

Part 2

Control of ciliary beating by
neurotransmitters
in isolated ciliated cells

Summary

In order to investigate the effect of neurotransmitters on the ciliary movement of isolated cells, individual cells were held by a micropipette. Response to dopamine on *Pseudocentrotus depressus* and *Hemicentrotus pulcherrimus* was tested for the cells bathed in three different media; dispersion medium, Ca²⁺-free ASW and normal ASW. When dopamine was applied in the dispersion medium, which includes EDTA and no Ca²⁺ added, or Ca²⁺-free ASW, cilia showed the BPTR similar to one observed previously in the epithelial cilia of tethered larvae. The BPTR under conditions where cell rotation was not allowed by suction indicates that it can be caused by changes in the ciliary motile apparatus itself. The BPTR induced under Ca²⁺-free conditions suggests that this response does not necessarily require the Ca²⁺ influx. Ciliary reversal, which has been argued not to occur in the absence of external Ca²⁺, was observed after suction even under Ca²⁺-free conditions described above.

A kind of symmetrical beating, which has two recovery strokes in the opposite directions, one replacing the effective stroke, was observed under experimental conditions in *Hemicentrotus pulcherrimus*, and *Pseudocentrotus depressus*. This symmetrical beating appears different from flagella-type beating in that a cilium propagates only one bend at the same time during each recovery stroke, and hence was named R- R beating. The R- R beating has never been seen in epithelial cilia and seems restricted in isolated cells. This fact indicates that asymmetric wave propagation, which is a major feature of ciliary beating, is not necessarily inherent to

ciliary structures.

Instability of beat period has been known not to change significantly after isolation of cells. The instability did not appear to increase to a much greater extent after suction. It undergoes the same control by neurotransmitters. In the presence of dopamine, beat period is elongated by insertion of pauses mainly at the end of the effective stroke. This means that the angular velocity and stability of beat period were controlled by independent systems as in intact larvae. Thus, ciliary beat period can be modulated at a cellular level by two mechanisms, i. e., control of angular velocity and changes in the location of switching from the effective stroke to the recovery stroke or inserting pauses between the effective and recovery strokes, and the latter may be regulated by neurotransmitters.

Introduction

In Part 2, responses in single cilia were studied using isolated ciliated cells isolated from sea urchin larvae. Considering the results of Part 1, effects of dopamine in *Pseudocentrotus depressus* and carbamylcholine in *Hemicentrotus pulcherrimus* were studied. As previously described (Baba, 1975), epithelial cells of sea urchin larvae have only one cilium per cell, isolation was useful to examine responses within a single cilium. Rotation movements of free single ciliary cells have been studied (Mogami *et al.*, 1993). It is demonstrated that ciliary beating was guided by a glass surface and its plane always parallel to the focal plane. It was thought that beat-plane turning response (BPTR, described in Part 1) could not be detected if it occurred under free rotating conditions, because upon BPTR the cell body itself would rotate with the beat plane being guided by the surface. In this study, ciliary cells were held by micropipette not to rotate. Under these condition, this enabled changing of external medium keeping cells in position.

Beating of an isolated cilium was analyzed from the same points of view as in Part 1, i. e., beat configuration and individual beat period. Cilia maintain the same ability of response to neurotransmitters after isolation. This system is available for studying function of cells and its regulatory mechanisms.

Materials and methods

Isolation of ciliary cells from sea urchin larvae

Sea urchins, *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus* were used. Eggs and sperms were obtained by injection into the body cavity with 0.55 mol l^{-1} KCl or $10^{-5} \text{ mol l}^{-1}$ carbamylcholine. Eggs were fertilized in Jamarin U artificial sea water (Jamarin Laboratory, Osaka) and developed at 17°C .

Procedure of isolation followed a method described previously (Mogami *et al.*, 1993). Pluteus larvae (55- 72h after insemination) were collected by hand centrifuge and suspended in a dispersion medium which consisted of (in mmol l^{-1}) 900 glycine, 60 KCl, 40 KOH, 2 EDTA (pH=8.0). This medium had been modified for preparation of cell models. The original dispersion medium including Na^{+} instead of K^{+} was also used. After incubation for 15 min in these media, they were pipetted softly to dissociation. After dissociation, isolated cells were kept in the dispersion medium or transferred to Ca^{2+} - free ASW (465 NaCl, 10 KCl, 25 MgCl_2 , 28 MgSO_4 , 10 Tris- HCl (pH=8.0). The method of transfer followed Mogami *et al.*, (1993). After spicule and aggregated cells were sedimented by hand centrifuge, supernatant were further centrifuged at 1000 rpm for 5 min. Cells in pellet were resuspended in Ca^{2+} -free ASW.

