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LOCALIZATION OF CALCIUM CHANNELS IN *PARAMECIUM CAUDATUM*

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SUMMARY

1. Electrical recordings from *Paramecium caudatum* were made after removal of the cilia with chloral hydrate and during ciliary regrowth to study the electrical properties of that portion of the surface membrane enclosing the ciliary axoneme.

2. Removal of the somatic cilia (a 50% reduction in membrane surface area) results in an almost complete elimination of the regenerative Ca response, all-or-none Ba^{2+} spike, and delayed rectification.

3. A twofold increase in input resistance resulted from the 50% reduction in membrane surface area.

4. The electrical properties remained unchanged, despite prolonged exposure to the chloral hydrate, until the cilia were mechanically removed.

5. Restoration of the Ca response accompanied ciliary regrowth, so that complete excitability returns when the cilia regain their original lengths.

6. It is concluded that the voltage-sensitive Ca channels are localized to that portion of surface membrane surrounding the cilia.

7. Measurements of membrane constants before and after deciliation and estimations of the cable constants of a single cilium suggest that the cilia of *Paramecium* may be fully isopotential along their length and with the major cell compartment.

INTRODUCTION

The locomotor behaviour of the ciliated protozoan *Paramecium* is mediated by the electrical properties of the surface membrane (Eckert, 1972; Naitoh & Eckert, 1974). Depolarizing stimuli (mechanical or electrical) produce graded, regenerative potential changes, the amplitudes of which depend on the external calcium ion concentration (Naitoh & Eckert, 1969; Eckert, Naitoh & Friedman, 1972). Reversal in the direction of ciliary beat accompanies this regenerative potential change, causing the free-swimming ciliate to back up.

Depolarization of the surface membrane under voltage clamp produces a transient increase in Ca^{2+} conductance followed by a delayed, maintained conductance increase for an outward current (Naitoh, 1974). In the unclamped membrane the increase in the calcium permeability results in a quasi-regenerative inward Ca^{2+} current. The addition of Ba^{2+} to the medium converts the graded response to an all-or-none spike (Naitoh & Eckert, 1968*b*). This suggests that the normally graded response is qualitatively similar to all-or-none responses of nerve and muscle membranes, in which it has been proposed that currents are carried through ion-selective channels, the permeability of which is altered by the electric field across the membrane (Hodgkin & Huxley, 1952).

Evidence supporting the direct involvement of Ca^{2+} in the regulation of ciliary reversal was provided by experiments in which paramecia were extracted with Triton X-100 and reactivated with ATP and Mg^{2+} (Naitoh & Kaneko, 1972). The extracted models swam forward in solutions containing less than 10^{-6} M free Ca^{2+} and in reverse if the Ca^{2+} concentration exceeded this level. Saiki & Hiramoto (1975) reported that ciliary reversal was produced by pressure injection of Ca^{2+} into paramecia in a concentration comparable to that effecting reversal in extracted models. A membrane mutant of *P. aurelia* generated by Kung (1971) is unable to undergo ciliary reversal. Electrophysiological experiments on 'pawn' (Kung & Eckert, 1972) showed the membrane to be inexcitable (i.e. it lacked the Ca response). Since extracted models of pawn were able to reverse in solutions containing 10^{-6} M-Ca^{2+} (Kung & Naitoh, 1973) it was concluded that the membrane Ca conductance is necessary for ciliary reversal.

On the basis of these findings, then, it is logical to make the assumption that the cytoplasmic Ca^{2+} concentration of a forward-swimming, live *Paramecium* is below 10^{-6}M. It has been calculated that the amount of Ca^{2+} crossing the surface membrane to depolarize the membrane by 1 mV would raise the intraciliary Ca^{2+} concentration to about 10^{-6}M (Eckert, 1972). According to the extraction experiments, this is just sufficient to effect a ciliary reversal.

Similar calculations indicate that the same amount of Ca^{2+} distributed throughout the entire cytoplasm would be ineffective in producing the increase in concentration required for ciliary reversal. It seemed reasonable to suppose, then, that part or all of the Ca^{2+} influx may occur through channels in the membrane surrounding the axoneme, thereby resulting in an effective concentration increase in the immediate environment of the ciliary apparatus.

The present study considers both the distribution of Ca channels in the membrane of *Paramecium*, and the electrical properties which the cilia

contribute to the cell. Information was derived through a monitoring of electrical behaviour before and after deciliation and during the process of ciliary regrowth. The results indicate that the voltage-sensitive Ca^{2+} channels reside primarily in those portions of the uninterrupted surface membrane enclosing the axoneme rather than being uniformly distributed throughout the surface membrane. Estimates of ciliary length constant suggest that the cilia are isopotential with only negligible cable loss. Some of these findings have been reported elsewhere in preliminary form (Dunlap, 1976; Dunlap & Eckert, 1976; Ogura & Takahashi, 1976).

