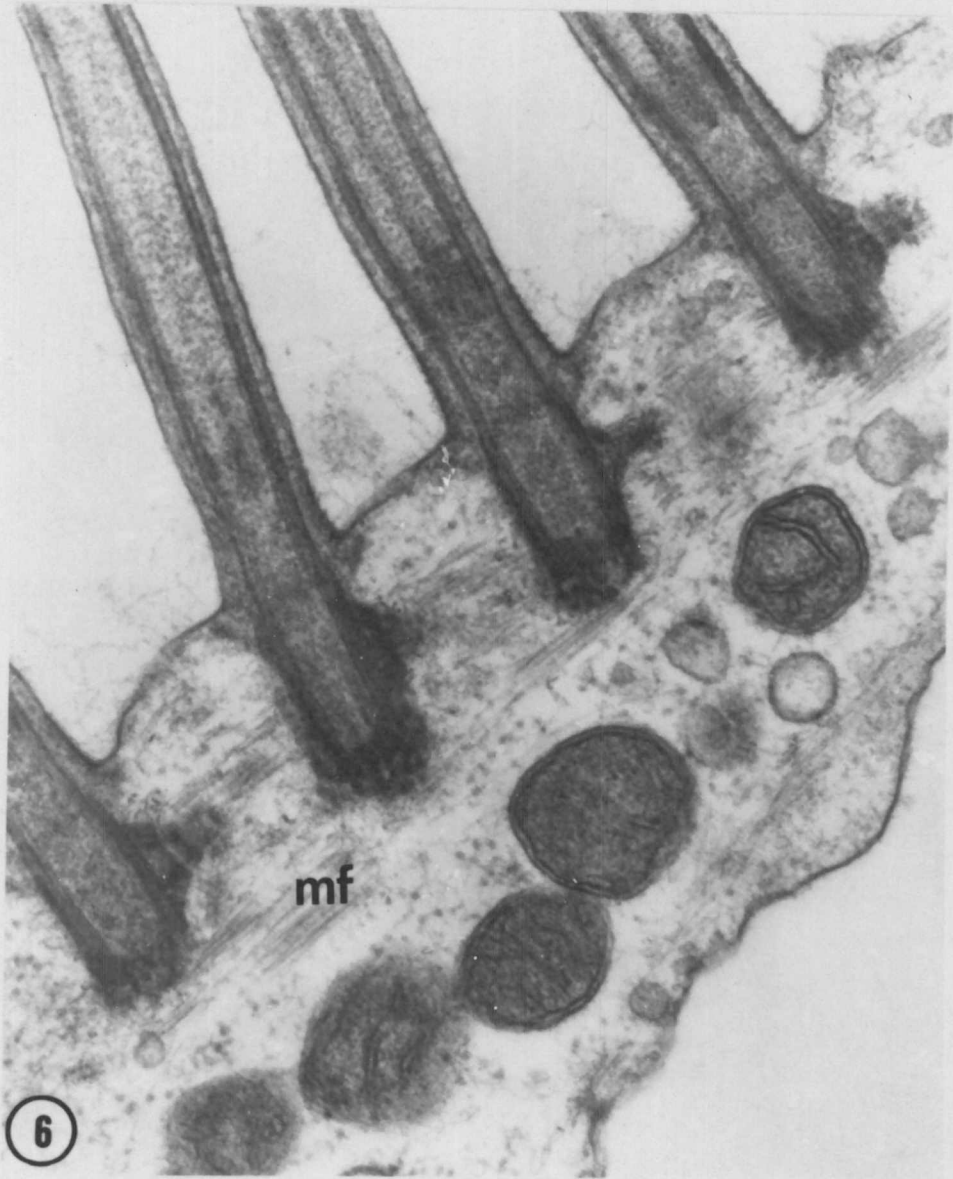


Fig. 6. Higher magnification of a ciliated outgrowth cell showing microfilamentous network (mf) present among the basal bodies and ciliary rootlets. x53,000



(Hanks balanced salt solution was used in these experiments) in which the ciliated epithelial explants were maintained, was varied from 0.8 mM - 12.5 mM and frequencies measured.

Fig. 7 a-d (lower curves) shows the effect of 0.8, 2.75, 5.4, and 12.5 mM Ca^{2+} on ciliary frequency of explants. Initial mean frequencies of the cilia varied but were generally in the range of 9 to 17 beats/sec. At all 4 concentrations employed, most explants showed a slight decrease in frequency during the 30 min. observation period. In those which did show a decrease, it did not exceed 3 beats/sec. There appeared to be no difference in the effect on ciliary activity between the four Ca^{2+} concentrations employed. Thus it is apparent that changes in extracellular Ca^{2+} concentration in the range of 0.8 mM to 12.5 mM does not significantly alter ciliary beat.

Changes in intracellular Ca^{2+} have been reported to alter ciliary activity in a variety of cells and tissues. In order to determine whether changes in intracellular Ca^{2+} levels altered ciliary motility, tissue was exposed to calcium ionophore A23187. This carboxylic acid antibiotic is lipid soluble and facilitates the movement of divalent cations across membranes (Reed and Lardy, 1972). It has been shown to affect many cellular processes involving Ca^{2+} , particularly those which appear to be dependent on a rise in cytosolic Ca^{2+} .

Fig. 7 a-d (upper curves) shows the results of the presence of 10^{-5}M A23187 in medium (HBSS) containing various Ca^{2+} concentrations (0.8, 2.75, 5.4, and 12.5 mM) on the frequency of explant cilia. Addition of A23187 generally led to an immediate significant increase ($p < 0.001$) in ciliary activity at all of the Ca^{2+} concentrations tested. In 0.8 mM Ca^{2+} , increases ranged from 2-6 beats/sec. In 2.75-12.5 mM Ca^{2+} ciliary activity generally showed an increase of 5-7 beats/sec.

Fig. 7. The lower curves of each graph show the effect on ciliary frequency of various concentrations of Ca^{2+} in the culture medium. Each curve represents the data obtained from one animal, with frequency readings from ten cells at each time point. Upper curves show the effect of the addition of $1 \times 10^{-5}\text{M}$ A23187 to the medium in the presence of Ca^{2+} concentrations of a) 0.8 mM, b) 2.75 mM, c) 5.4 mM, d) 12.5 mM.

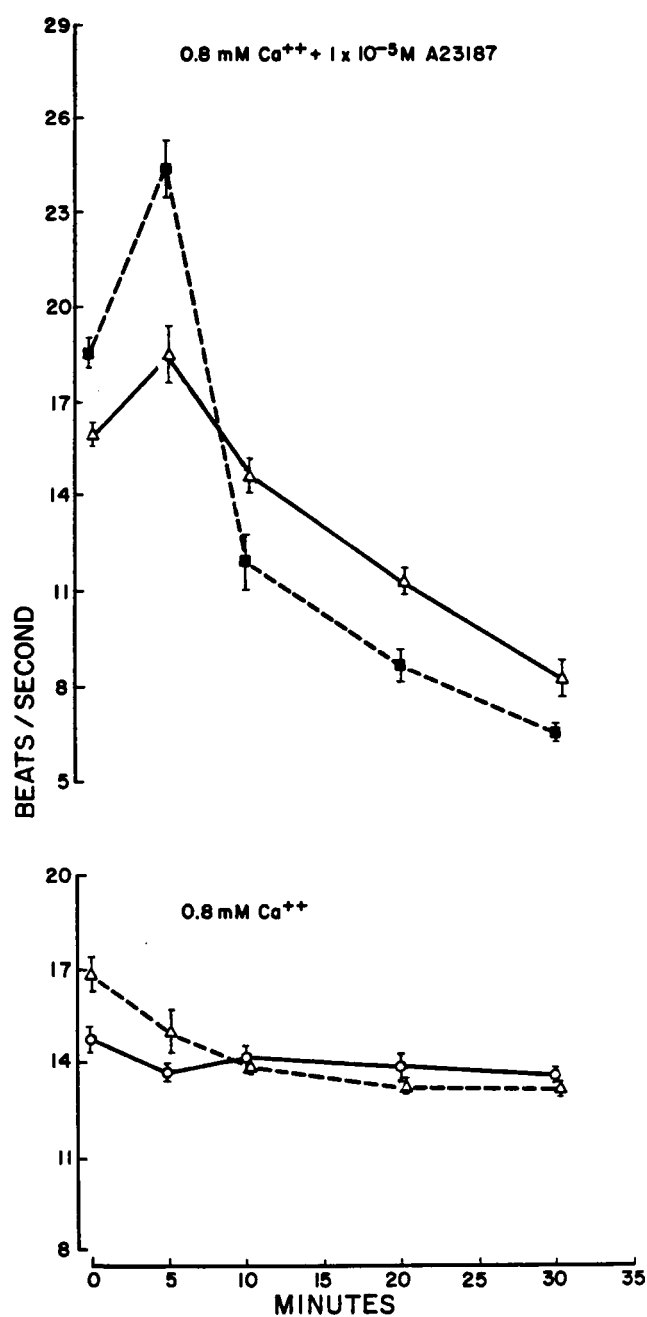
**a**

Fig. 7.

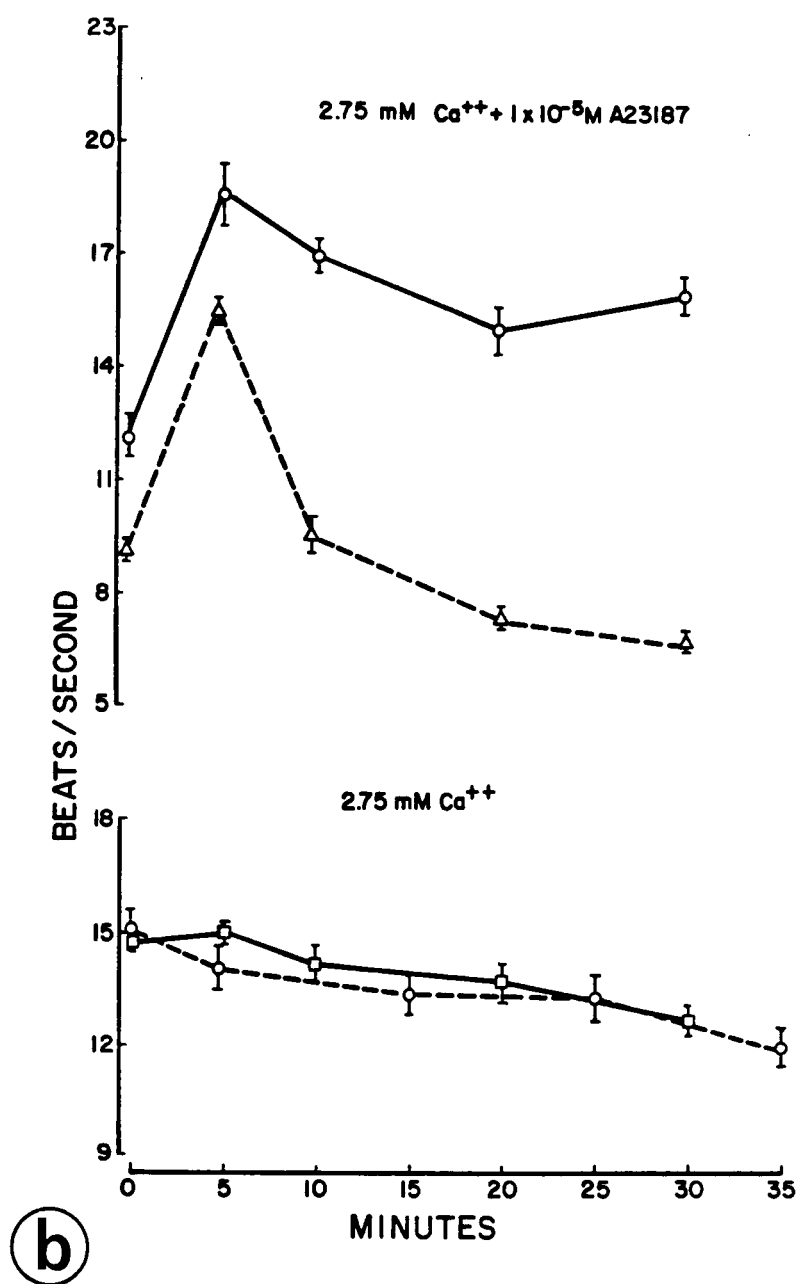


Fig. 7. (Continued)

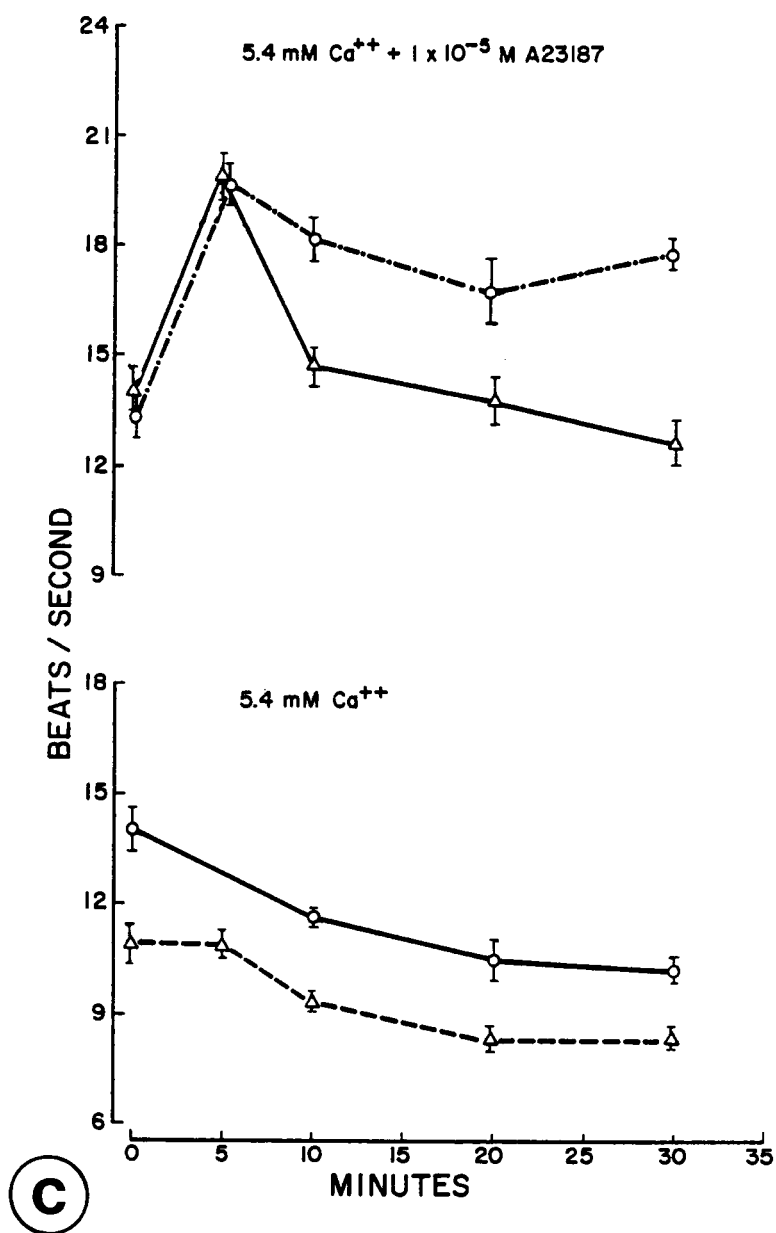


Fig. 7. (Continued)

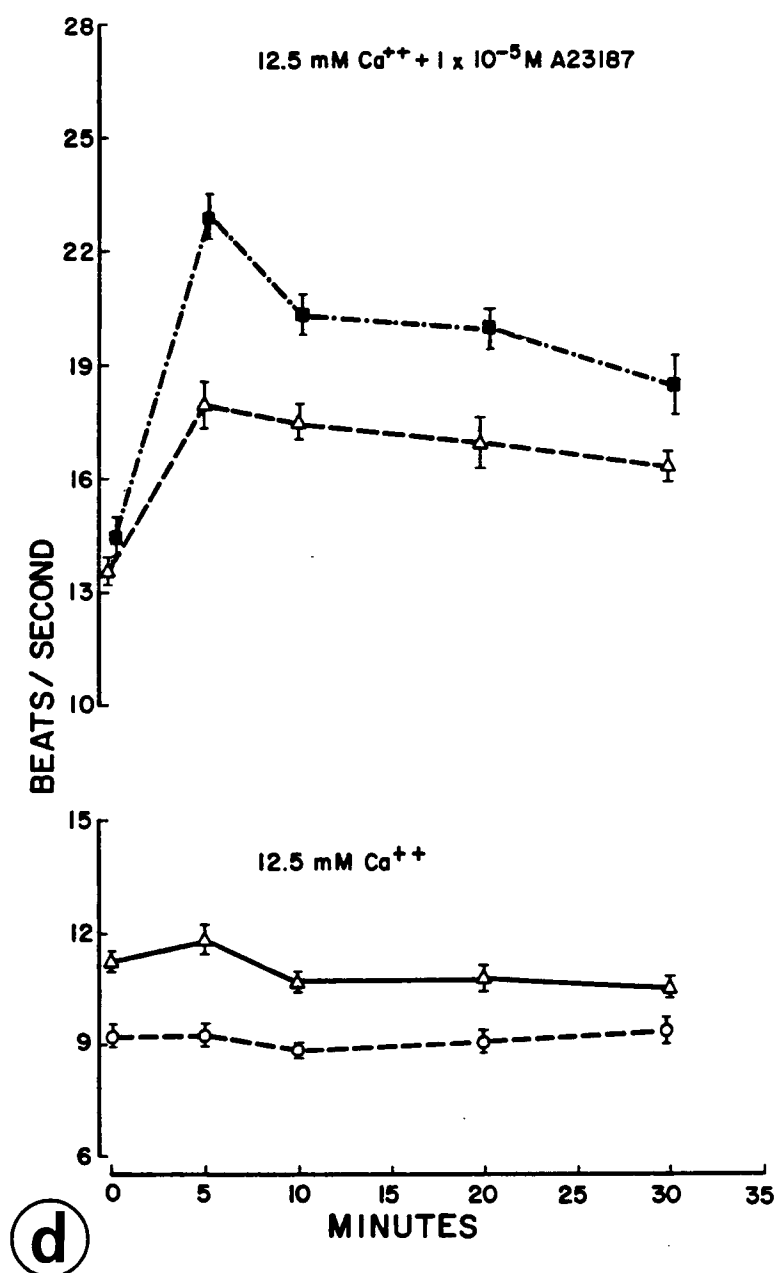


Fig. 7. (Continued)

At intervals after 5 min. the pattern changed depending on the Ca^{2+} concentration employed. The frequency in tissue exposed to 0.8 mM (Fig. 7a) dropped below initial levels after 10 min. In 2.75 and 5.4 mM Ca^{2+} (Fig. 7b,c) frequencies in some instances stayed higher than initial values for the 30 min. test period. In other cases, frequencies returned to or dropped below initial activity. At the highest Ca^{2+} concentration tested (12.5 mM - Fig. 7d) ciliary activity generally remained high during the entire 30-35 min observation period.