Observation and recording of ciliary movement

About $300 \mu\text{l}$ of cell suspension was loaded into an experimental chamber

made by 0.5 mm thick silicone spacer placed between slide and cover slip; slide was coated by 0.05% agarose to prevent cell adhesion. The chamber had three openings, two as circular holes on spacer that located outside the cover slip connected via thin canals to the main chamber for perfusion of solution, and the other that was made by removing the wall of the main chamber on one side and was used for insertion of a micropipette. Micropipettes were made from cored glass tubule of 1 mm diameter (GD-1, NARISHIGE, Japan) by heating and pulling with a needle-puller (NARISHIGE, PP-83) to a final tip diameter of about 5 μm . They were inserted to a holder that was connected via Teflon tubule to a syringe and were exchanged for every preparation.

Observation was done through a microscope with phase-contrast long working distance optics (CDPlan 40PL) under stroboscopic illumination from a xenon flash tube (STROBEX) triggered by VD signals from video camera (XC-77RR, SONY, Japan). Date and time of experiments generated by a character generator (Victor, CG-V60) were superimposed on VTR. After starting video tape recording (Panasonic S-VHS NV-CX1) a ciliary cell was held by suction with a micropipette, and then the timer was started. After a minute from the start of timer, test solutions including 10^{-5}M neurotransmitters (dopamine hydrochloride for *P. depressus* and carbamylcholine chloride for *H. pulcherrimus*) were perfused using two pipettes placed on two circular openings, one for influx and another for drain. The amount of perfused solution was about of the same volume as chamber. In the case that basic solution was normal ASW, ASW replaced Ca^{2+} -free ASW in which cell was captured and after further few minutes, test solution was perfused. After 3 to 10 min bathing in the test solution, basic

solution was perfused again to remove neurotransmitter used.

Analysis of ciliary configuration

Ciliary beat forms were traced on transparency sheets from 100 successive VTR fields. Coordinates of at least 30 points along ciliary shaft and basal line were entered to a personal computer by a digitizer (OSCON). Shear angles at 5 μm from the base of cilium were calculated from the coordinates as previously described (Mogami and Baba, 1985). Curvatures at 10 μm from the base of cilium were also calculated.

Analysis of beat period

Successive beat periods were measured from VTR were analyzed by the method of short-term analysis described in Part 1.

Results

Changes in beating patterns

BPTR in isolated cells

In all basic solutions tested here, the BPTR similar to that in intact larvae described in Part 1 was observed in about 10% of isolated cells of *P. depressus* (Figs. 1,2,3) in response to dopamine. Figs. 1 and 2 show the BPTRs in dispersion medium and Ca²⁺- free ASW both including dopamine, respectively. When ASW replaced Ca²⁺- free ASW to test dopamine in ASW, cilia stopped and remained motionless before and after application of dopamine. It was found that ciliary beating continued in ASW after passing Ca²⁺- free ASW when dispersion medium was replaced by one including Na⁺ instead of K⁺ as stated in **Materials and methods**. In experiments using Na⁺- dispersion medium, the BPTR in response to dopamine was observed in ASW (Fig. 3). The BPTR is not frequently observed in isolated cells as in intact larvae. In some cases, cells showed shrinkage and the surface became rugged after addition of dopamine. This contraction by dopamine occurred even when the BPTR was not observed. In isolated cells, time to start the BPTR from addition of dopamine was varied largely, but turning duration to complete turned beating was shorter (~ 1 min) than in intact larvae (~ 3 min).

To examine whether turned beating in isolated cells has the same characteristic configuration as that in intact larvae, time sequences of shear angles at

the point near the ciliary base (5 μm from the base) were compared. Fig. 4 shows plottings of shear angles obtained from 100 sequential VTR fields of normal and turned beating in an isolated cell. The turned beating has the same feature as in intact larvae. In normal beating, fast transition corresponding to the effective stroke is from minus to plus and slow transition is to the opposite direction. In turned beating, the directions of changings in shear angle in the fast and slow phases are reversed.