METHODS

Morphology. Specimens of *Paramecium caudatum* (mating type I, syngen 1) reared in hay infusion (Naitoh & Eckert, 1972) were washed in control solution (5 mM-$MgCl_2$, 4 mM-KCl, 1 mM-$CaCl_2$, 1 mM Tris-HCl, 0·1 mM EDTA at pH 7·2). Deciliation was achieved by incubating the washed cells in control solution containing 4 mM chloral hydrate at 20° C for 20 hr (Alverdes, 1922; Grebecki & Kuźnicki, 1961; Kuźnicki, 1963). The denuded cells were then returned to control solution, in which regeneration of the cilia took place. At various times during the reciliation process cells were fixed and prepared for scanning electron microscopy using methods developed by Satir, Sale & Satir (1976) for *Tetrahymena*. Specimens were fixed in 2% glutaraldehyde, 75 mM-Na cacodylate buffer at pH 7·2 for 25 min and post-fixed in 1% OsO_4, 50 mM-Na cacodylate for 45 min. Fixation was followed by a series of alcohol dehydrations (50, 70, 95, 100%) and Freon TC substitutions (25, 50, 75 and 100%). Cells were then critical-point dried in Freon 13, sprinkled on to a cover-slip affixed to a stub with silver paste, coated with either gold or gold–palladium and viewed on a Coates and Welter or an ETEC scanning electron microscope.

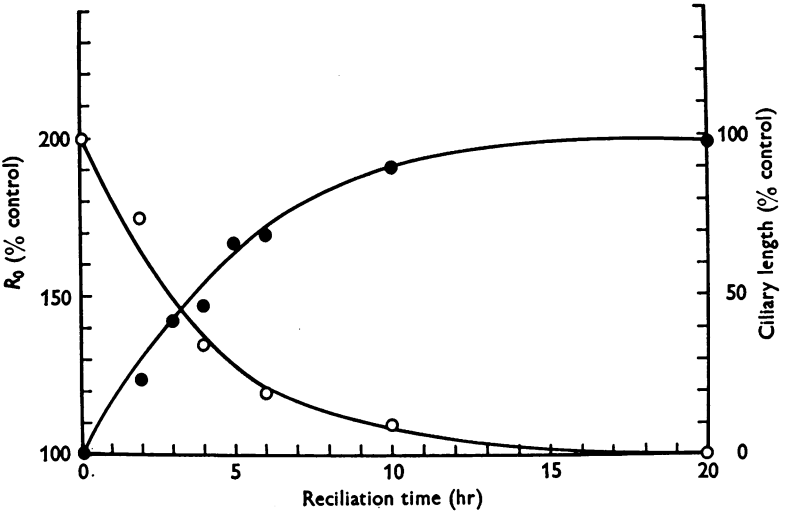
Electrophysiology. Paramecia were deciliated as described above. An alternative deciliation technique was occasionally employed in which cells exposed to chloral hydrate were agitated with fine air bubbles (Y. Naitoh, personal communication). In some experiments, regrowth of cilia was halted by the addition of colchicine 10 mg/ml. to the control solution. For this purpose 1 mM Tris-HCl was replaced by 1 mM-HEPES (Margulis, Banerjee & White, 1969). HEPES appeared in no way to affect either the action of chloral hydrate or the rate of ciliary regrowth.

Electrical properties of control, deciliated, reciliating, and reciliated cells were monitored with intracellular microelectrode techniques described elsewhere (Naitoh & Eckert, 1968*a*). Membrane responses were elicited by 200 and 800 msec rectangular and 8 sec ramp current pulses from a constant current device (New, 1972). The responses were recorded in control and 4 mM-$BaCl_2$ solutions. A slow current ramp was employed to allow the membrane to approach steady state. For the ramp experiments voltage was displayed on the oscilloscope as a function of current, producing a quasi steady-state I–V plot which was recorded on film. Membrane responses to rectangular current pulses were displayed as a function of time.

The regenerative response during the early part of a rectangular current pulse is due to a graded Ca current (Naitoh, Eckert & Friedman, 1972; Naitoh, 1974). The strength of the Ca current (I_{Ca}) was estimated from the first derivative of the voltage (voltage differentiated with 20 msec time constant). Assuming that inward (Ca) and outward (K) currents are separated in time, dV/dt_{max} (if it occurs early in time) is directly proportional to the maximum amplitude of the inward current (Hodgkin

& Rushton, 1946). Actual measurements of I_{Ca} were made by subtracting the current through the leakage resistance (i.e. the membrane resistance which is voltage independent) from the total membrane current. Since relative rather than absolute values for I_{Ca} are required, any error introduced by overlap of inward and outward currents is of little consequence as long as any changes in time courses for currents remain insignificant as suggested by the differentiated recordings.

I_{Ca} did not saturate within the range of evoked voltage displacements. Therefore the ratio of I_{Ca} to membrane potential (calculated from the product of applied current and leakage resistance) is used as an index assumed to be proportional to channel density.



Text-fig. 1. Time course for regrowth of cilia (filled circles) and the change in resting resistance (open circles) accompanying ciliary regrowth. Both plotted as percentage of control values.