The spatial organization of cilia on outgrowth cells, (described above and shown in Figs. 3-5), allowed observation of the activity of individual ciliated cells in a way not possible with tracheal explants. Also, these cells generally seemed to be more sensitive to the agents tested in this study, perhaps due to the increased surface area exposed to the external medium. Ciliary frequency was monitored for up to 6 hours in these experiments. The tissue remained in a healthier state over a longer period of time in Waymouth's medium compared to HBSS, so these experiments were performed in Waymouth's medium (Waymouth's contains 0.8 mM Ca^{2+}).

The effect of A23187 on ciliary activity in outgrowth cells compared to explants is shown in Fig. 8. In explants, (Fig. 8, lower curve) the initial frequency increase (generally 3-6 beats/sec) which occurred during the first five minutes was followed by a slow decrease in frequency from these high values with a return to initial values by one hour. Some cell extrusion from the epithelial layer usually began to occur after 20-30 min. exposure to A23187 with concomitant ciliary cessation. By 5-6 hours, 30-50% of the cells had been extruded.

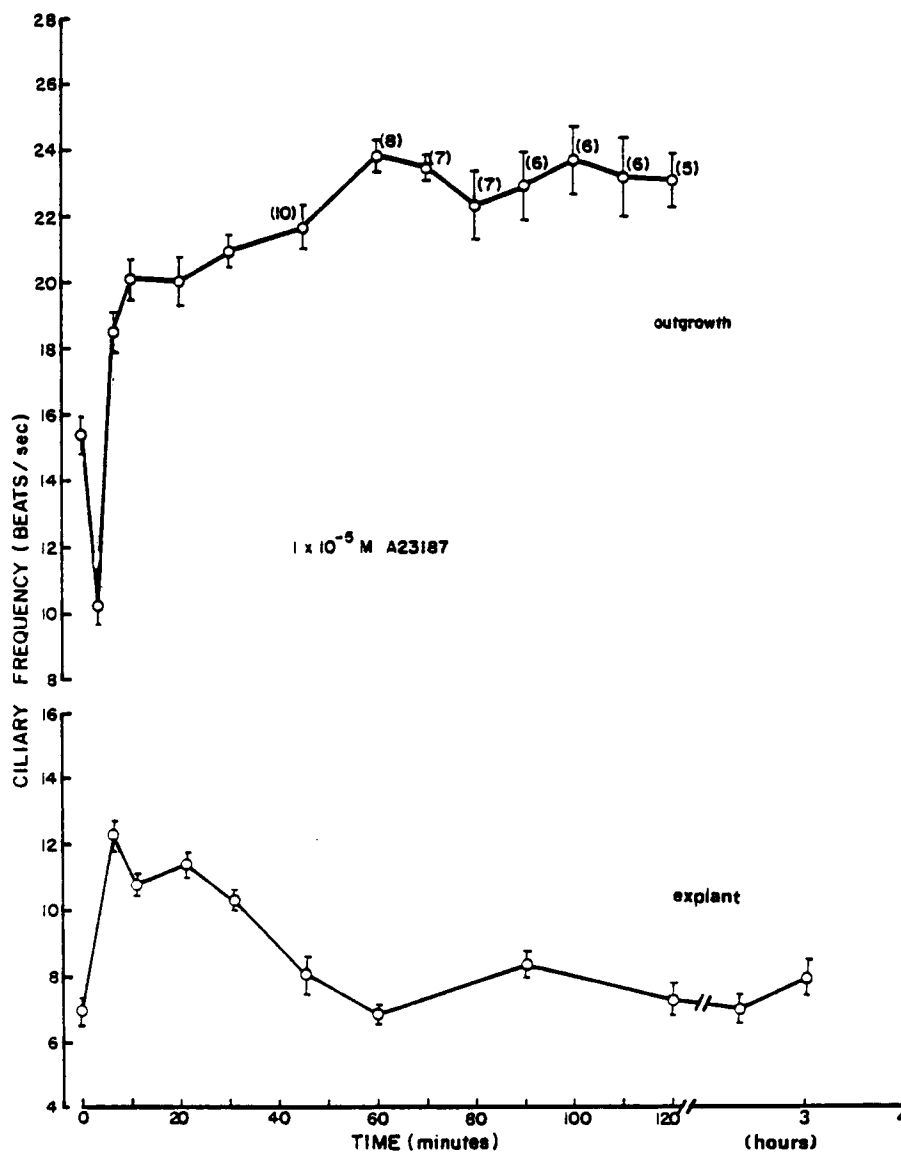


Fig. 8. Comparison of the effects of 1×10^{-5} M A23187 on the frequency of cilia in explant (lower curve) and outgrowth (upper curve) cells. Each curve shows one experiment with the mean frequency of 12 cells plotted at each time point. The numbers in parentheses on the upper curve show the number of cells still beating out of the 12 being monitored.

An initial drop in frequency was noted upon addition of ionophore to the outgrowth. However, it was discovered that a similar brief frequency drop occurred when the medium was drawn off the culture and replaced with fresh medium. This decreased frequency was not detected in explants although the transfer of the tissue to a new slide for the addition of A23187 prevented observation of a response for approximately one minute. Immediately following the initial drop in frequency of cilia in outgrowth cells, there was a dramatic increase in rate of ciliary beat (4-8 beats/sec increase from initial values) (Fig. 8b). The frequency of cilia remained high until the cilia on individual cells ceased beating, an event that occurred by 15 minutes for some cells, or several hours later for other cells. During the last 5 minutes before ciliary arrest, the frequency dropped steadily and ciliary movement became dyskinetic. In Fig. 7a, the numbers in parentheses indicate the number of cells with active cilia out of 12 cells monitored. Dye exclusion studies using 0.5% trypan blue indicated that the cells were still viable at the time of ciliary cessation. Cell death usually occurred from 30 min. to 1.5 hours after cessation.

In some experiments, after the cilia from 3 or 4 outgrowth cells had stopped beating, the medium containing A23187 was removed. The culture was washed several times in medium without A23187 and the outgrowth monitored for several hours to determine whether ciliary arrest was reversible. The frequency of those ciliated cells that were still active generally remained high for at least an hour before returning to initial levels. No recovery of arrested cilia was detected, and no additional ciliated cells were inhibited.

$^{45}\text{Ca}^{2+}$ Uptake and Efflux in the Presence of A23187

For experiments using $^{45}\text{Ca}^{2+}$, tracheal epithelial cell suspensions were used in order to have tissue free of adhering connective tissue. The isolated ciliated cells responded to all experimental conditions with identical responses to that of the outgrowth and explant preparations.

$^{45}\text{Ca}^{2+}$ uptake was measured in the presence of physiological levels of calcium (Waymouth's 752/1 medium). Fig. 9 shows the $^{45}\text{Ca}^{2+}$ uptake into a cell suspension of tracheal epithelial cells in the presence and absence of A23187. Kinetic analysis of the uptake curves (Table I) shows at least two distinct exchangeable components. The faster phase may reflect movement of calcium across the plasma membrane while the slower component may be associated with an intracellular calcium sequestering organelle such as the mitochondria. There also appears to be a faster component occurring within the first two minutes that likely represents binding of Ca^{2+} to the cell surface (Borle, 1970; 1972). As not enough time points could be taken during this period to obtain a measurement of the component with an acceptable precision, only the fluxes of the slow phase and the relatively faster phase were considered in this paper. Kinetic analysis of the faster phase shows that the rate of calcium exchange in the A23187-treated cells is approximately 1.5 times that of the control. Analysis of the slower component shows a 4.5 fold increase in the rate of calcium exchange.

Fig. 10 a,b shows representative $^{45}\text{Ca}^{2+}$ efflux curves in the presence and absence of A23187. The points represent pmoles $^{45}\text{Ca}^{2+}$ mg protein⁻¹ left in the cells at each time point. Both curves are graphically resolved into two exponential components (solid lines) with slopes of k_1 and k_2 . Kinetic analysis

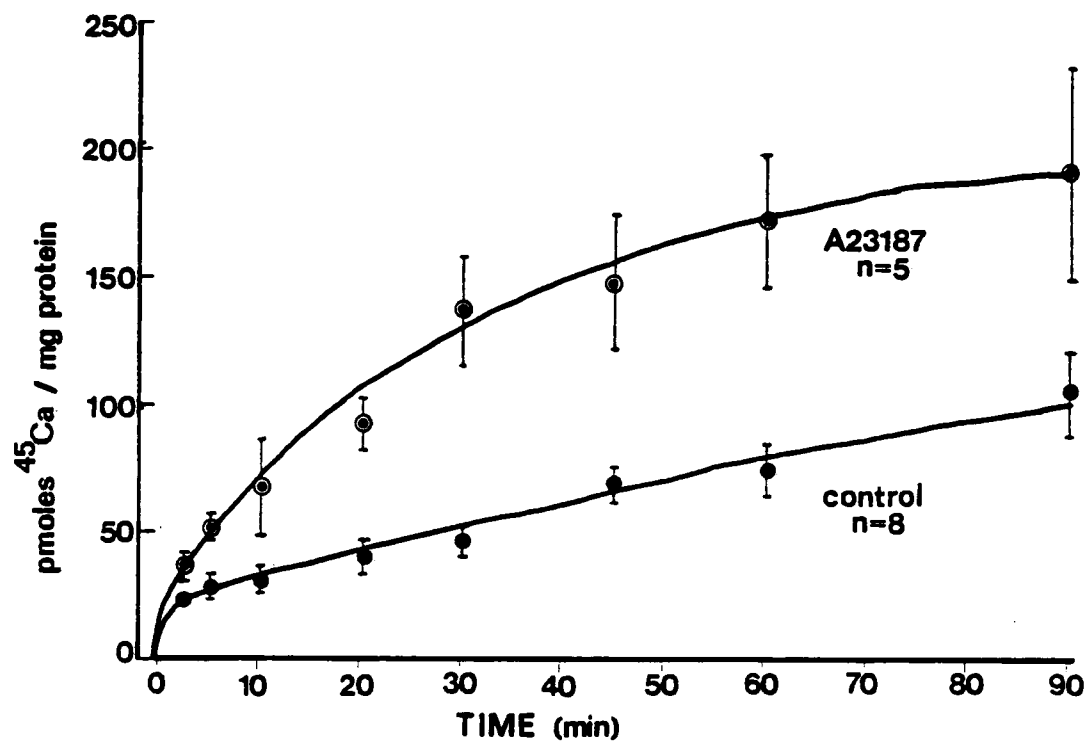


Fig. 9. ^{45}Ca uptake by isolated tracheal epithelial cells in the presence and absence of $1 \times 10^{-5}\text{M}$ A23187. Each point on the control curve represents the mean of 8 experiments $\pm$ S.E. The points for the A23187 curve represents the average of 5 experiments $\pm$ S.E.

TABLE I
KINETIC ANALYSIS OF $^{45}\text{Ca}^{2+}$ UPTAKE CURVES

		<u>COMPONENTS</u>	
		FAST	SLOW
CONTROL	Rate constant (min^{-1})	0.4704 ± 0.0313	0.0011 ± 0.0001
	$^{45}\text{Ca}^{2+}$ exchange (pmol $^{45}\text{Ca}^{2+}$ $\text{mg}^{-1} \text{min}^{-1}$)	15.765 ± 1.212	0.890 ± 0.056
A23187	Rate constant	0.3995 ± 0.0333	0.0219 ± 0.0122
(10^{-5}M)	$^{45}\text{Ca}^{2+}$ exchange	23.408 ± 2.601	4.156 ± 0.374

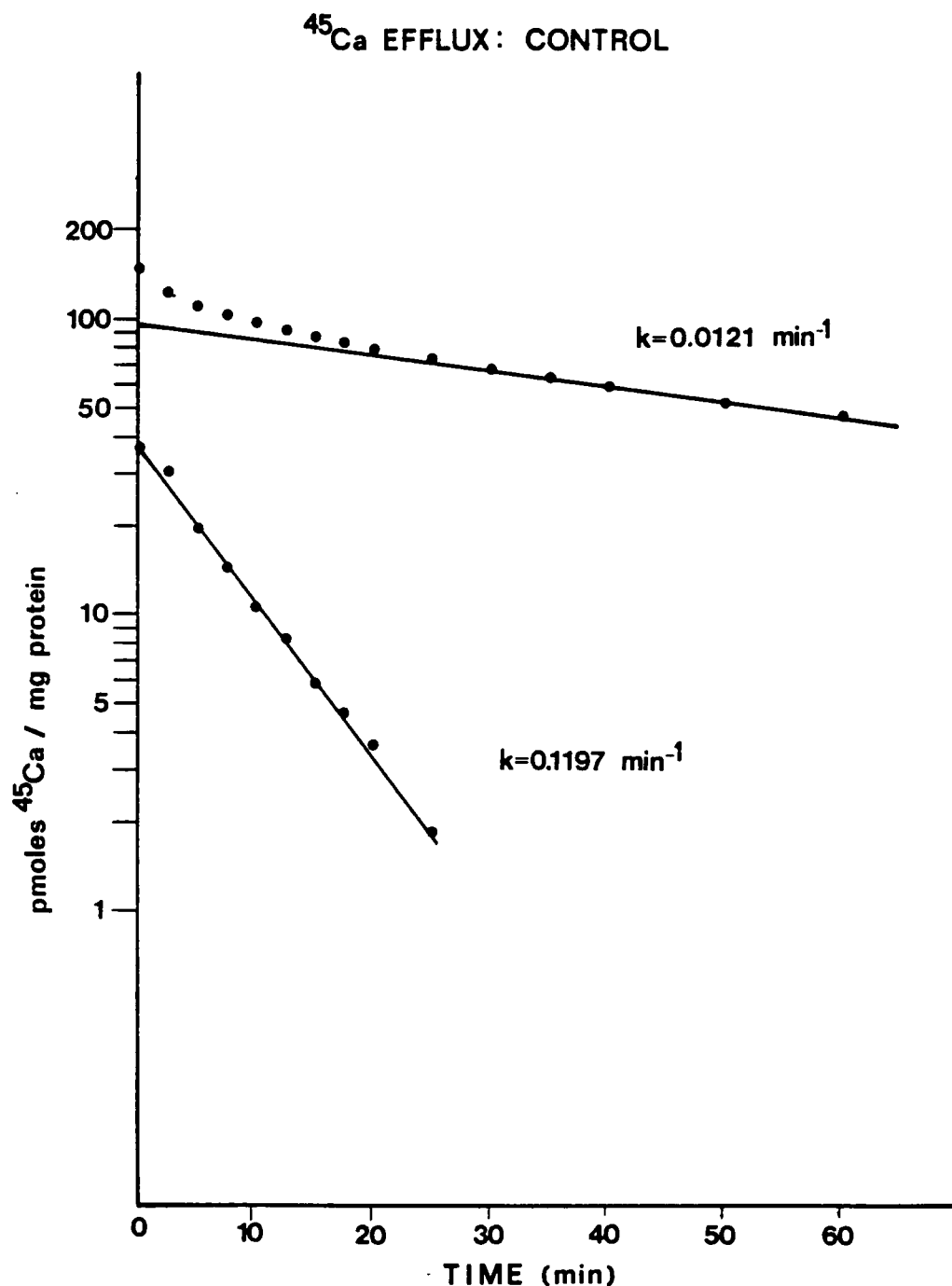


Fig. 10. a) ^{45}Ca efflux from an untreated tracheal epithelial cell suspension. This representative control experiment (of 13) shows the existence of 2 phases. b) ^{45}Ca efflux from a tracheal epithelial cell suspension in the presence of $1 \times 10^{-5} \text{ M}$ A23187. This representative experiment (of 4) shows the presence of a fast and slow component of runoff.