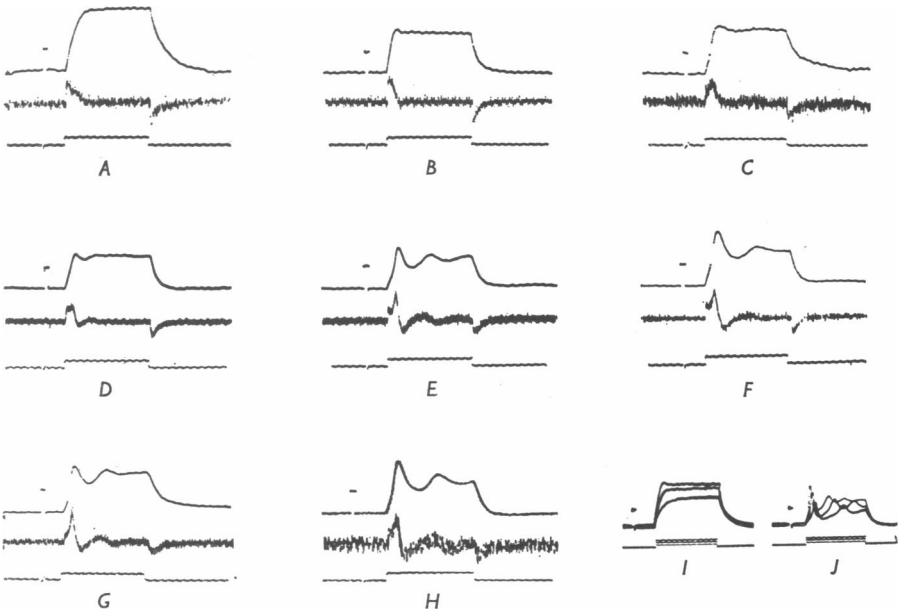
RESULTS

Morphology

Scanning electron micrographs of representative control, deciliated and reciliating cells are shown in Pl. 1. Twenty hours incubation in 4 mM chloral hydrate results in the elimination of all somatic cilia (i.e. cilia covering the general surface outside the oral groove). The cilia in the oral groove, on the other hand, remain intact with this deciliation procedure, perhaps because they are protected from environmental shearing forces. Approximately 25% non-specific shrinkage resulted from fixation. The somatic cilia, 8 μm in length and 0·2 μm in diameter, were found to occur at a density of 1 cilium per 1·5 μm^2 in fixed cells. In an average-sized, fixed cell (130 × 30 μm) in which the membrane surface area is taken as $A = m^2\psi 4{\cdot}5$ (where m = cell length and ψ is $\frac{1}{2}$ cell diameter/cell length)

deciliation results in a 50% loss of membrane surface area, if one takes into account the doubling effect of surface sculpturing present between cilia (Fortner, 1925).

Breakage occurs very close to the base of the cilium (Pl. 2*B*), confirming earlier results from transmission electron microscopy (Kennedy & Brittingham, 1968). Reciliation proceeds unevenly over the surface of the cell. Cilia grow first on the anterior and posterior ends of the cell as well as that area anterior to the oral groove on the ventral surface of the cell (Pl. 1*C*). The cilia covering the rest of the cell lag in their growth behind these fast starters but eventually reach equivalent lengths. Asynchronous regrowth of cilia has been found to occur also in *Tetrahymena* after deciliation with dibucaine (Satir *et al.* 1976).



Text-fig. 2. Potential responses of representative deciliated (*A*), 2, 4, 6, 8, 10 and 24 hr reciliating (*B–G* respectively), and control (*H*) cells to outward current pulses of 0·35 nA. Centre traces are the differentiated voltage records. (*I*, *J*): superimposed traces showing relation of voltage to current strength in deciliated and control cells. Calibration pulses = 10 mV, 10 msec.

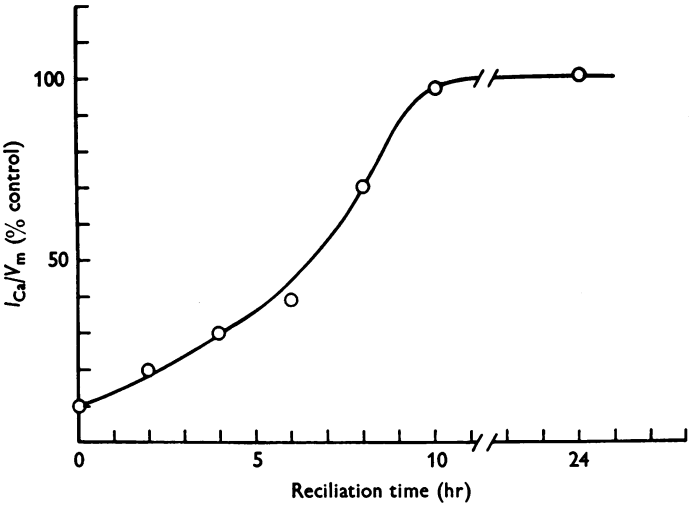
At any given time during the period of reciliation there is a wide range of ciliary lengths between close neighbours; that is, asynchronous regrowth occurs not only in the cilia from different regions of the cell but also between cilia in the same region. For example, in 4 hr reciliated cells the lengths of cilia ranged from 0·5 to 5 μm. Cilia were measured at random, averaged

from cells in 1 hr time blocks, and plotted against time from onset of reciliation (Text-fig. 1). Average ciliary length is assumed to be directly proportional to area of membrane surrounding the cilia. Reciliation is more than 90% complete in 10 hr.