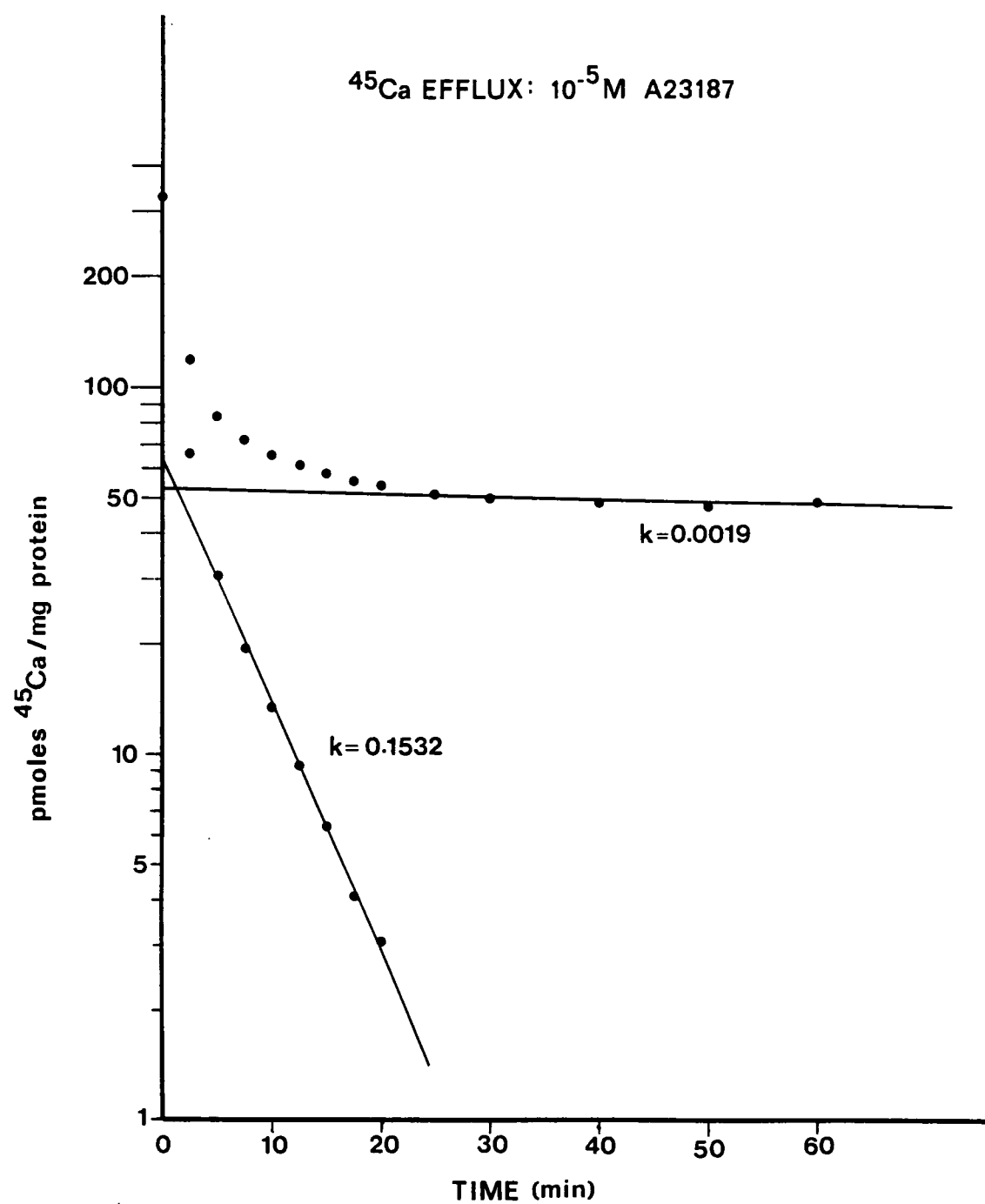


Fig. 10. (Continued)

of the $^{45}\text{Ca}^{2+}$ efflux curves (Table 2) also shows at least two components. The rates of efflux from the fast and slow compartments are reduced 30 and 20% respectively. These data indicate that A23187 leads to increased net calcium levels in both cellular compartments analyzed (probably represented by cytosolic and mitochondrial pools). The increased intracellular calcium levels coincide with increased ciliary motility. The ultimate cessation of cilia may be due to detrimental effects of the ionophore on the metabolic machinery of the cell. When mitochondrial function was disrupted by exposing explants and outgrowths to 10^{-5}M antimycin A, an antibiotic which blocks mitochondrial electron transport and thus inhibits ATP production, ciliary frequency was decreased immediately (Fig. 11). Within 5 min., frequency was reduced by at least 50% in both outgrowths and explants. Most cilia had ceased beating by 60 min.

Conditions which would likely act to lower intracellular levels of calcium were also employed and the effects on ciliary activity examined. Fig. 12 compares the effect of Ca^{2+} -free medium containing 1 mM EGTA in the presence and absence of 10^{-5}M A23187 on ciliary frequency. The EGTA effectively lowers the concentration of Ca^{2+} in the medium and reverses the normal inward Ca^{2+} gradient across the membrane. Addition of A23187 to the medium caused a rapid movement of Ca^{2+} down its concentration gradient, out of the cells (see below). The frequency of cilia in both instances was reduced. Explants exposed to A23187 and EGTA showed extensive cell extrusion and mucus release after 5-10 min. in some cases making frequency readings difficult beyond 20-30 min. Outgrowth cells would generally round up beginning 15-20 min. after exposure to A23187 plus EGTA but otherwise followed the

TABLE 2
 $^{45}\text{Ca}^{2+}$ EFFLUX: KINETIC ANALYSIS

		<u>COMPONENTS</u>	
		FAST	SLOW
CONTROL	Rate constant (min^{-1})	0.1577 ± 0.0173	0.0120 ± 0.0012
	$^{45}\text{Ca}^{2+}$ efflux ($\text{pmol } ^{45}\text{Ca}^{2+}$ $\text{mg protein}^{-1} \text{ min}^{-1}$)	7.202 ± 1.072	1.001 ± 0.1296
N=13			
A23187	Rate constant	0.1294 ± 0.0141	0.0020 ± 0.0005
(10^{-5}M)	$^{45}\text{Ca}^{2+}$ efflux	5.177 ± 0.857	0.084 ± 0.030
N=4			

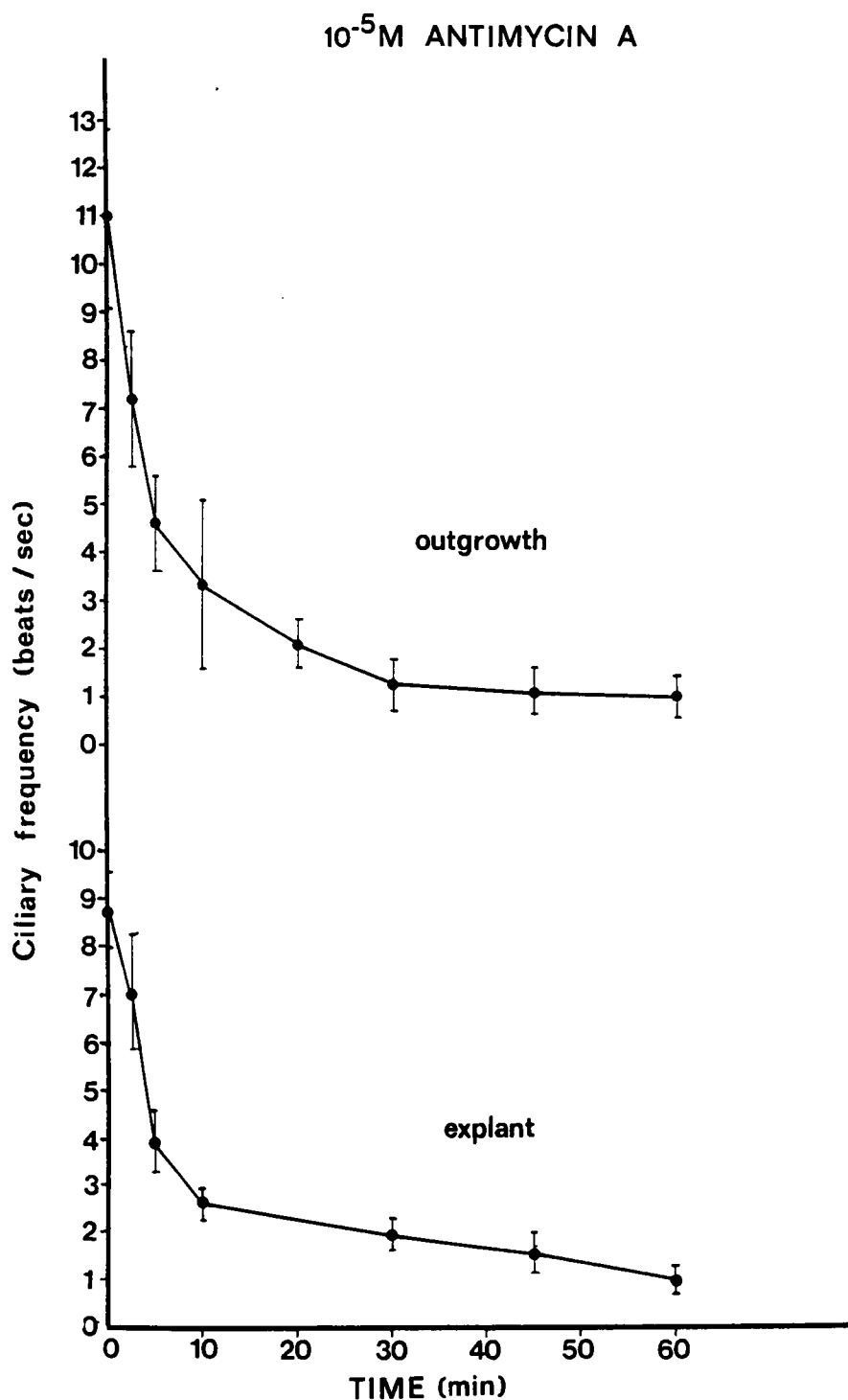


Fig. II. The effects of 1×10^{-5} M antimycin A on ciliary frequency in outgrowth (upper curve) and explant (lower curve) cells. Each curve shows the results from a representative experiment (of 3), with the mean frequency of 12 cells shown at each time point.

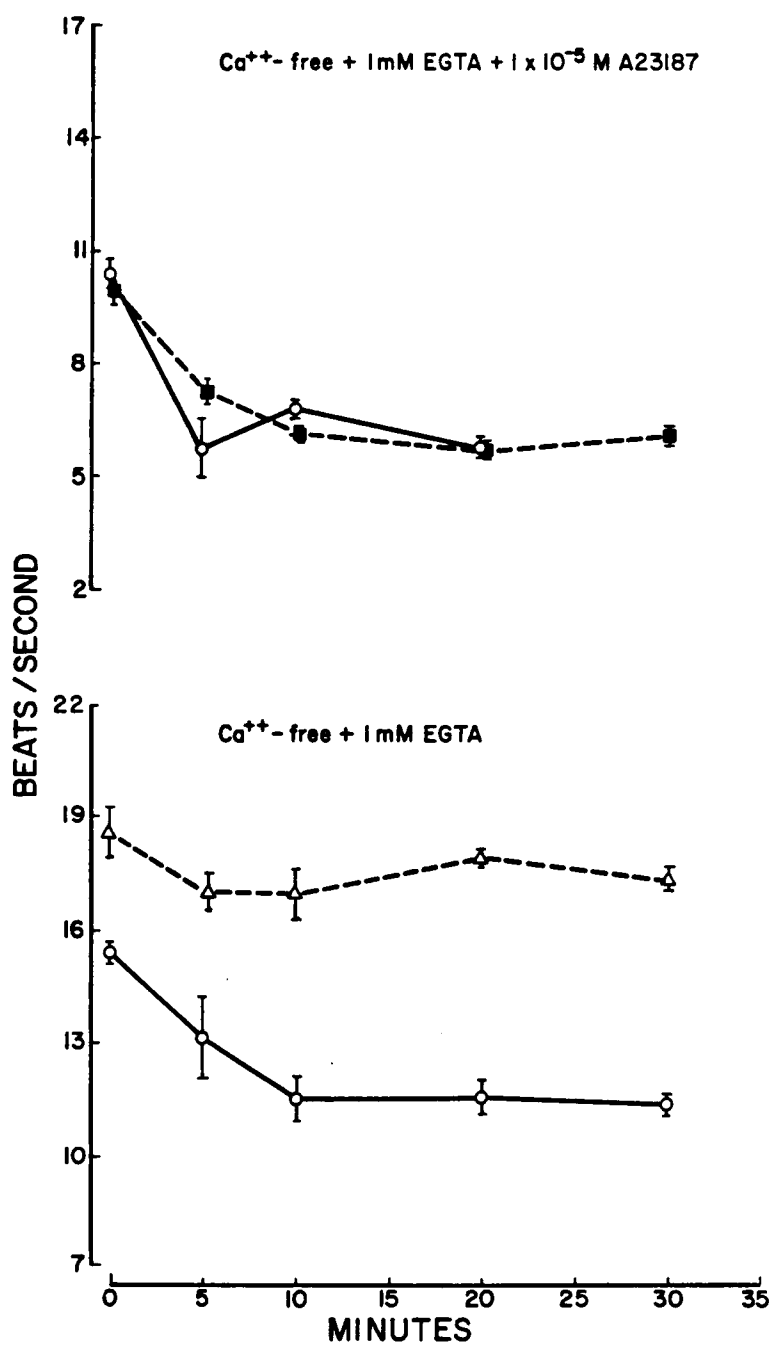


Fig. 12. Effects of Ca^{2+} -free medium containing 1mM EGTA on explant ciliary frequency (lower curves) and effects of Ca^{2+} -free medium containing EGTA plus 1×10^{-5} M A23187 (upper curves). Each curve depicts a representative experiment (of 4) with the frequency of ten cells being monitored at each time point.

same pattern of frequency decrease observed in explants. In the presence of EGTA alone, ciliary activity was reduced but no cell or tissue damage was evident during the period of observation.

Figure 13 shows the uptake of $^{45}\text{Ca}^{2+}$ in cells exposed to both A23187 and EGTA compared to uptake in controls. Kinetic analysis of the graph (Table 3) shows a 4-fold decrease in $^{45}\text{Ca}^{2+}$ exchange in the fast phase of A23187-EGTA treated cells compared to controls. There is no slow component for the uptake curve implying that there is little or no calcium uptake occurring after the first few minutes of exposure to the ionophore and EGTA.

The efflux of $^{45}\text{Ca}^{2+}$ in the presence of A23187 + EGTA is shown in Fig. 14. The curve can be resolved into 3 distinct components: fast, intermediate, and slow (Table 4). The initial fast efflux shown in the first 8 min. (Fig. 14) is not present under control conditions suggesting a rapid depletion of calcium is occurring upon exposure to A23187 and EGTA compared to controls.

Trifluoperazine, a phenothiazine that antagonizes the action of calmodulin (Levin and Weiss, 1976), was employed to probe for possible calmodulin-related events involved in the motility of cilia. Fig. 15 shows the effects of 10^{-4}M trifluoperazine on ciliary activity in outgrowths and explants. In outgrowths, ciliary frequency was reduced by at least 50% at 10 min. By 15-30 min., many cells had rounded up and by 25-30 min., some ciliary arrest was observed. Explant cilia appeared to be less sensitive to the effects of trifluoperazine, with a 50% reduction in ciliary frequency occurring only after 60 min. of exposure to the drug. However, some ciliary inhibition was noted by 45-60 min. By 2 hrs., only 5-20% of cilia were still beating, although at very low frequencies.

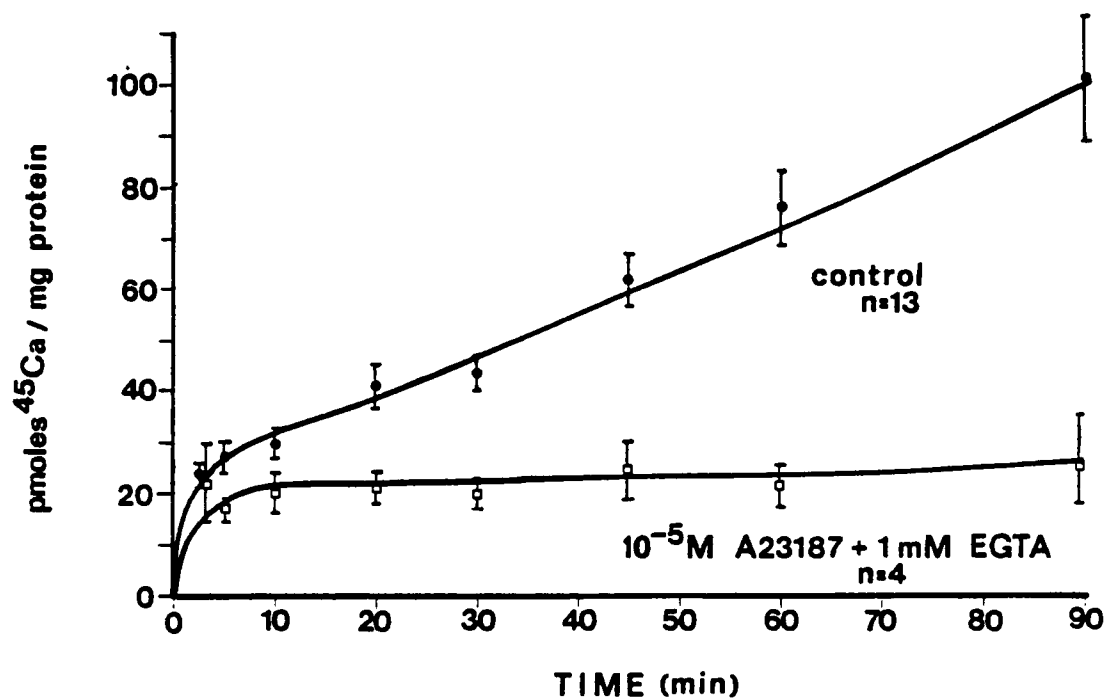


Fig. 13. ^{45}Ca uptake by a suspension of tracheal epithelial cells with and without 1 mM EGTA and $1 \times 10^{-5}\text{M}$ A23187 in the medium.