Electrophysiology

Ca response

Deciliation and subsequent ciliary regrowth were unaccompanied by any changes in resting potential. Removal of the cilia did, however, result in an almost complete elimination of both the regenerative Ca response and



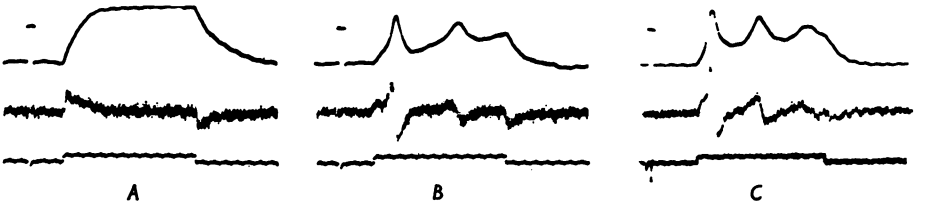
Text-fig. 3. Return of the calcium response with time as the cilia regrow. See text for methods of calculating I_{Ca}/V_m.

delayed rectification (i.e. the time-dependent sag in potential with maintained outward current), as is shown in Text-fig. 2. Small Ca responses could be seen with relatively large depolarizations (Text-fig. 2*I*). These responses may arise in the oral cilia, which remain intact with the deciliation procedure. Changes in ciliary beat appear to follow these small Ca responses (exact description of changes in ciliary activity was not feasible with the techniques employed). The regenerative response returns as the cilia regrow, reaching control levels in 10 hr. Removal of cilia results in a 90% reduction in I_{Ca} (see Methods for calculations) as is shown in Text-fig. 3 in which the ratio I_{Ca}/V_m (assumed proportional to channel density) is plotted against reciliation time. The residual 10% I_{Ca} is thought to arise from Ca channels in the oral cilia as the proportion of membrane present in the 1000 cilia in the oral groove (Ehret & McArdle, 1974) was

found to be 9% of the total membrane. As the cilia grow, I_{Ca} increases. Control responses are reached in approximately 10 hr.

Possible pharmacological effects of chloral hydrate

To determine whether the loss of excitability can be attributed to a pharmacological effect of chloral hydrate, a deciliation technique (Y. Naitoh, personal communication) was employed in which cells exposed to the chloral hydrate were subjected to mechanical shearing by air bubbles



Text-fig. 4. Voltage responses of representative (*A*) deciliated, (*B*) ciliated, and (*C*) control cells. *A* and *B* were exposed to chloral hydrate for 10 hr. Unlike *B*, *A* was agitated with air bubbles and thereby deciliated. Calibration pulse = 10 mV, 10 msec.

passed through a porous filter at the bottom of an incubation flask. Deciliation was complete in only 10 hr with this procedure. Membrane responses of the deciliated cells were compared to cells incubated an equivalent time in chloral hydrate but without agitation so as to avoid deciliation. Text-fig. 4 shows that removal of cilia eliminated both the Ca response and delayed rectification while cells incubated an equivalent time in chloral hydrate but retaining their cilia exhibited a Ca response equivalent to the control cells. Thus, it is the removal of cilia rather than a pharmacological effect of the chloral hydrate which is responsible for loss of the Ca response.

Membrane constants and cable properties

Steady-state *I–V* characteristics (from both rectangular and ramp current pulses) were analysed during regrowth of cilia. Deciliation resulted in a doubling of the input resistance (R_0), calculated as the slope resistance through zero current. As the cilia regrew, R_0 decreased exponentially with time becoming asymptotic at the control resistance and following a time course similar to that of ciliary regrowth (Text-fig. 1).

Experimentally determined values of R_0, R_i (Gelfan, 1926–7), and membrane surface area allowed the calculation of specific membrane resistance (R_m), resistance of a unit length of ciliary membrane (r_m) resistance of a unit length of cytoplasm in a cilium (r_i), and length constant (λ) of a

cilium from the equations which follow. Average values are shown in Table 1.

Removal of the somatic cilia results in a 50% reduction in cell membrane surface area and a doubling of the input resistance. This suggests that the membrane resistance is uniform throughout the cell surface and can be calculated from cells with or without cilia.

$$R_m = R_0 . A,$$

where A = membrane surface area. Specific cytoplasmic resistance (R_i) was converted from direct measurements of cytoplasmic conductance (0·0068 mho/cm) made by Gelfan (1926–7). The resistance of a unit length of cytoplasm in the cilium is most probably much higher than that calculated for a cylinder of diameter equal to that of a cilium, since a substantial proportion of intraciliary space is occupied by the axoneme and associated structures.