TABLE 3
KINETIC ANALYSIS $^{45}\text{Ca}^{2+}$ UPTAKE CURVES

		<u>COMPONENTS</u>	
		FAST	SLOW
CONTROL	Rate constant (min^{-1})	0.4704 ± 0.0313	0.0011 ± 0.0001
	$^{45}\text{Ca}^{2+}$ exchange (pmol $^{45}\text{Ca}^{2+}$ $\text{mg}^{-1}\text{min}^{-1}$)	15.765 ± 1.212	0.890 ± 0.056
A23187 (10^{-5}) + 1mM EGTA	Rate constant	0.2499 ± 0.0178	-
	$^{45}\text{Ca}^{2+}$ exchange	3.877 ± 0.242	-

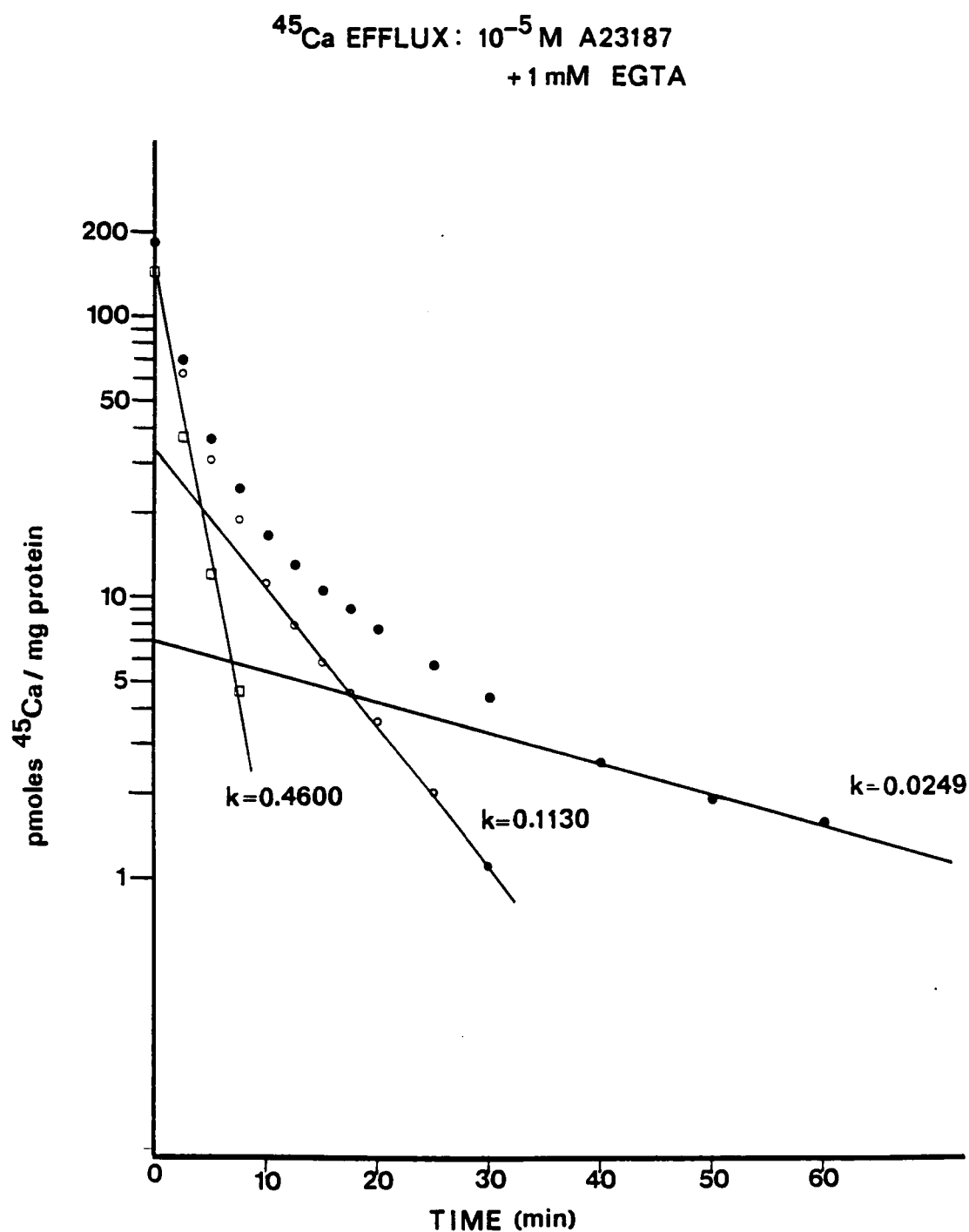


Fig. 14. ^{45}Ca efflux from tracheal epithelial cells in the presence of 1mM EGTA and $1 \times 10^{-5}\text{ M}$ A23187 showing a fast (□), intermediate (○), and slow (●) component of runout.

TABLE 4
 $^{45}\text{Ca}^{2+}$ EFFLUX: KINETIC ANALYSIS

		<u>COMPONENTS</u>		
		FASTEST	FAST	SLOW
CONTROL	Rate constant (min^{-1})		0.1577 ± 0.0173	0.0120 ± 0.0012
N=13	$^{45}\text{Ca}^{2+}$ efflux (pmol $^{45}\text{Ca}^{2+}$ mg protein $^{-1}$ min $^{-1}$)		7.202 ± 1.072	1.001 ± 0.1296
A23187	Rate constant	0.5645 ± 0.1046	0.1046 ± 0.0084	0.0269 ± 0.0010
+ EGTA	$^{45}\text{Ca}^{2+}$ efflux	80.931 ± 15.378	2.887 ± 0.571	0.305 ± 0.0895
N=3				

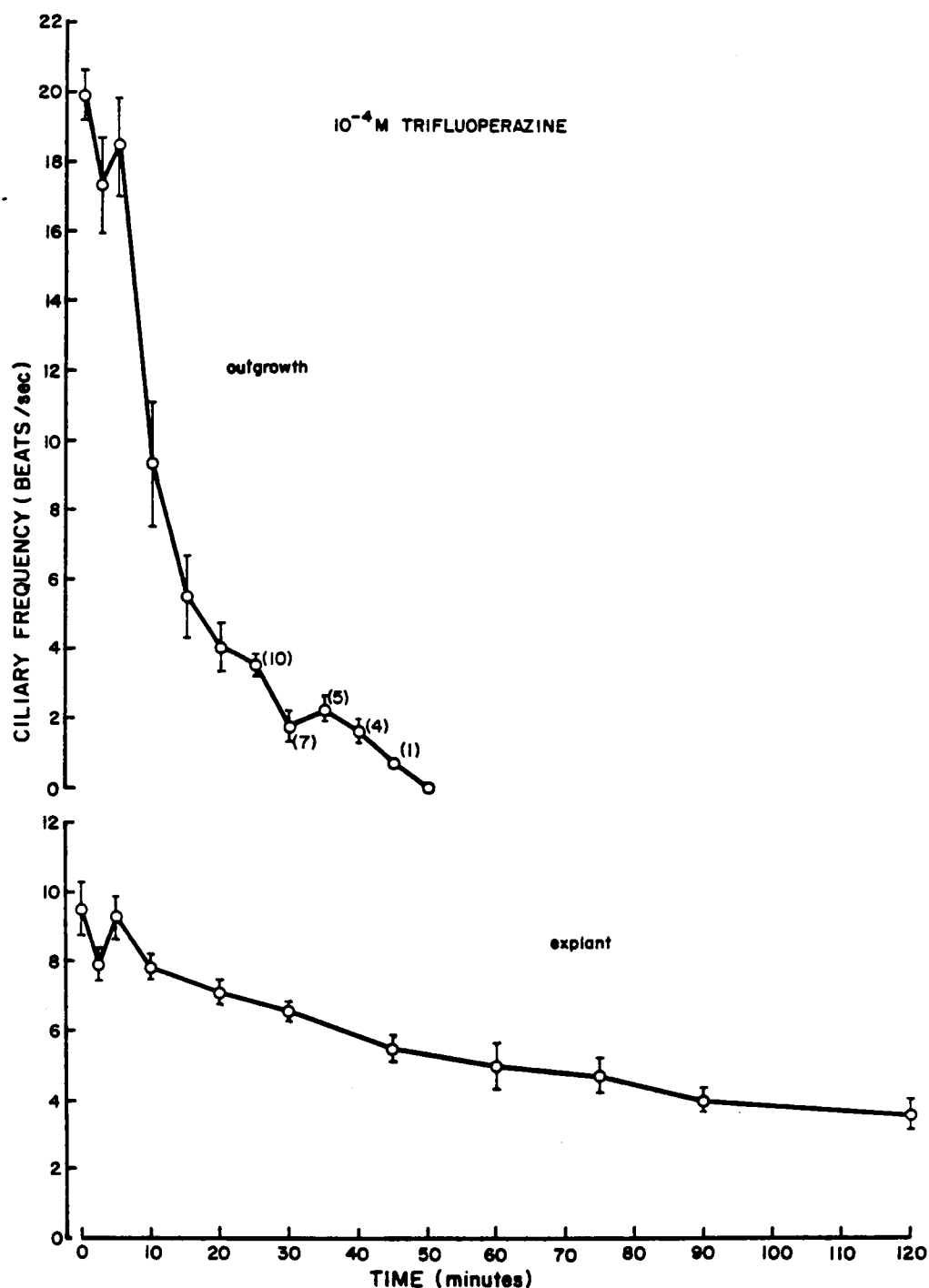


Fig. 15. Effects of 1×10^{-4} M trifluoperazine on ciliary frequencies in explants (lower curve) and outgrowths (upper curve). Each curve depicts the results of a representative experiment (of 5) with the mean frequency $\pm$ S.E. of 12 cells shown at each time point. The numbers in parentheses above the top curve show the number of cells still beating at that time period.

Reactivation Studies

Studies using demembranated cilia allow examination of the effects of various agents directly on the axoneme without having to consider possible effects on the plasma membrane. Fig. 16 shows a micrograph of the apical portions or cortices of ciliated cells produced following the demembration procedure. The ciliated cortices were immotile until reactivated with ATP, at which time 95% of all cilia become motile. The presence of calcium was not necessary for the reactivation. At all Ca^{2+} concentration tested (10^{-8} M- 10^{-2} M), reactivation of motility occurred in 95% of the cilia. In the presence of lower concentrations of Ca^{2+} (0, 10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} M) reactivation of cilia with a normal amplitude occurred lasting 10-40 sec. A less vigorous movement (reduced amplitude) persisted for 2-10 min. with little or no movement detected at 3-4 hrs. At 10^{-4} , 10^{-3} and 10^{-2} M Ca^{2+} , a strong reactivation was evident for only 4-10 sec. with less vigorous movement present for up to 40 more secs. Cessation occurred after 1-7 min. At all concentrations employed, ciliary movement was kinetic for only the first few seconds, after which movement appeared to be more or less asynchronous. Addition of trifluoperazine (25 or 100 μM) to the reactivation solution had no effect on the reactivation or duration of movement following reactivation at any of the Ca^{2+} concentrations tested (0- 10^{-7} M).

It appears then that calcium is not necessary for initiation or maintenance of ciliary motility but probably acts to modulate ciliary frequency. Elevated levels of intracellular calcium stimulate ciliary activity initially but ultimately it appears that high concentrations of calcium are inhibitory. The experiments reported here using demembranated cilia support this observation since exposure of the axoneme to high concentrations of Ca^{2+} also causes ciliary arrest.

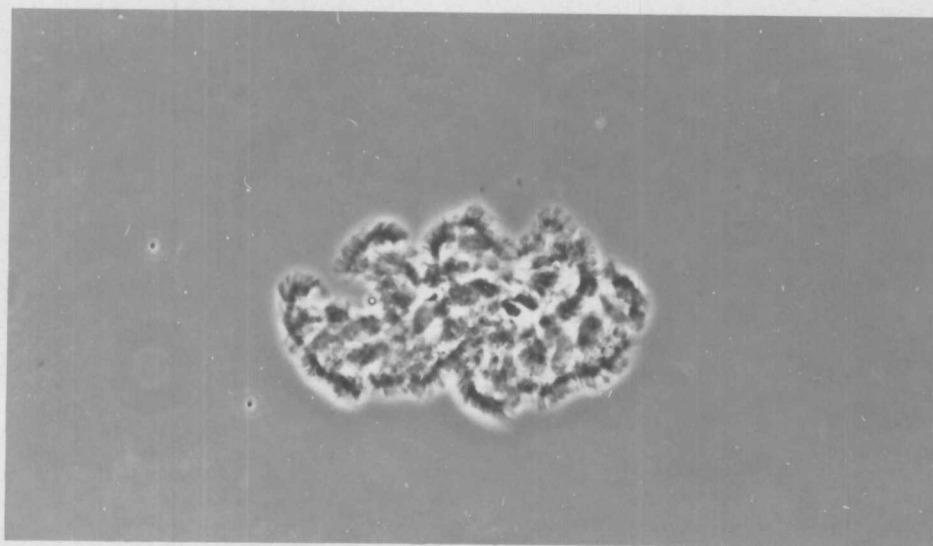


Fig. 16. Light micrograph showing ciliated apical cortices from the tracheal epithelium.

IV. DISCUSSION

The present findings indicate that high levels of intracellular Ca^{2+} as well as a reduction below physiological levels of cellular Ca^{2+} cause an inhibition of ciliary activity. However, changes in extracellular calcium alone, in the range of 0.8 mM-12.5 mM, did not affect the ciliary frequency of explant cilia. This differs from the effect of extracellular calcium on sperm motility in certain species. For example, chimpanzee, bull (McGrady, et al., 1974) and dog (see Tash and Means, 1983) sperm were inhibited by calcium in a dose-dependent manner at concentrations of Ca^{2+} of 1 mM and higher. However, ram, mouse, rabbit, and guinea pig sperm showed little or no change in motility in the presence or absence of Ca^{2+} at less than 5 mM (Morita and Chang, 1970; Quinn, et al., 1970; Bredderman and Foote, 1971; Hyne and Garbers, 1981).

Exposure of tracheal explant cells to 1×10^{-5} M A23187 in the presence of various extracellular Ca^{2+} concentrations (0.8 mM-12.5 mM) led to an increase in ciliary frequency in all cases. At the lowest concentration of Ca^{2+} , there was a tendency for frequency to fall below initial values after 10 min. In contrast, at 12.5 mM Ca^{2+} , ciliary frequency remained high after 30 minutes exposure. Response of cilia to 2.75 and 5.4 mM Ca^{2+} varied: in some cases ciliary beat remained above initial levels, while in others there was a return to initial values after 30 min. In experiments employing outgrowths, similar results were obtained. An initial increase in frequency of cilia was followed by arrest beginning after 45-60 min. of exposure to ionophore.

These results suggest that varying intracellular levels of calcium act to modulate ciliary activity. The ciliated epithelium appears to be able to adjust

and perhaps overcompensate for the lower levels of calcium introduced to the cell. It may be that at higher external Ca^{2+} concentrations, Ca^{2+} is moving down its chemical gradient faster than it can be pumped out or sequestered in internal organelles such as the mitochondria and ciliary motility remains modified. These results are in contrast to those obtained in the lateral cilia of freshwater mussel gills (Satir, 1975) where complete inhibition is obtained upon exposure to 1×10^{-5} M A23187 and 6.25 or 12.5 mM Ca^{2+} , and partial inhibition is obtained in the presence of A23187 and 1.25 mM Ca^{2+} . It appears that the calcium thresholds in these different cilia are quite different. Inhibition of ciliary activity does occur in explants but not until the tissue has been exposed to A23187 for at least 1.5-2 hrs. This may be due to detrimental effects of the ionophore induced on mitochondrial activity as has been reported by Friedmann, et al., (1979) in liver tissue and by Tamarit-Rodriguez (1977) for pancreatic islets.

The effect of A23187 on calcium levels in cells varies. Many investigators have reported that A23187 increases the uptake of $^{45}\text{Ca}^{2+}$ in tissues (Reed and Lardy, 1972; Prince, et al., 1973; Stolze and Schulz, 1980). However, other reports have shown that this ionophore can induce cellular events which are dependent on a rise in cytosolic Ca^{2+} activity in the absence of extracellular Ca^{2+} (Chambers, et al., 1974; Karl, et al., 1975; Borle and Studer, 1978). These authors suggest that the ionophore mobilizes Ca^{2+} from intracellular compartments such as the mitochondria. Babcock, et al., (1976) and Stolze and Schulz (1980) suggest that at low concentrations, A23187 causes a movement of Ca^{2+} from an intracellular Ca^{2+} pool. Under this condition, the authors observed a cellular $^{45}\text{Ca}^{2+}$ efflux since, they suggest, the Ca^{2+} extrusion

mechanism of the plasma membrane was not yet sufficiently bypassed by A23187. At higher ionophore concentrations, cellular Ca^{2+} extrusion mechanisms are overcome and net Ca^{2+} uptake occurs. Babcock, *et al.*, (1976) also proposed that low levels of A23187 may produce mitochondrial calcium efflux followed by influx from the medium. The same concentration of A23187 can result in seemingly opposite effects on Ca^{2+} movements in different cells. For example, 5 μM A23187 has been reported to produce Ca^{2+} efflux from hepatocytes (Kleineke and Stratman, 1974) and influx into oocytes (Steinhardt and Epel, 1974). It was therefore important to study in detail the Ca^{2+} fluxes which occurred upon addition of A23187. The $^{45}\text{Ca}^{2+}$ flux studies reported here suggest that 10^{-5} M A23187 produced a net increase in cellular Ca^{2+} . The initial increase in cellular Ca^{2+} level corresponds with an increase in ciliary frequency but is ultimately inhibitory. These results agree in part with those of Eckert and Murakami (1972), where iontophoretic injection of Ca^{2+} led to a stimulation of ciliary frequency.

Friedmann, *et al.*, (1979) demonstrated that A23187 stimulated calcium uptake into rat liver but also markedly reduced hepatic ATP levels. It has been reported that mitochondrial sequestering of calcium supercedes ADP phosphorylation (Rossi and Lehninger, 1964). Decreased ATP levels have also been shown in pancreatic islets exposed to A23187 (Tamarit-Rodriguez, *et al.*, 1977). Since ciliary activity has been shown to be directly related to cellular ATP content in hamster tracheal epithelium (Gabridge and Polisky, 1977), and is suggested by our studies using antimycin A, the eventual arrest of ciliary beat in this study may be due to the detrimental effects of the ionophore on ATP production.

In the studies using demembranated ciliated cortices, reactivation occurred both in the absence of Ca^{2+} and in the presence of Ca^{2+} concentrations as high as 10^{-2} M. At lower Ca^{2+} levels (0 , 10^{-8} , 10^{-7} and 10^{-6} M), movement could be detected after addition of ATP for at least 4 hours. Higher concentrations of Ca^{2+} (10^{-4} , 10^{-3} , 10^{-2} M), however, caused an inhibition of motility by 1-7 min. These results differ from the motility responses in other demembranated ciliated and flagellated cells. For example, reactivated *Mytilus* and *Elliptio* cilia become quiescent at 10^{-6} M Ca^{2+} (Tsuchiya, 1976, 1977; Walter and Satir, 1978). Ca^{2+} concentrations causing inhibition of reactivated motility for other species are 100-200 μM for sea urchin sperm (Gibbons and Gibbons, 1980), 200-600 μM for hamster sperm (Mohri and Yano, 1980; Mohri and Yanagimachi, 1980; Gibbons and Gibbons, 1980), 9 μM for dog sperm (Tash and Means, 1982) and 60 μM for Tetrahymena cilia (Blum and Hayes, 1977).

It appears then, that the Ca^{2+} -dependent responses of rabbit trachea epithelial cilia in both intact cells and demembranated preparations differ from other ciliated cells including Paramecium, Tetrahymena, and molluscan gills. The fact that the molecular constitution of cilia from different species is different may offer an explanation for the various responses that are observed. For example, different dynein ATPases have been isolated from several ciliated cells: Tetrahymena cilia contain 30S and 14S dyneins (Gibbons, 1963), whereas bovine tracheal and oviduct ciliary dynein show a sedimentation coefficient of 12S, with a minor 18S peak (Pallini, et al., 1982). Calcium binding proteins in these tissues may also play a role in mediating differing effects (see below).

The addition of 10^{-5} M A23187 to Ca^{2+} -free HBSS containing 1 mM EGTA led to an immediate decrease in ciliary frequency and ultimate arrest.

Studies utilizing $^{45}\text{Ca}^{2+}$ indicate that the ionophore acted to equilibrate the free Ca^{2+} across the cell membrane, resulting in significantly reduced intracellular calcium levels. One mM EGTA in Ca^{2+} -free medium without the addition of A23187 also resulted in reduced ciliary activity. This is consistent with Lee and Verdugo's study (1976) showing a decrease in ciliary beat of the ciliated cells of rabbit fimbria equilibrated in a Ca^{2+} -free Hanks solution containing 2 mM EGTA. Our studies and others (Dirkson and Zeira, 1981; Torres, et al., 1977; Naitoh and Kaneko, 1972) using demembranated cilia suggest, however, that calcium is not essential for ciliary motility in vitro. Current hypotheses suggests that there are two systems responsible for generating motility in cilia and eukaryotic flagella: 1) the Mg^{2+} -dependent sliding of microtubules, and 2) a Ca^{2+} -sensitive system that controls and modulates the sliding and bending (see recent review by Satir, 1982). Mg^{2+} appears to be essential for motility whereas Ca^{2+} acts in a modulatory manner. The ciliary arrest produced when cellular calcium was depleted with either EGTA or A23187 could be due to some detrimental effect of EGTA and/or A23187 on the metabolic machinery of the cell. Another possibility is that a soluble calcium-binding protein normally present in the cell, which plays a role in the control of ciliary activity, is lost in the preparation of demembranated cortices.

Since Ca^{2+} appears to play a role in the control of ciliary activity, it is likely that Ca-binding proteins are involved in mediating the action of Ca^{2+} . The presence of calmodulin has been reported in Tetrahymena (Jamieson, et al., 1979; Kumagai, et al., 1980; Blum, et al., 1980), Parmecium (Maihle, et al., 1979; Walter and Schultz, 1981), in scallop gill epithelial cilia (Stommel,

et al., 1982) and in rabbit tracheal epithelium (Gordon, 1982). Trifluoperazine (TFP), a phenothiazine which inhibits the action of calmodulin was found to inhibit ciliary activity in both outgrowths and explants. However, several studies have indicated that TFP may perturb membranes (Seeman and Weinstein, 1966; Landry, et al., 1981) and may disrupt mitochondrial energy production (Rubin and Rasmussen, 1981). The inhibition of ciliary beat by TFP detected in intact cells did not occur in demembranated models. Addition of either 25 μM or 100 μM TFP did not affect reactivation of ciliated cortices at any of the Ca^{2+} concentration tested ($0\text{--}10^{-3}$ M). This suggests that the effects of TFP in intact cells may be the result of a membrane or mitochondrial disturbance. It is also possible that the detergent treatment results in the loss of a calcium-sensitive component involved in the regulation of ciliary function. There are at least three possible functions for ciliary calmodulin (see Stommel, et al., 1982): (1) regulation of microtubule sliding, (2) regulation of a membrane Ca^{2+} -ATPase system and (3) calmodulin may act in part as a calcium "sink" if it is present in high concentrations as reported for scallop gill cilia (Stommel, et al., 1982). The different Ca^{2+} -dependent motile responses observed in various ciliated cells may be governed in part by the relative amounts of calmodulin associated with the cilia.

Thus it appears that calcium plays a modulatory role in the control of ciliary activity. Elevated levels of cellular calcium stimulate motility, although continued exposure of cells to A23187 leads to ciliary arrest. This may be a result of impaired mitochondrial function. However, the investigations reported here using demembrated ciliated cortices lends support to the possibility that high levels of Ca^{2+} may exert inhibitory effects directly on the axoneme since reactivation was of very short duration at the higher Ca^{2+} concentrations

tested. Ca^{2+} does not appear to be necessary for ciliary motility in demembranated preparations. The reduction in ciliary frequency observed when cells are depleted of calcium may be due to detrimental effects of this treatment on the metabolic activity of the cell. More biochemical studies are needed in order to elucidate the molecular mechanisms underlying the various motile responses in ciliated cells. It is important to identify all ciliary Ca^{2+} receptors. As calmodulin has been implicated in the regulation of ciliary activity, the identification of possible calmodulin-acceptor proteins (see Means and Dedman, 1980) in cilia is also important in order to determine the mechanism by which the Ca^{2+} -calmodulin complex may mediate ciliary responses.

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PART II

THE MODULATION OF CILIARY ACTIVITY BY CYCLIC ADENOSINE
3',5'-MONOPHOSPHATE IN RABBIT TRACHEAL EPITHELIAL EXPLANTS,
OUTGROWTHS, AND DEMEMBRANATED MODELS

I. INTRODUCTION

The basic mechanochemical mechanism proposed for ciliary and flagellar motility is the ATP-induced sliding of axonemal microtubule doublets (Satir, 1968; Gibbons, 1968; Brokaw, 1968; Summers and Gibbons, 1971). Both calcium and cyclic adenosine 3',5'-monophosphate (cAMP) have been implicated as primary regulators in axonemal motility (see reviews by Blum and Hines, 1979; Satir, 1982; and Tash and Means, 1983). Calcium has been shown to be responsible for the reversed swimming response of ciliated protozoa (Naitoh and Kaneko, 1972; Eckert, 1972; Naitoh and Eckert, 1974), changes in waveform in Chlamydomonas (Bessen et al., 1980) and sperm (Gibbons and Gibbons, 1973) ciliary arrest in mussel gill cilia (Satir, 1975) and stimulation of ciliary frequency in salamander oviduct cells (Eckert and Murakami, 1974) and rabbit tracheal epithelium (See Part I).

Cyclic AMP has also been implicated in the stimulation of sperm motility (for reviews, see Garbers and Kopf, 1980; and Tash and Means, 1983). Direct evidence obtained by adding cAMP to ATP-reactivated demembranated sperm models showed that cAMP produced increases in both flagellar frequency and proportion of motile cells (Lindemann, 1978; Morisawa and Okuno, 1982).

Cyclic AMP acts to stimulate protein phosphorylation through activation of cAMP-dependent protein kinase (Rosen, et al., 1975). In vivo pulse ^{32}P incorporation experiments by Piperno, et al. (1981) on Chlamydomonas flagella provide evidence that more than 80 axonemal polypeptides are phosphorylated. Although the role of these phosphorylated proteins is yet to be assessed, cAMP may modulate ciliary and flagellar activity via such protein phosphorylation.

The interrelationship of Ca^{2+} and cAMP in the regulation of cellular function is an even more complex problem. In some cell types, increased cAMP levels results in a mobilization of cellular calcium, possibly by release of sequestered calcium from mitochondria (Borle, 1974; Putney, et al., 1977). Cyclic AMP has also been shown to stimulate uptake of $^{45}\text{Ca}^{2+}$ by kidney, heart, pancreatic beta-cells and parotid glands (Meinertz, et al., 1973; Miller and Nelson, 1977; Borle and Uchikawa, 1979). Conversely, there have been many reports describing regulation of cellular cAMP levels by Ca^{2+} . For example, Ca^{2+} has been found to inhibit adenylate cyclase (Johnson and Sutherland, 1973; Steer and Levitzki, 1975) and stimulate phosphodiesterase (Teo and Wong, 1973; Brostrom and Wolff, 1974), conditions which result in a decrease in cAMP. However, investigations using other tissues show Ca^{2+} activation of adenylate cyclase (Brostrom, et al., 1975). Several studies suggest that interplay between cAMP and Ca^{2+} is also involved in the regulation of axonemal function. For example, bovine, sea urchin, and guinea pig sperm adenylate cyclase activities are stimulated by Ca^{2+} (Braun, 1975; Watkins, et al., 1978; Hyne and Garbers, 1979). In dog sperm, on the other hand, cAMP stimulates motility and protein phosphorylation while Ca^{2+} inhibits motility and the overall phosphorylation of endogenous proteins (Tash and Means, 1982).

Sperm (Hoskins, et al. 1972; Tash and Means, 1983), Tetrahymena (Murofushi, 1973; Jamieson, et al. 1979), and Paramecium (Lewis and Nelson, 1981; Walter and Schultz, 1981) have been found to contain cAMP-dependent protein kinases and calmodulin, major components involved in the action of cAMP and Ca^{2+} in the control of cell motility in both muscle and nonmuscle cells (Dedman, et al., 1979). It may be that the activity of flagella and cilia

is modulated by these components through changes in the phosphorylation state of proteins via the action of cAMP.

While most of these studies deal with sperm flagellar or invertebrate ciliary regulation little is known concerning the role of cAMP in mammalian ciliary function. The present studies were undertaken to determine whether cAMP is involved in the regulation of ciliary activity in rabbit tracheal epithelium.

II. MATERIALS AND METHODS

Tissue Preparation

Ciliated explant and outgrowth cultures were prepared as described in Part I. Briefly, tracheas were removed from rabbits and cut into four strips. The epithelial layer with adhering connective tissue was dissected away from the cartilagenous rings. The strips of epithelial tissue were cut into small pieces and placed in petri dishes containing Waymouth's 752/1 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% horse serum. A monolayer outgrowth composed of both ciliated and nonciliated cells generally grew out from the explant within 4-5 days.

Quantitation of Ciliary Frequency

The effects of 10 mM theophylline, 0.5 mM 8-bromo cyclic adenosine 3',5'-monophate (8-Br cAMP), and 50 or 100 ng/ml cholera toxin (all purchased from Sigma Chemical Co., St. Louis) on ciliary frequency of explants were determined employing a photomultiplier and spectrum analyzer system as described by Kennedy and Duckett (1981) and in Part I. For measurement of outgrowth ciliary frequency, a modification of this system was employed as described in Part I.

Reactivation Studies

Demembranated ciliated cortices were obtained following the procedure of Dirkson and Zeira (1981). Pieces of tracheal epithelial tissue were vortexed in a test tube intermittently for 40 min. in an isolation solution (4°C) consisting of 0.25 M sucrose, 0.02 M Hepes, pH 7.5, 2 mM EDTA, 25 mM KCl and 0.05% Triton X-100 (Calbiochem-Behring Corp., LaJolla, CA). After 40 min. the

pieces of tissue were removed and an equal volume of isolation solution containing 1% Triton X-100 was added to the suspension. Following a 60 sec. incubation and a 2-3 sec. vortex, the contents were centrifuged for 10 min. at 500 g in a Sorvall SP/X centrifuge. The pellet, containing cortices with demembrated cilia as well as some whole cells and nuclei was resuspended in isolation solution without Triton X-100 and stored at 4°C until ready to use.

For each reactivation experiment twenty-five μ l of ciliated cortices were mixed with 25 μ l of a reactivation solution consisting of 75 mM KCl, 2.5 mM MgSO_4 , 0.1% β -mercaptoethanol in 20 mM Tris-HCl buffer (pH 8.0) and containing Ca^{2+} concentration from 0 – 10^{-2} M. A 10 μ l aliquot of this solution was placed on a slide and an aliquot of 2 mM ATP in the appropriate reactivation solution was added to the edge of the coverslip and drawn across the suspension of cortices. In order to determine the effect of cyclic adenosine 3',5'-monophosphate (cAMP) (Sigma Chemical Co., St. Louis) on the motility of exposed axonemes, cAMP was present in the reactivation solutions in some of the experiments at a concentration of 10^{-4} M.

III. RESULTS

Changes in Ciliary Frequency

Theophylline and cholera toxin have been shown to raise cellular cAMP levels in many different cell types including guinea pig tracheal epithelium (Adler, et al., 1981). These compounds, and 8-Br cAMP, a cAMP analog, were employed to examine the effects of this cyclic nucleotide on ciliary activity in rabbit tracheal epithelium. As shown in Fig. 1, 10 mM theophylline significantly increased the ciliary frequency of both explant and outgrowth cells ($P < 0.025$). There was an average increase of 2-3 beats/sec within the first 5 min of exposure. In both types of preparation, ciliary activity remained above initial levels for at least 3 hrs.

Addition of 8-Br cAMP (0.5mM) to the culture medium also resulted in a significant increase ($P < .025$) in rate of ciliary beat (Fig. 2). Explants showed a 30% increase in ciliary frequency by 10 min. This elevated frequency was maintained for at least one hour. The activity of outgrowth cilia also increased an average of 30% after 10 min in the presence of 8-Br cAMP and continued to increase another 30% during the next 60 min. However the most significant increase was between time zero and 30 min which showed a 50% increase in frequency.