TABLE 1. Cable constants calculated for cells and individual cilia at various times during the reciliation process. Average cell dimensions of 180×40 μm were used in the calculations. Averaged values of ciliary length and R_0 were made from a minimum of five cells per category. $R_i = 150$ Ω.cm

Category	Average ciliary length (μm)	Total membrane area (10^4 μm^2)	R_0 (10^6 Ω)	R_m (10^4 Ω cm^2)	r_m of cilium (10^9 Ω cm)	r_i of cilium (10^{12} Ω/cm)	λ of cilium (μm)
Control	10	16	40	6·4	1	2·5	140
Deciliated	0	7·9	80	6·3	—	—	—
4 hr reciliated	5	12	54	6·5	—	—	—
10 hr reciliated	9	15	44	6·6	—	—	—

If that portion of cytoplasm occupied by the axoneme is taken as 80% (most likely an over-estimate favouring a shorter λ), any additional resistance offered by associated structures assumed negligible, and the resistance of the external medium assumed equal to that of the cytoplasm in the cilium, the length constant of a cilium can then be calculated from the standard cable equation (Hodgkin & Rushton, 1946) to be 140 μm

$$\lambda = \left(\frac{r_m}{r_i + r_0}\right)^{\frac{1}{2}},$$

where

$$r_m = R_m / 2\pi\rho$$

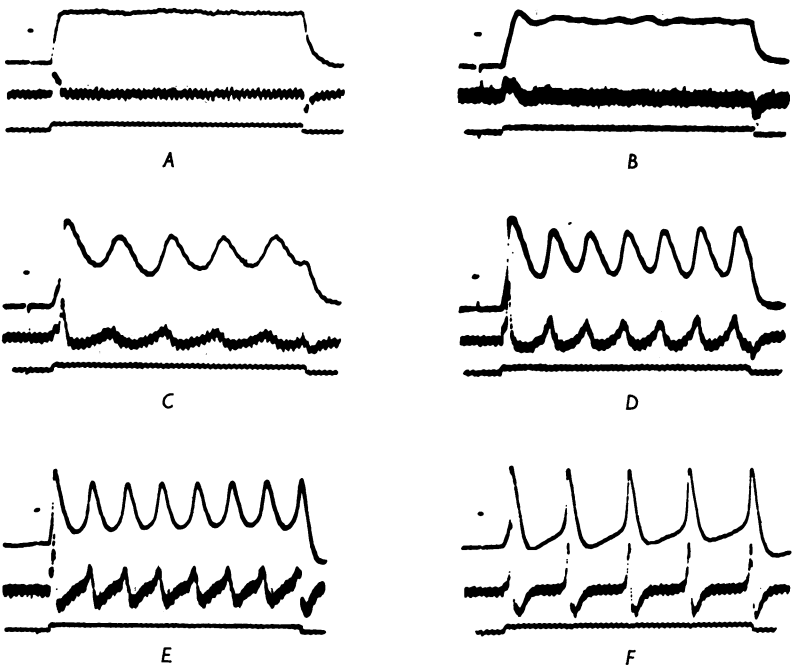
$$r_i = \frac{5R_i}{\pi\rho 2}.$$

r_m = resistance of a unit length of ciliary membrane, r_i = resistance of a unit length of cytoplasm within cilium, r_0 = resistance of a unit length of external solution, and ρ = radius of cilium = 0·1 μm.

Results are shown in Table 1.

All-or-none spike

In *Paramecium* Ba^{2+} converts the graded Ca^{2+} response to an all-or-none action potential as seen in Text-fig. 5*F* (Naitoh & Eckert, 1968*b*). Deciliation eliminates the current-evoked all-or-none barium spike (Text-fig. 5*A*). A graded Ba response returns during ciliary regeneration. All-or-none Ba spikes (i.e. amplitude of spike is independent of current intensity) were not observed before 8 hr of reciliation. After this, a single all-or-none spike followed by several spikes of decreased amplitude were produced by the 800 msec pulse.

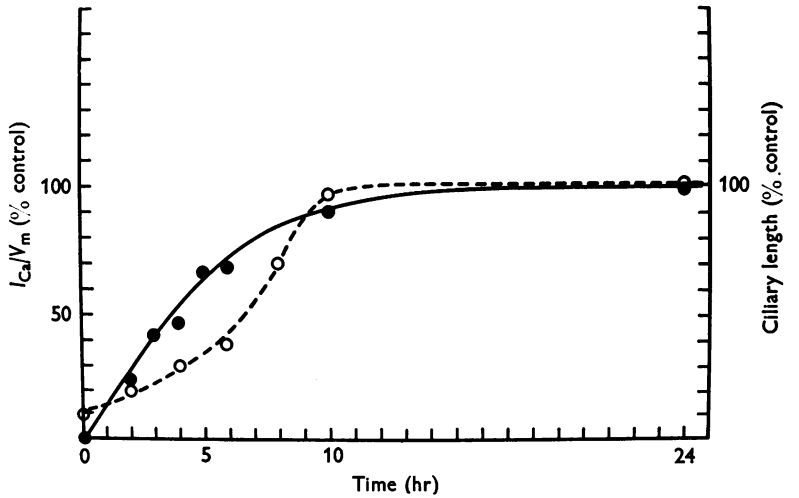


Text-fig. 5. Voltage responses of representative cells in control solution containing 4 mM-Ba^{2+} to 800 msec outward current pulses of 0·17 nA. *F*, control; *A*, deciliated; *B–E*, 3, 5, 8 and 24 hr reciliated cells respectively. Calibration pulse = 10 mV, 10 msec.

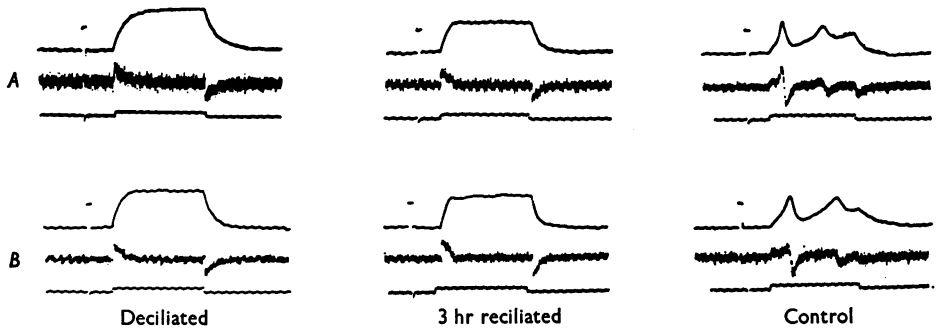
Relationship of Ca response to regrowth of cilia

The return of the Ca response follows a different time course than that of ciliary regrowth (Text-fig. 6). To test the possibility that development of excitability is independent of ciliary elongation, cilia were halted at given times during reciliation by the addition of colchicine, 10 mg/ml. Any further development of the Ca response could then be followed in time

in the absence of ciliary growth. Exposure of control cells to colchicine for extended periods of time (up to 24 hr) did not result in either deciliation or changes in the electrical properties of the surface membrane (resting potential, R_0, Ca response). No significant changes in electrical



Text-fig. 6. A comparison of the time courses of ciliary regrowth (continuous line) and return of the Ca response (dashed line).



Text-fig. 7. Voltage responses of representative deciliated, 3 hr reciliated and control cells to outward current pulses of 0·17 nA. *A*, responses from cells whose ciliary regrowth was uninterrupted. *B*, responses from cells whose ciliary regrowth was interrupted with colchicine 10 mg/ml. for 8 hr. Records were taken after the 8 hr incubation period in colchicine. Calibration pulse = 10 mV, 10 msec.

properties took place while regrowth was arrested by colchicine. The membrane properties of partially reciliated cells and cells in which reciliation had been suspended for 8 hr could not be distinguished electro-

physiologically (Text-fig. 7). It can be concluded, therefore, that restoration of lost membrane function is associated with the process of ciliary growth, and that the cilia must reach full length before these functions are fully restored.

DISCUSSION

Deciliation of *Paramecium* with chloral hydrate produced a 90% reduction in the regenerative calcium response to depolarizing stimuli. This response returned as the cilia were allowed to regrow to their original lengths. If it is assumed that the Ca current which generates the response is carried through specific membrane channels as in other excitable membranes, it would appear that these channels are largely confined to those portions of the cell membrane removed by deciliation, namely those portions of surface membrane surrounding the ciliary axoneme.

Action of chloral hydrate

The mechanism of chloral hydrate deciliation in *Paramecium* is unclear. Kennedy & Brittingham (1968) reported that the point of scission of the cilium occurs just distal to the basal body, in a region of depolymerized microtubules. The fact that agitation significantly reduced the time course of deciliation suggests that the shearing forces encountered by the cell in its environment may be responsible for the actual breakage and removal of the cilia weakened by exposure to the chloral hydrate. This would explain the retention of the oral cilia which lie protected in the oral groove.

It has been shown in a number of systems (Thompson, Baugh & Walker, 1974; Kiehart & Inoué, 1976; Schliwa, 1976) that Ca^{2+} plays a major role in the depolymerization of microtubules. Whether or not an increase in intracellular Ca^{2+} concentration is responsible for deciliation by chloral hydrate remains unclear. Forward-swimming velocity is decreased after several hours in chloral hydrate, which could result from a loss of cilia (Kennedy & Brittingham, 1968) and/or a slight increase in cytoplasmic Ca^{2+} (Machemer, 1974; Eckert & Machemer, 1975) produced by the application of the drug.

Lateral diffusion of channels

Considering the fluid nature of plasma membranes at 20° C (Frye & Edidin, 1970) the findings presented here, that ion-specific membrane channels are confined to the ciliary membrane, suggest that the channels are in some way either anchored to those portions of surface membrane or are in some other way physically restrained and prevented from diffusing to areas of interciliary membrane. Evidence for such an anchoring

system has been observed in cilia of *Tetrahymena* (Satir *et al.* 1976), where intramembrane particle arrays thought to be involved in Ca^{2+} pumping at the ciliary base appear to be attached via connexions to microtubule doublets. These particle patches, observed also in *Paramecium* (Plattner, Miller & Bachman, 1973), may provide the necessary obstacle to diffusion of the channels. On the other hand, either the channels themselves may be anchored in some way, or freely diffusible channels may have a high turnover rate, newly synthesized channels being inserted only in the ciliary membrane.