Fig. 3 shows the effect of cholera toxin (50 ng/ml) on ciliary frequency of outgrowths. A gradual increase in rate of beat occurs over 60 min. at which time the frequency (50% higher than initial levels) plateaus and remains at this elevated level for at least 1.5 hrs. Characteristically, after exposure to cholera toxin there is a time lag before increased levels of cellular cAMP

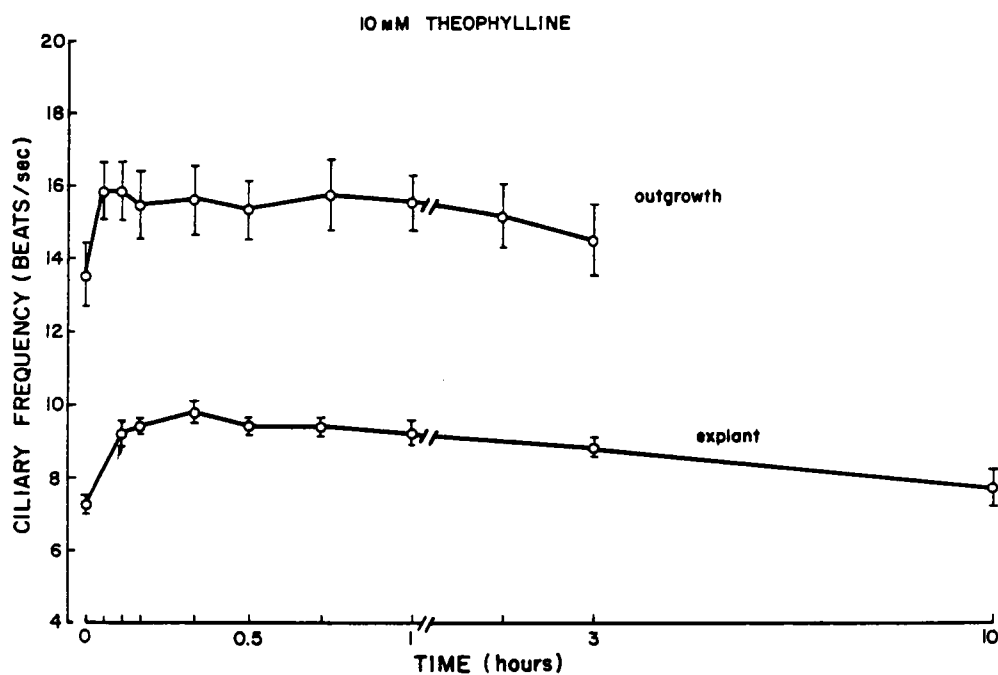


Fig. 1. Effects of 10mM theophylline on ciliary frequency in explant (lower curve) and outgrowth (upper curve) cells. Each curve shows the results of a representative experiment (of 4) with the mean frequency $\pm$ S.E. of 12 cells shown at each time point.

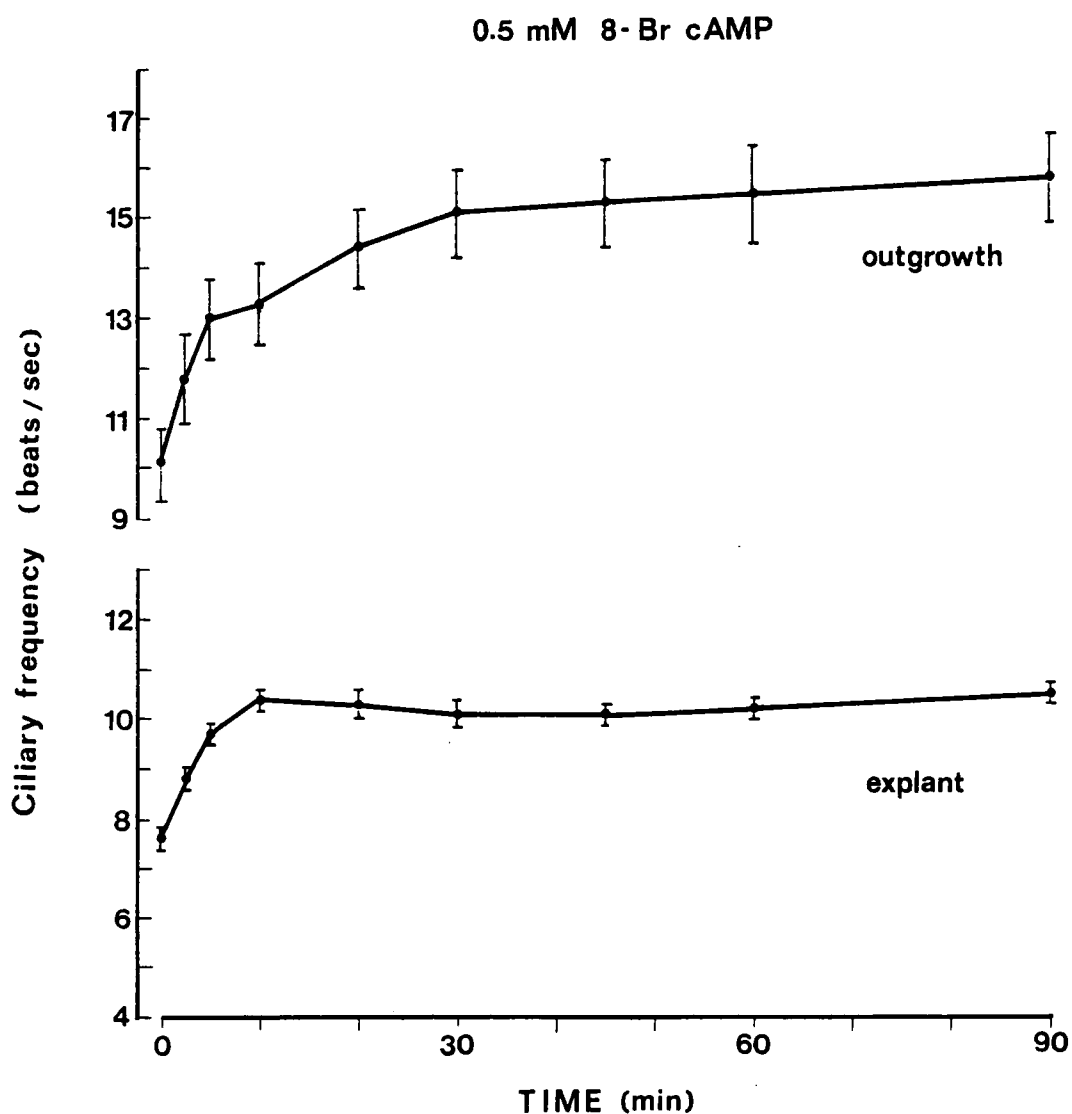


Fig. 2. Effects of 0.5 mM 8-Br cAMP on the frequency of cilia in explant (lower curve) and outgrowth (upper curve) cells. Each curve depicts the results of a representative experiment (of 4) with the frequency of 12 cells monitored at each time point.

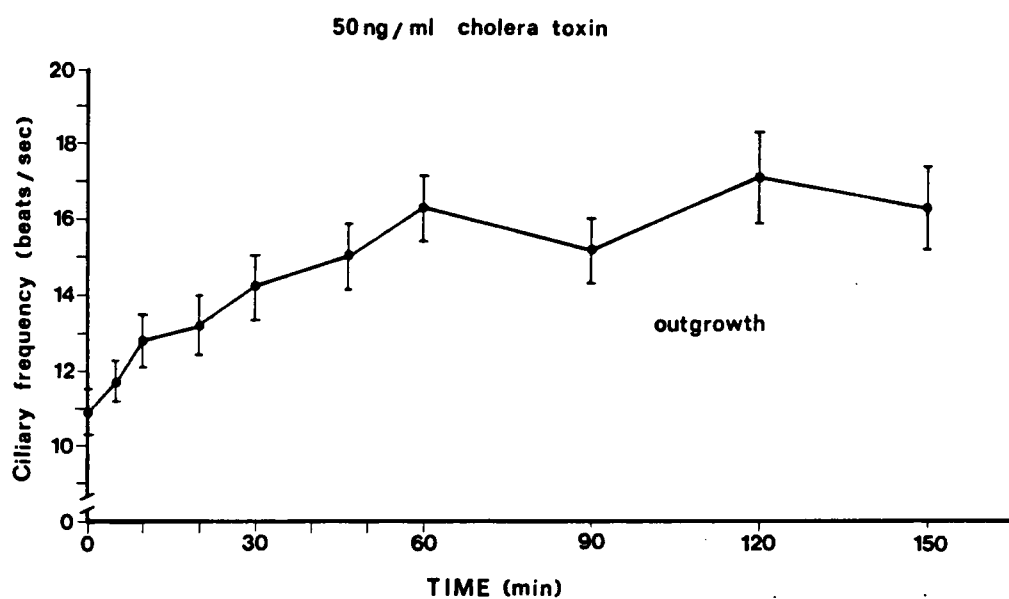


Fig. 3. Effects of 50 ng/ml cholera toxin on ciliary frequency in outgrowth cells. The curve shows a representative experiment (of 5) with the average frequency $\pm$ S.E. of 12 cells shown at each time point.

can be detected in various tissues (Gill, 1977). The results show a 30-55 min delay in the time at which a maximal frequency response occurs in the presence of cholera toxin compared to theophylline (10 min to maximum response) and 8-Br cAMP (10-30 min to max. response). Explants showed little or no increase in ciliary frequency during exposure to cholera toxin over a 2 h. observation period at concentration of 50 ng/ml and 100 ng/ml (Fig. 3b).

Reactivation Studies

Experiments reported in Part I using demembranated models of ciliated tracheal epithelial cells indicated that the cilia reactivated upon addition of ATP both in the presence and absence of Ca^{2+} . Cilia reactivated at all Ca^{2+} concentrations tested in the range of 10^{-8} M to 10^{-2} M. However, as the Ca^{2+} levels were increased from 10^{-6} to 10^{-2} M, the duration of motility decreased significantly.

Addition of 10^{-4} M cAMP to the reactivation solutions led to a more vigorous reactivation at all Ca^{2+} concentrations tested (0 - 10^{-7} M). The frequency of cilia upon reactivation (generally in the range of 2-4 beats/sec) was not changed but the amplitude of ciliary beat appeared greatly enhanced. Cilia reactivated without cAMP present, generally showed only weak movements beyond 3-4 min in the presence of Ca^{2+} in the range of 0 - 10^{-5} M and 5-30 sec at higher levels of Ca^{2+} (10^{-4} - 10^{-2}). In the presence of the lower range of Ca^{2+} concentration (0 - 10^{-5} M), the addition of 10^{-4} M cAMP to the reactivation solution resulted in greatly enhanced amplitude of beat for up to 10 min with activity continuing for at least 4 more hrs. At the higher levels of Ca^{2+} (10^{-4} - 10^{-2} M), initial reactivation, as mentioned above, was much more vigorous

in the presence of cAMP. However, the duration of axonemal motility (only 1-10 sec.) was not increased by the addition of cAMP.

IV. DISCUSSION

The modulation of motility of tracheal epithelial cilia by cAMP has been demonstrated in the present study in both intact cells and demembranated models. Ciliary frequency was stimulated in outgrowth and explant cells following exposure to 10 mM theophylline, a phosphodiesterase inhibitor. This concentration has been reported to double cellular cAMP levels in guinea pig tracheal epithelium (Adler, et al., 1981). 8-Br cAMP, an analog of cAMP that is readily transported across the cell membrane, also stimulated ciliary frequency in both outgrowth and explants.

Cholera toxin has been shown to activate the adenylate cyclase system of epithelial cells lining the gastrointestinal tract as well as in a great variety of other tissues (see Gill, 1977) including guinea pig tracheal epithelium (Adler, et al., 1981). Exposure of rabbit trachea epithelial outgrowths to cholera toxin (50 ng/ml) led to an increase in ciliary frequency. However, cholera toxin at concentrations of both 50 and 100 ng/ml caused little or no increase in ciliary frequency in explants. Adler et al. (1981) have reported that 5 μ g/ml cholera toxin did not increase cAMP content in guinea pig tracheal explants but that 50 μ g/ml resulted in doubling of cAMP content. Thus the concentrations employed for explants in this study may have been too low. Outgrowth cultures appear to be much more sensitive to cholera toxin than explants, perhaps due to increased surface area available to the toxin. Studies are now in progress to determine the effect of higher concentrations of cholera toxin on explant ciliary frequency.

Liedtke, et al. (1982) reported that cAMP levels in rabbit tracheal epithelium were increased by the β -adrenergic agonist (-)-isoproterenol. Verdugo, et al. (1980) found that the same concentration of (-)-isoproterenol increased the frequency of cilia in rabbit trachea epithelial outgrowth cells. These reports taken together are in accord with the present findings that substances which mimic or increase cAMP levels also increase ciliary frequency.

Many investigations employing sperm have also demonstrated a correlation between changes in cAMP content and motility (for reviews see Garber and Kopf, 1980; Tash and Means, 1983). Studies employing demembranated sperm have shown that cAMP is not required for motility (Lindeman, 1978) but rather acts to increase beat frequency and the proportion of motile cells. The ciliated cortices employed in this study were shown to reactivate in the absence of added cAMP, supporting the hypothesis that cAMP-dependent phosphorylation is not required for the production of motility, but rather is involved in the modulation of motility. At all of the concentrations of Ca^{2+} tested ($0 - 10^{-2}$ M), when reactivation occurred in the presence of cAMP, an increase in ciliary wave amplitude was observed. However no increase in frequency compared to reactivation without cAMP was detected. Whether the increased wave amplitude noted in the demembranated cilia relates to elevated ciliary frequencies observed in intact cells exposed to agents which increase cAMP levels, remains to be determined. Increase in wave amplitude may also be the mechanism responsible for the increased swimming velocity that has been reported in sperm in the presence of cAMP (See Garbers and Kopf, 1980). Bovine sperm were shown to increase beat frequency in response to cAMP (Lindemann, 1978), but whether wave amplitude was affected was not mentioned.

An increase in the longevity of sperm activity in the presence of phosphodiesterase inhibitors has also been reported (Schoefeld et al., 1975; Haesungcharern and Chulavatuatol, 1973). The present results support these findings since an increased duration of motility was also observed after reactivation of demembranated ciliated cortices in the presence of cAMP.

A major problem now being investigated is the molecular site of action of cAMP and the biochemical mechanism involved in modulating axonemal function. The discovery of cAMP-dependent protein kinase in sperm (Hoskins et al., 1972; Garbers et al., 1973) was followed by many reports of phosphorylation of sperm proteins (see review by Tash & Means, 1983). Investigations with Chlamydomonas (Piperno et al., 1981) of in vivo pulse ^{32}P incorporation into flagellar proteins have demonstrated that more than 80 axonemal components are phosphorylated. Lewis and Nelson (1981) have found that approximately one-third of the ciliary peptides in Paramecium tetraurelia were phosphorylated in vitro and that the phosphorylation of specific polypeptides was enhanced by cAMP. Schultz and Jantzen (1980) have reported the presence of 2 protein kinases in Paramecium cilia dependent on cAMP. Isolated axonemes of Tetrahymena cilia have also been reported to contain a cyclic AMP-dependent protein kinase (Murofushi, 1973). It appears then that the control of ciliary and flagellar motility may be defined in part by cAMP-dependent protein phosphorylation.

Little is known regarding the interaction between calcium and cAMP in the regulation of ciliary and flagellar activity. In the present study, cAMP enhanced the amplitude and duration of ciliary beat in demembranated models both in the presence and absence of Ca^{2+} . Addition of Ca^{2+} to the exposed

axonemes in the absence of added cAMP did not appear to stimulate motility in reactivated cilia compared to reactivation without Ca^{2+} present (see Part I). However, ciliary frequency in intact cells was stimulated when cellular Ca^{2+} levels were elevated. It is possible that increased intracellular Ca^{2+} may stimulate cellular cAMP production and in turn increase ciliary frequency. In addition to the cAMP that would be washed away during the preparation of demembranated ciliated cortices, all soluble and membrane proteins regulated by Ca^{2+} would be removed. Regulation of various protein kinases, adenylate cyclase, and dynein and membrane associated ATPase activities are possible mechanisms which may be involved in the control of axonemal function by Ca^{2+} .

In summary, cAMP appears to be involved in modulating ciliary frequency, amplitude and duration of beat in the rabbit tracheal epithelium. Exposure of intact ciliated cells to agents which have been reported to elevate intracellular levels of cAMP in a variety of cells and tissues led to an increase in ciliary frequency. Evidence supporting these results were obtained using demembranated cilia whereupon direct exposure of cAMP to the axoneme caused enhanced ciliary amplitude and increased the duration of reactivation.

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PART III

CILIARY ACTIVITY AND THE INTERRELATIONSHIP OF SODIUM AND CALCIUM MOVEMENTS IN THE TRACHEAL EPITHELIUM OF RABBITS

I. INTRODUCTION

The activity of cilia in tracheal epithelium depends on both the intrinsic regulatory mechanism affecting the ciliated cells as well as the physical properties of the fluid in which the movement of cilia takes place. Since water moves across epithelia in response to osmotic gradients, changes in permeability of the epithelium should be reflected in changes in water secretion. An important factor in determining the rheological properties of respiratory tract secretions may be the balance between the amount of water secreted and the amount mucus secreted in the airways.