Uneven distribution of channels

Whether or not the discrepancies between the time course for return of excitability and the elongation of the cilia in fact represent an uneven distribution of channels along the entire length of ciliary membrane remains unclear. A uniform distribution of Ca channels along the length of ciliary membrane seems to be contradicted by the fact that when regrowth of cilia was halted for extended periods of time with colchicine no further development of the Ca current beyond that measured immediately before interruption of regrowth was observed. Large doses of colchicine (10 mg/ml.) were essential for inhibition of reciliation. Comparable concentrations have been necessary in other ciliates and flagellates (Margulis *et al.* 1969; Rosenbaum & Carlson, 1969). The latter investigators, using colchicine, 4 mg/ml., to inhibit flagellar regrowth in *Chlamydomonas*, found no effect of the inhibitor on protein or RNA synthesis. If this is also the case in *Paramecium*, and if the drug does not interfere with the insertion of the channels in the membrane, the results from the colchicine experiments reported here suggest that the channels are distributed somewhat non-uniformly all along the length of the cilia. The additional and somewhat esoteric possibility that the time course for development of excitability is determined by some property of the channels themselves cannot be ruled out by these experiments. The present alternatives, that the conductance of an individual channel is variable with respect to (1) time after its insertion into the membrane, (2) the total number of channels in the membrane, or (3) its location in the membrane cannot be disputed on the basis of information reported here.

Isopotentiality of paramecium

A 50% reduction in membrane surface area would be expected to lead to a twofold increase in input resistance if it is assumed that membrane resistance is uniform throughout the surface and the cell is isopotential. That this proportionality was found upon removal of the cilia suggests that each cilium is isopotential along its length as well as with the major

intracellular compartment. That is, there is little cable loss along the ciliary shaft for d.c. potentials. This conclusion receives support from the similarity between the restoration of input resistance and ciliary length during regrowth (Text-fig. 1).

Calculations of the length constant of a cilium, using the measured membrane resistance of the deciliated cell and estimates of longitudinal resistances, indicate a length constant several times longer than the cilium. The error introduced in these calculations by ignoring any resistance resulting from ciliary substructure associated with the axoneme may be substantial. In particular, the transverse plate, the uppermost section of the basal granule, may be a significant barrier to current flow as it spans the base of the cilium. Since it is not removed with deciliation, whether or not it is associated with a large resistance cannot be determined by time constant measurements, which would reflect a change in series resistance. A large resistance at the cilium's base could result in a potential difference between the cilium and the major cell compartment. The isopotential nature of this major compartment has been demonstrated (Eckert & Naitoh, 1970).

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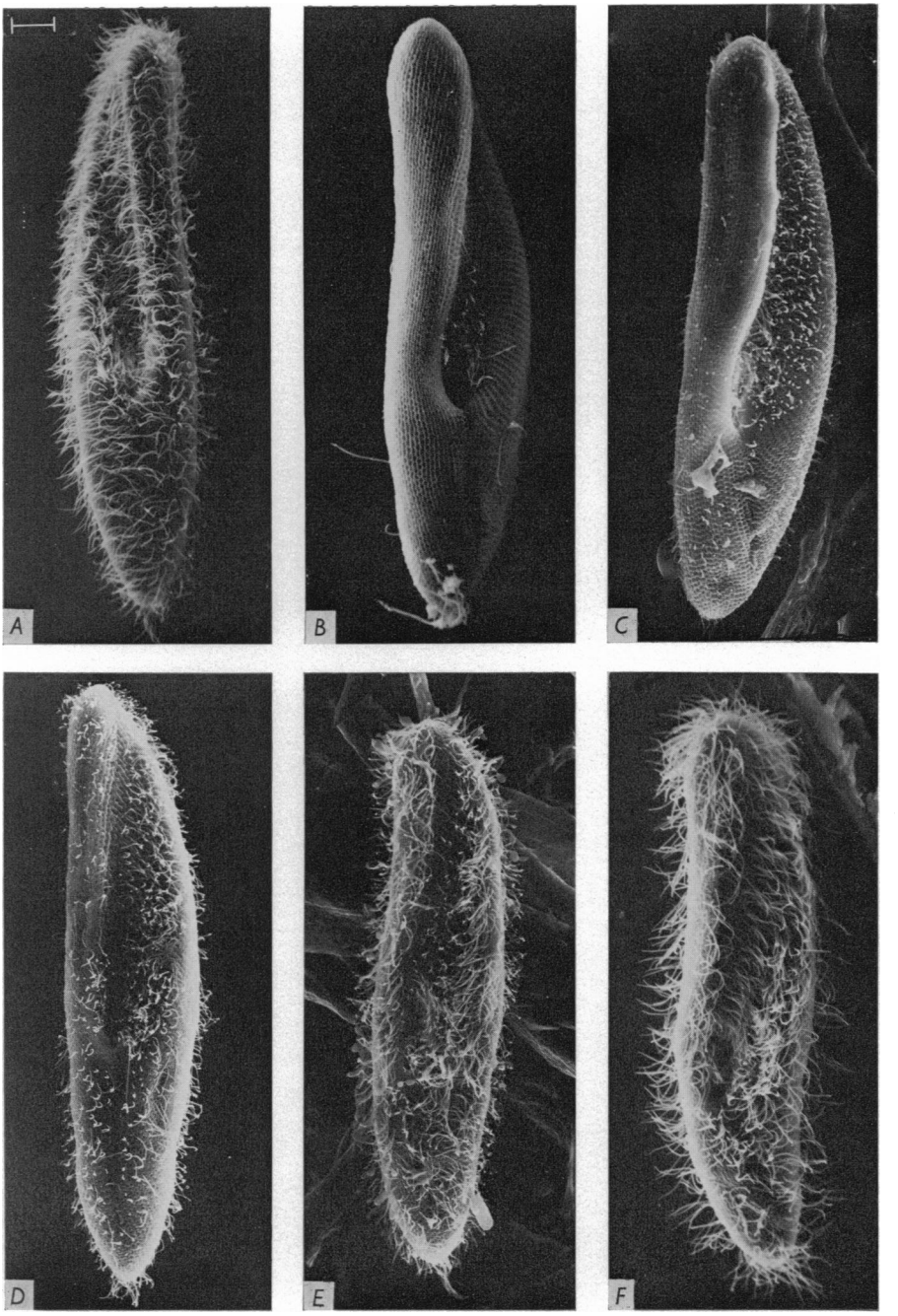
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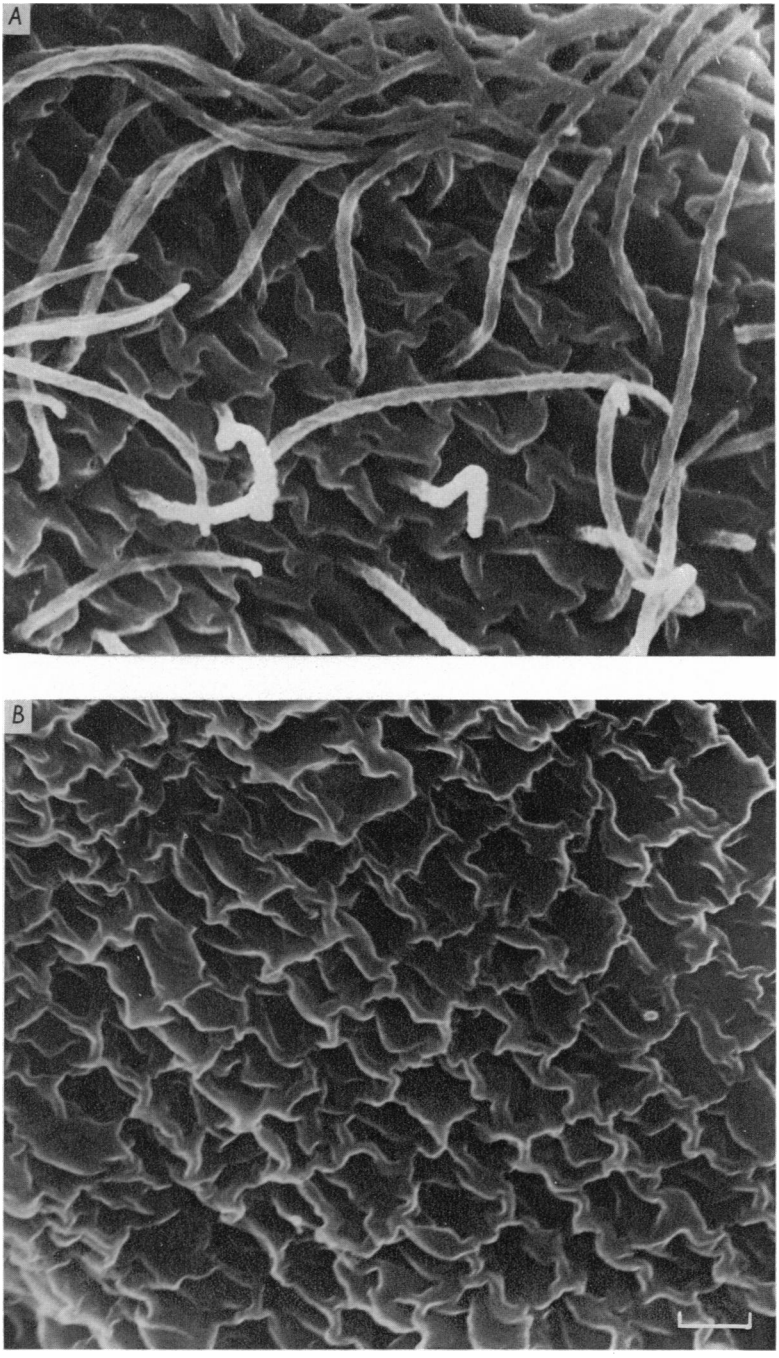
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EXPLANATION OF PLATES

PLATE 1

Scanning electron micrographs of (*A*) control, (*B*) deciliated, (*C–F*) 2, 4, 10 and 24 hr reciliated cells respectively. Calibration bar = 10 μm.

PLATE 2

High power scanning electron micrographs of (*A*) control and (*B*) deciliated surface membrane. Note that scission point of the cilium is very near the base. Calibration bar = 1 μm.