In studies using Ussings short-circuit current technique, Oliver, et al. (1975) investigated the active transport of Na^+ and Cl^- across dog tracheal epithelium. They found that the short-circuit current was accounted for by a net movement of Cl^- toward the lumen and by a smaller net movement of Na^+ toward the submucosa. Evidence has been provided to suggest that Cl^- is actively transported toward the lumen in rats (Mangos, 1976) and that Na^+ and Cl^- are actively transported in a similar manner to dog trachea in cats (Phipps, et al. 1978) and rabbits (Mellon, 1968). Widdicombe, et al. (1979), using autoradiographic localization of ^3H -ouabain, determined that the $\text{Na}^+ \text{K}^+$ -ATPase is found exclusively on the basolateral cell membranes in both surface and glandular epithelium. Studies by Al-Bazzaz and Jayaram (1981) suggest that an increase in intracellular calcium levels generated by calcium ionophore A23187, increases Cl^- secretion and abolishes Na^+ transport in dog tracheal epithelium. Several investigators have also shown that intracellular Ca^{2+} levels appear to play a role in modulating ciliary activity in a variety of cells and

tissues (Eckert and Murakami, 1972; Satir, 1975; Machemer, 1974) including rabbit tracheal epithelium (see Part I).

Calcium is normally maintained at low levels intracellularly (10^{-6} - 10^{-7} M) compared to extracellularly (10^{-3} M). This could be accomplished by a plasma membrane Ca^{2+} -ATPase which would transport Ca^{2+} out of the cell as is the case for erythrocytes (Schatzmann, 1975). In some tissues, the extrusion of Ca^{2+} against its electrochemical gradient occurs via a Na/Ca exchange (Blaustein, 1974). This system depends on an inward Na^{+} gradient which is maintained by the activity of the Na^{+} pump (Na^{+} K^{+} -ATPase). A change in intracellular sodium concentration could lead to an increase in cytosolic Ca^{2+} and thus changes in ciliary activity by the following mechanisms: 1) it could contribute to the total intracellular positive charge that causes depolarization and open voltage-sensitive Ca^{2+} channels, 2) a disruption of the Na/Ca counter transport system could occur involving an increase in Ca^{2+} influx due to stimulation of Na^{+} efflux as well as an inhibition of Ca^{2+} efflux resulting from increased competition by intracellular Na^{+} for the common Na-Ca carrier, 3) it could cause a mobilization of Ca^{2+} from intracellular stores as suggested by Lowe (1976) who demonstrated that release of Ca^{2+} from isolated heart mitochondria occurs in the presence of elevated levels of Na^{+} .

In the present study, substances which disrupt Na^{+} and Ca^{2+} fluxes were employed in order to ascertain the role of these ion movements in ciliary activity. Ouabain has been shown to elevate cytosolic Ca^{2+} levels by membrane depolarization and consequent opening of voltage-dependent Ca^{2+} channels, through an inhibition of Na/Ca exchange, or through mobilization of intracellular Ca^{2+} stores (see Borle, 1981). Veratridine has also been used to increase the

Na⁺ permeability of excitable cells (Ulbricht, 1969) and nonexcitable cells such as pancreatic cells (Donatsch, et al., 1977).

Membrane potential has also been shown to affect ciliary and flagellar motility in a number of different cells and tissues including Paramecium, (Machemer, 1976; DePeyer and Machemer, 1978) and sperm (McGrady and Nelson, 1973, McGrady, 1979). In the present study, the role of membrane potential on ciliary frequency in rabbit tracheal epithelium was also examined by observing the effects of raised extracellular K⁺ or Na⁺-free medium.

II. MATERIALS AND METHODS

Tissue Preparation

Explants, outgrowths and cell suspensions were prepared as described in part I. Briefly, tracheas were removed from young adult rabbits from the cricoid cartilage to the bifurcation to the lungs. The epithelial layer was dissected away from the tracheal rings, cut into pieces to provide explants or placed in petri dishes containing Waymouth's MB 752/1 medium supplemented with 10% horse serum to obtain outgrowths. After several days in culture outgrowths of ciliated cells were present adjacent to the explants.

For studies using cell suspensions a modification of the technique of Tandler et al. (1980) was used. Strips of epithelial tissue were incubated in Ca^{2+} - Mg^{2+} -free Earles balanced salt solution containing 20 mM EDTA at 37°C. Following a one hour incubation, the epithelial cell layer was separated from adhering connective tissue with a scalpel. The cells were centrifuged and washed several times with Waymouth's medium to rinse the cells of EDTA. The cells were then equilibrated for 1.5 hrs. in Waymouth's to reestablish intracellular levels of calcium. Ciliated cells in these suspensions retain their normal frequencies and show the same responses to the test substances used in this study as explants and outgrowths.

Quantitation of Ciliary Frequency

Fresh explants, outgrowths, and cell suspensions were exposed to veratridine, ouabain, and depolarizing concentrations of K^{+} or to Na^{+} -free medium. Ciliary frequency was monitored as described previously (see Part I). Briefly, light signals from beating cilia were enhanced with a photomultiplier

system and frequency data obtained from the analog signal using a spectrum analyzer.

$^{45}\text{Ca}^{2+}$ Uptake and Efflux

The methods used for measurements of $^{45}\text{Ca}^{2+}$ uptake and efflux are described in detail in Part I. For uptake, 5 $\mu\text{Ci/ml}$ $^{45}\text{Ca}^{2+}$ and 10 $\mu\text{Ci/ml}$ ^3H -inulin (used to correct for extracellular space) were added to the cell suspension. For some experiments, veratridine or ouabain was present. Aliquots of cells were taken at appropriate time intervals and centrifuged through oil to separate them from the incubation medium. The severed tip of the tube with the pellet was transferred to a scintillation vial and radioactivity measured with a Beckman LS 8100 liquid scintillation counter under double isotope settings. Aliquots of cells were also taken during the course of the uptake experiment for determination of protein (Lowry, 1951).

For efflux studies, cell suspensions were preloaded for 1.5 hrs. in Waymouth's medium containing 10 μCi ^{45}Ca and 20 μCi ^3H -inulin. The cells were then centrifuged and resuspended in medium without isotope and, for some experiments, with ouabain or veratridine present. At appropriate intervals up to 60 min. the cell suspension was centrifuged, the medium removed and placed in a scintillation vial. Fresh medium was added and the pellet resuspended after each interval. The amount of $^{45}\text{Ca}^{2+}$ and ^3H -inulin present in the runout solution at each interval was measured by liquid scintillation spectroscopy and $^{45}\text{Ca}^{2+}$ content corrected for extracellular space (as measured by ^3H -inulin). Efflux curves were constructed as described in part I. Methods for analysis of $^{45}\text{Ca}^{2+}$ uptake and efflux curves have also been described previously. (See Section I).

III. RESULTS

In order to determine whether increases in internal Na^+ concentration affected ciliary frequency the effects of veratridine, an alkaloid known for its ability to increase Na^+ permeability in nerve cells (Ohta et al., 1976) were examined on tracheal epithelium. The graphs shown in Fig. 1 show that veratridine stimulated ciliary activity in both outgrowth and explant preparations. The initial increase in frequency in explants was statistically significant ($P < 0.005$) but not as dramatic as the increase observed in outgrowths. In explants, there was generally a return to initial frequencies by 5 min, whereas in outgrowths, ciliary activity remained high for at least 1-2 hours before frequencies returned to initial levels.

Since increased intracellular Na^+ has been found in some cells to influence cellular Ca^{2+} movements, calcium fluxes were monitored using $^{45}\text{Ca}^{2+}$. Fig. 2 shows that $^{45}\text{Ca}^{2+}$ uptake is stimulated in cells exposed to 10^{-4} M veratridine. Analysis of the uptake curve reveals two components. The faster component may represent movement of Ca^{2+} into the cell whereas the slower phase may reflect an intracellular sequestering of Ca^{2+} into organelles such as the mitochondria. Kinetic analysis of these components (Table I) shows that $^{45}\text{Ca}^{2+}$ exchange in the faster compartment is increased ($p < 0.05$) in the veratridine-treated cells compared to controls. In the slower phase, there is a two-fold increase in $^{45}\text{Ca}^{2+}$ exchange in cells exposed to veratridine.

Analysis of $^{45}\text{Ca}^{2+}$ efflux curves in control and veratridine-treated cells also shows two distinct kinetic phases (Fig. 3). The data obtained from these graphs (Table 2) show a 30% and 20% decrease of $^{45}\text{Ca}^{2+}$ efflux in the fast

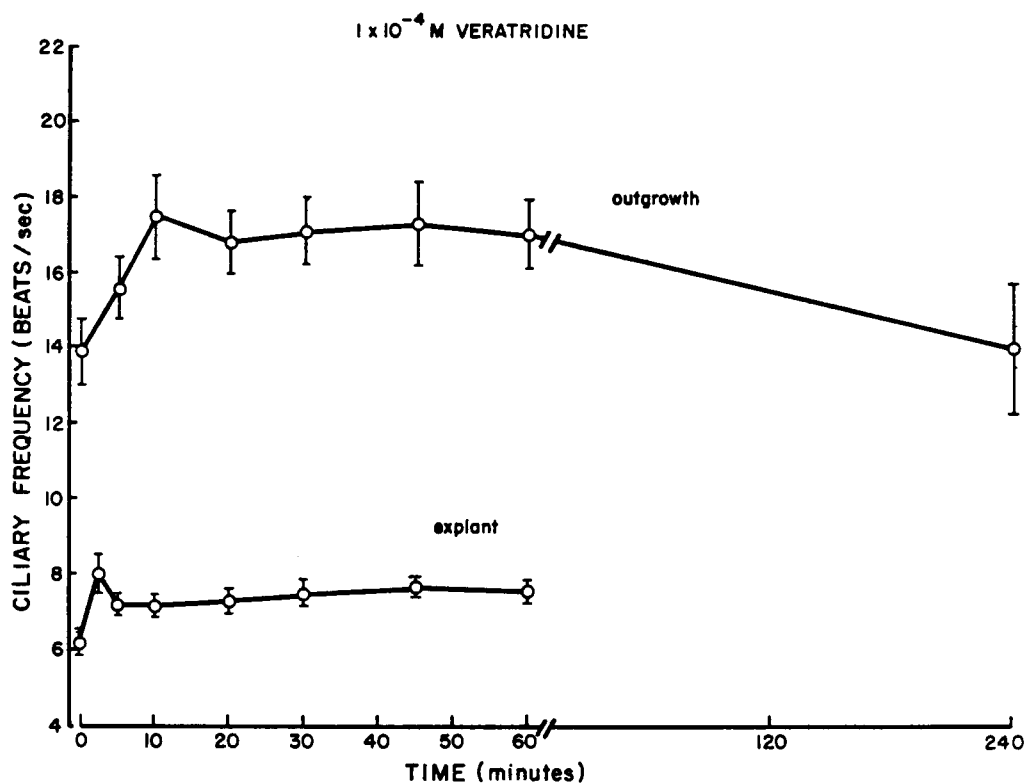


Fig. 1. Effect of 1×10^{-4} M veratridine on ciliary frequency in explant (lower curve) and outgrowth (upper curve) cells. Each curve shows a representative experiment (of 4) with mean frequencies $\pm$ S.E. shown at each time point.

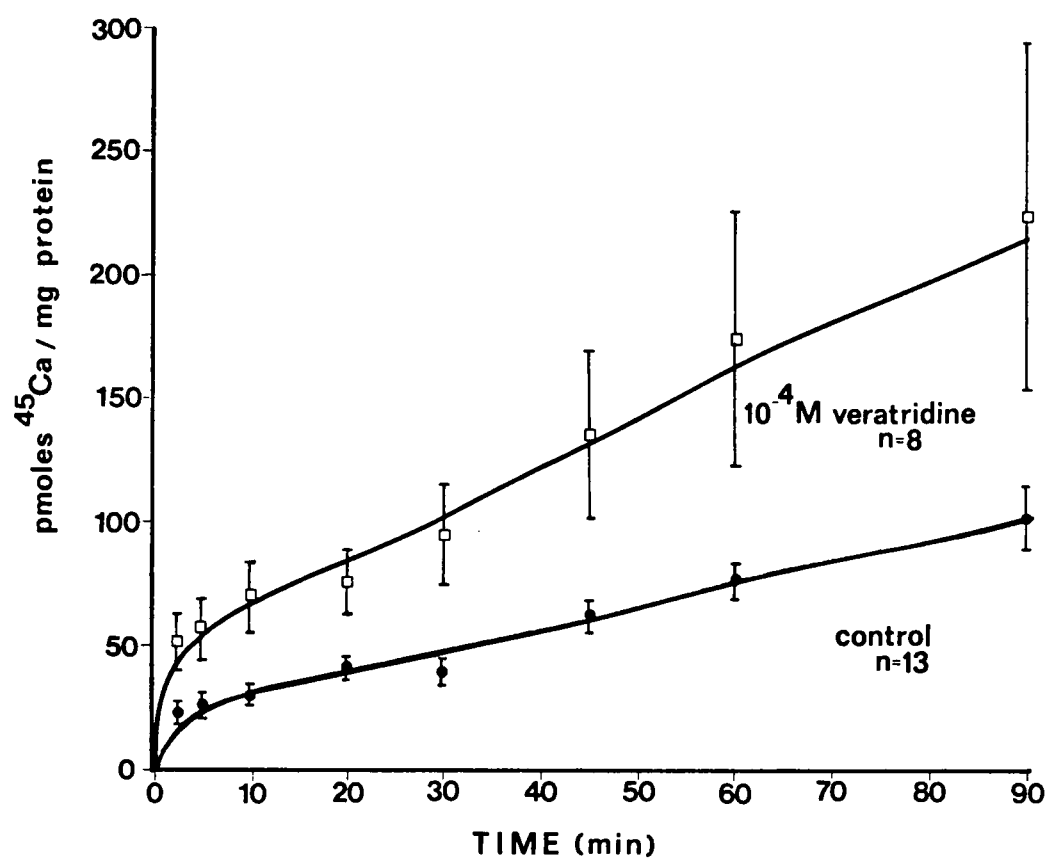


Fig. 2. ^{45}Ca uptake by a cell suspension of tracheal epithelial cells in the presence and absence of $1 \times 10^{-4}\text{ M}$ veratridine. The points on the control curve represent the mean of 13 experiments $\pm$ S.E. The points on the veratridine curve represent the mean of 8 experiments $\pm$ S.E.

TABLE I
KINETIC ANALYSIS OF $^{45}\text{Ca}^{2+}$ UPTAKE CURVES

		<u>COMPONENTS</u>	
		FAST	SLOW
CONTROL	Rate constant (min^{-1})	0.4707 ± 0.0313	0.0011 ± 0.0001
	$^{45}\text{Ca}^{2+}$ exchange ($\frac{\text{pmol}}{^{45}\text{Ca}^{2+} \text{ mg}^{-1} \text{ min}^{-1}}$)	15.765 ± 1.212	0.890 ± 0.056
VERATRIDINE (10^{-4}M)	Rate constant	0.2936 ± 0.2914	$.0028 \pm 0.0002$
	$^{45}\text{Ca}^{2+}$ exchange	18.2564 ± 1.0142	1.8528 ± 0.1089

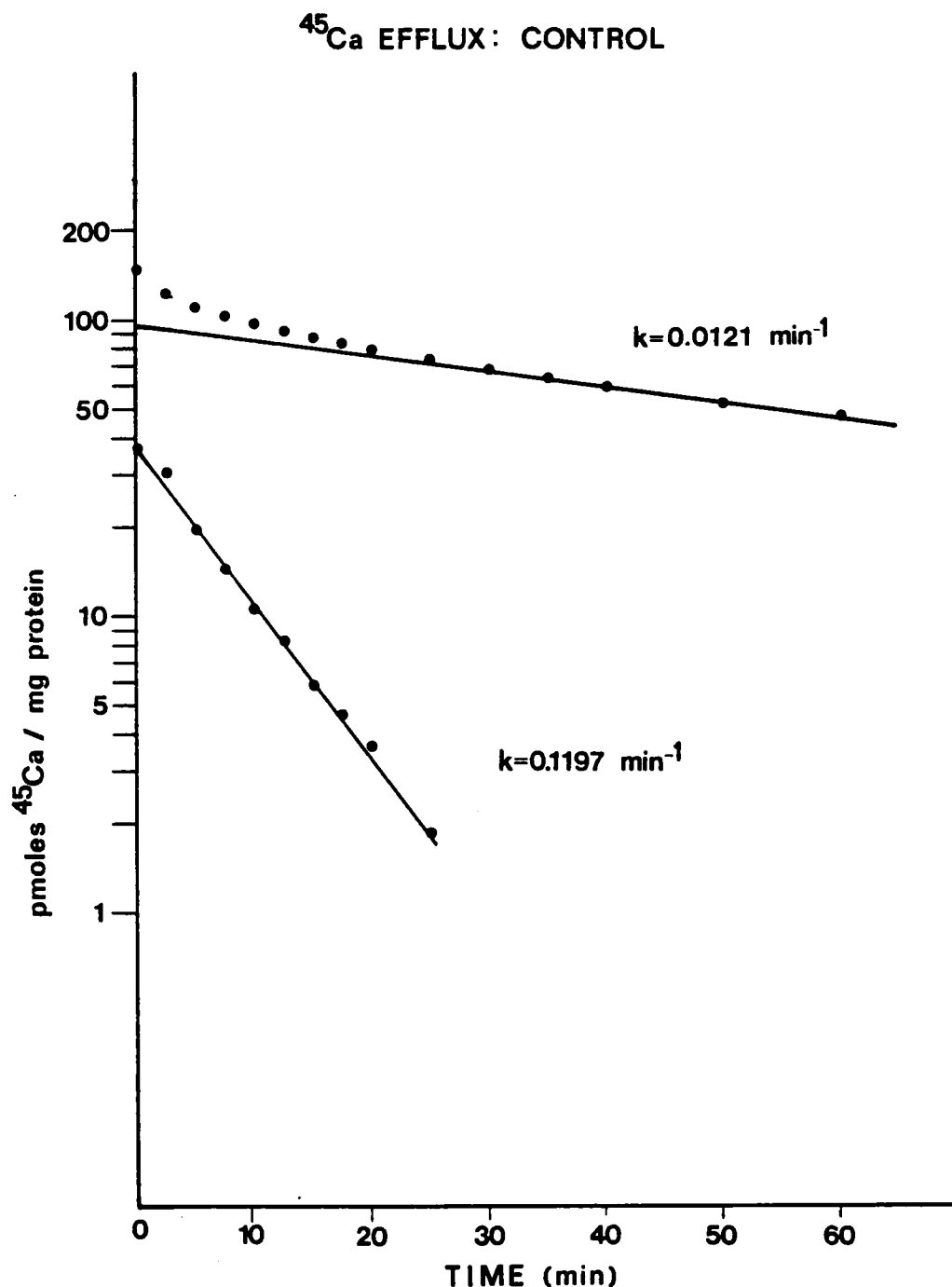


Fig. 3. a) ^{45}Ca efflux from tracheal epithelial cells. Representative control experiment (of 13) showing the presence of two phases of ^{45}Ca runout. b) ^{45}Ca efflux from tracheal epithelial cells in the presence of 10^{-4} M veratridine. Representative experiment (of 5) showing the presence of a fast and slow component of ^{45}Ca runout.

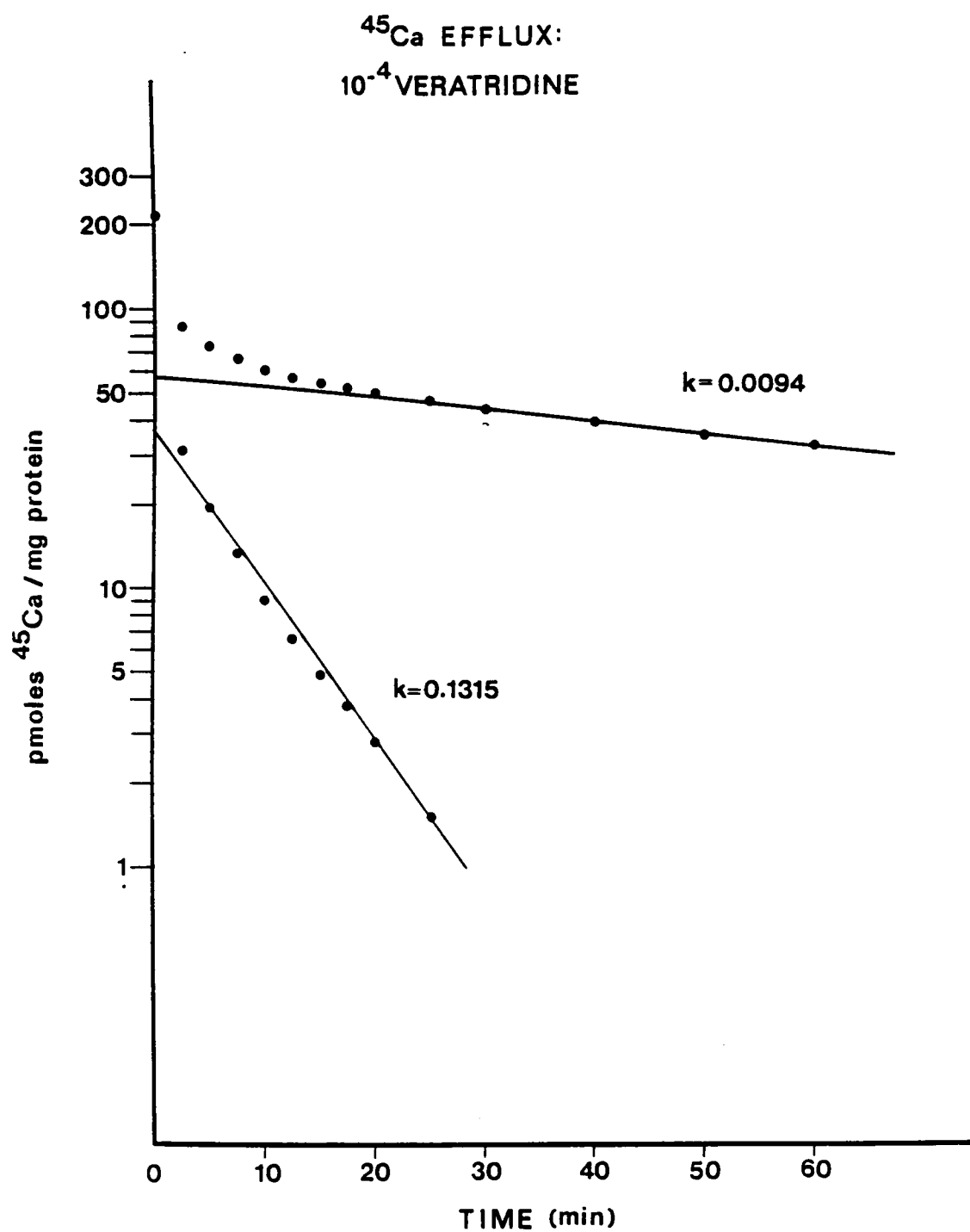


Fig. 3. (Continued)

TABLE 2
 $^{45}\text{Ca}^{2+}$ EFFLUX: KINETIC ANALYSIS

		<u>COMPONENTS</u>	
		FAST	SLOW
CONTROL	Rate constant (min^{-1})	0.1577 ± 0.0173	0.0120 ± 0.0012
N=13	$^{45}\text{Ca}^{2+}$ efflux ($\text{pmol } ^{45}\text{Ca}^{2+} \text{ mg protein}^{-1} \text{ min}^{-1}$)	7.202 ± 1.072	1.001 ± 0.1296
VERATRIDINE	Rate constant	0.1211 ± 0.0093	0.0094 ± 0.0002
(10^{-4}M)			
N=5	$^{45}\text{Ca}^{2+}$ efflux	5.159 ± 1.069	0.822 ± 0.101

and slow compartments, respectively, in tissue exposed to veratridine. Taken together the data obtained for $^{45}\text{Ca}^{2+}$ uptake and efflux experiments suggests that intracellular levels of calcium may be elevated due to exposure to veratridine.

In many different cell types, intracellular levels of Na^+ increase in the presence of ouabain as a result of an inhibition of the $\text{Na}^+ \text{K}^+ \text{ATPase}$ (Donatsch et al. 1977; Russo et al., 1977; Baker, 1978). The effect of 1 mM ouabain on ciliary frequency in explants and outgrowths is presented in Fig. 4. In both cases, rate of beat is significantly reduced below initial levels by 10 min and thereafter continues to decrease. Some cell desquamation was evident in explants by 30-60 min. Approximately 10% of the cells showed ciliary arrest by 1.5-2 h., 50% by 4-5 hr., and 90% had ceased beating by 6-7 hrs. Many of the extruded cells appeared swollen. Ciliated outgrowth cells tended to round up by 4 hr and generally by 6-8 hrs., all outgrowth cells had rounded up. In most cases, cilia stopped beating within an hour after the cells had swollen. Uptake and efflux of $^{45}\text{Ca}^{2+}$ was not affected by the presence of ouabain.

In order to determine whether membrane depolarization would affect the frequency of cilia, outgrowths and explants were exposed to high levels of extracellular K^+ (25 mM) or Na^+ -free solutions with isoosmotic replacement with choline. Ciliary activity was not affected during an hour observation period under either of these conditions.

The results above suggest that under certain conditions, elevated intracellular Na^+ stimulates ciliary frequency. This increased ciliary activity coincides with and may be due to elevated levels of intracellular Ca^{2+} . Changes in membrane potential itself, on the other hand, appears to have no effect on ciliary frequency.

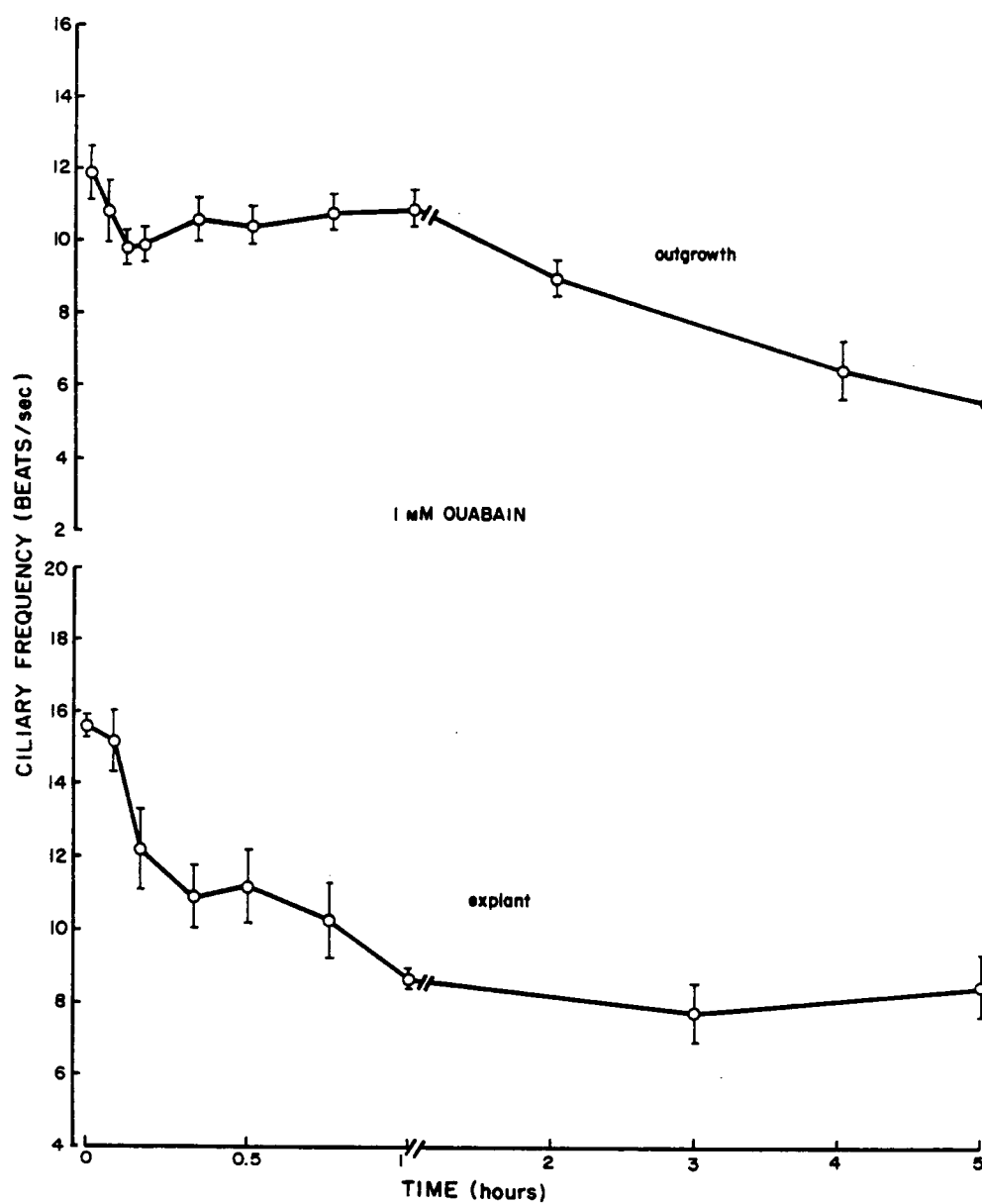


Fig. 4. Effect of 1mM ouabain on ciliary frequency in explant (lower curve) and outgrowth (upper curve) cells. Each curve depicts a representative experiment showing mean frequency $\pm$ S.E. at each time point.

IV. DISCUSSION

The influence of sodium on cellular calcium metabolism varies greatly in different cell types. In muscle, nerve, and some secretory cells, the operation of a Na-Ca countertransport system is generally regarded as a mechanism for efflux of Ca^{2+} to maintain low cytosolic Ca^{2+} concentration. Accordingly, an elevation of cellular Na^+ due, for example, to a decreased activity of Na^+ K^+ ATPase reduces the Na^+ gradient across the cell membrane with a resultant increase in cytosolic Ca^{2+} ion activity. There is, however, little evidence of this mechanism occurring in other tissues such as liver (Van Rossum, 1970), kidney (Borle, 1982), or adrenal medulla (Rink, 1977). But even in tissues such as these, Na^+ may influence some aspects of cellular calcium transport and distribution, in turn affecting cellular function.

In the present study, exposure of rabbit tracheal epithelium to veratridine, an alkaloid which has been shown to increase sodium permeability in nerve and muscle cells (Ulbricht, 1969) and pancreatic β -cells (Donatsch et al., 1977; Pace, 1979) caused a stimulation of ciliary frequency in both explants and outgrowths. Coincident with this ciliary response there appeared to be an increased uptake of $^{45}\text{Ca}^{2+}$. Several studies have suggested that elevated cytoplasmic Ca^{2+} levels increase ciliary frequency (Eckert and Murakami, 1972; Verdugo, 1980; present report, Part I). In some cells, an increase in Na^+ permeability of the cell membrane concomitant with an increase in intracellular Na^+ leads to the opening of voltage-sensitive Ca^{2+} channels (Katz and Miledi, 1969; Baker and Glitsch, 1975; Donatsch et al., 1977). Ca^{2+} channel blockers such as cobalt, verapamil, and D600 antagonize this movement of Ca^{2+} .

Veratridine has also been shown to cause a release of Ca^{2+} from intracellular stores such as mitochondria (Donatsch et al., 1977; Lowe et al., 1976). The evidence reported here does not support this as a possibility in rabbit tracheal epithelial cells as ^{45}Ca efflux was unchanged in the presence of veratridine and also ouabain (see below).

The stimulation of ciliary activity in outgrowths and explants upon exposure to veratridine was not as striking as that which occurred in the presence of A23187 (see Part I). However, the kinetic data of $^{45}\text{Ca}^{2+}$ fluxes suggests that there may be a greater net uptake of Ca^{2+} into the cells in the presence of A23187 compared to veratridine which might account for the more substantial frequency increase of cilia.

In the present study, ouabain decreased ciliary activity in both explant and outgrowth cells. Ouabain, which increases intracellular levels of Na^+ has been shown to increase cellular Ca^{2+} levels in many tissues in which a Na/Ca exchange has been postulated (Borle, 1981). However in liver (Van Rossum, 1970; Van Rossum et al. 1971) ouabain appears to have no effect on Ca^{2+} fluxes or cell Ca^{2+} . In addition, in rat kidney (Matsushima and Gemba, 1979; Borle, 1982), ouabain has been shown to completely inhibit Na^+ transport without affecting Ca^{2+} influx or efflux. Similarly, in the present experiments, ouabain had no effect on uptake or efflux of $^{45}\text{Ca}^{2+}$ in tracheal epithelial cells. The finding that ciliary activity was not stimulated in the presence of ouabain is consistent with the fact that intracellular Ca^{2+} levels were apparently not increased. The observed decrease in ciliary frequency may reflect a requirement for normal levels of intracellular K^+ , since the inhibitory action of ouabain on $\text{Na}^+ \text{K}^+ \text{ATPase}$ would lead to decreased intracellular levels of K^+ . McGrady

(1979) also reported an inhibitory action of ouabain on motility in sperm. In other studies with sperm, Ablá et al. (1975) showed that the influx of ^{42}K correlated with sperm motility and that ouabain reduced uptake of ^{42}K .

Intracellular Na^+ levels have been shown to rise in a variety of cells in the presence of both ouabain and veratridine (See Blaustein, 1974). However, veratridine increases intracellular Na^+ in the presence of an intact Na^+ pump, thereby maintaining normal concentrations of intracellular K^+ . In the present study, increased $^{45}\text{Ca}^{2+}$ uptake occurred in the presence of veratridine, but not ouabain, reflecting the possibility that intracellular K^+ may be an important factor influencing the effectiveness of intracellular Na^+ in modulating Ca^{2+} uptake across the cell membrane.

Agents such as veratridine and ouabain should act to depolarize cells since they increase intracellular Na^+ levels and thus render the cell with increased positive charge intracellularly. In the present investigation, I tested whether depolarization itself influences ciliary activity. Eckert (1972) and Machemer (1974) suggest that depolarization of the cell membrane of *Paramecium* increases the Ca^{2+} conductance of the membrane causing a rise in intracellular Ca^{2+} which leads to reversal and frequency responses in cilia. However, the present results suggest that depolarization of rabbit tracheal epithelium by exposing the tissue to high extracellular K^+ or Na^+ -free medium does not alter ciliary activity.

In summary, ciliary activity appears to be stimulated in the presence of veratridine perhaps due to an increase in Ca^{2+} uptake. The lack of stimulation in the presence of ouabain may be attributed to the fact that Ca^{2+} fluxes in the epithelial cells are unchanged when exposed to ouabain. Since both ouabain

and veratridine have been shown to increase intracellular Na^+ but only ouabain inhibits K^+ extrusion from the cell, this suggests that K^+ may be an important factor influencing cellular Ca^{2+} levels and ciliary motility in response to elevated intracellular Na^+ .

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