Ciliary reversal in isolated cells

Reversed beating was also seen in isolated cells in ASW, Ca^{2+} - free ASW and even in dispersion medium. It often occurred when the medium was changed from Ca^{2+} - free ASW to ASW, and then spontaneously in ASW. In all solutions above, it was occasionally induced upon suction with the holding pipette (Fig. 5). Cilia sometimes stopped beating after they showed reversed beating several times and restored normal beating when they were released from the pipette. In these cases, there was a delay about 30 seconds before stopping from suction and restoring from release. It is interesting that reversed beating occurred even in the dispersion medium that contains no Ca^{2+} and includes EDTA, which chelates dications.

Effect of carbamylcholine on H. pulcherrimus

Primitive response-like responses to carbamylcholine as observed in intact larvae of *H. pulcherrimus* (see part.1) were not observed in isolated cells .

R-R beating

Symmetrical ciliary beating (Fig. 6) occurred spontaneously in isolated cells of *P. depressus* (A) and *H. pulcherrimus* (B) in all solutions. This symmetrical beating had two recovery strokes, one corresponding to the ordinary recovery stroke and the other replacing the effective stroke. They showed these alternatively recovery strokes directly from the end of the preceding one instead of corresponding effective stroke. This symmetrical beating appears different from flagella-type beating in that a cilium propagates only one bend at the same time during each recovery stroke, and also from the BPTR in which a cilium shows the effective stroke, and hence was named R- R beating. The R- R beating has never been seen in cilia of intact larvae. After an episode of the R- R beating that usually lasted for a few minutes, cilia restored the normal beating.

There are clear differences in time sequence of the curvature at the middle of the ciliary shaft between the R-R and the normal beatings (Fig. 7) as compared to those in shear angle at the base (data not shown). Curvature of the R-R beating varied both to the plus and minus values, while that of the normal beating was almost only of the plus value. This means that bends propagating along the cilium were bidirectional in the R-R beating.

Effect of dopamine on beat period

As in intact larvae, dopamine had an effect to lengthen the beat period. Fig. 8 shows changes in beat period by dopamine. The beat period fluctuated to the same

degree as before isolation. After addition of dopamine, the beat period became longer and fluctuated to a greater extent with occasional extremely long pauses as shown in Fig. 8. After removal of dopamine, the beat period gradually decreased with decreased fluctuation and restored its initial level (Fig. 8).

The rate of angular change measured at the middle of the effective stroke also fluctuated beat by beat in isolated cells. The rate of angular change fluctuated in Ca^{2+} -free ASW, to give a measurement of 32.03 ± 11.89 rad/s (mean \pm S.D., $N=529$) in a typical series of measurements. After application of dopamine the rate of angular velocity was deduced, producing one series of measurements a figure of 20.40 ± 9.72 rad/s (mean \pm S.D., $N=1025$). We found a low correlation between the beat period and the angular velocity as in intact larvae; $r= 0.0957$ before application of dopamine and -0.209 after application.

Discussion

Effects of isolation and suction

The fact that the BPTR was observed even after isolation indicates cilia could maintain its ability to respond to dopamine, i.e. the cells may have dopamine receptors. Characteristic features and the frequency of occurrence of this response in isolated cells are basically the same as those in intact larvae. This indicates that the ability of the axoneme for the BPTR was not damaged by isolation.

In contrast, responses to carbamylcholine were not observed in isolated cells. This might suggest that experimental procedures for dispersion selected out cells possessing responsibility to carbamylcholine. Alternatively some steps of response to carbamylcholine may be lost during isolation. It is more likely that carbamylcholine may affect indirectly on ciliated cells in intact larvae.

Cilia stopped beating in response to suction and restored it when released. This suggests that tension on cell membrane can initiate signal transduction resulting in ciliary cessation. It should be noted that the times for reaction and restoration are of the order of 30 s.

BPTR in isolated cells

In Part 1, two models were proposed for explaining ciliary beat plane rotation, model R and model A. The fact that the BPTR was observed in isolated ciliated cells

that were held by the pipette indicates no rotation of cell body occurred during the BPTR. This excludes model R1. Cell deformation by dopamine may reflect contraction of the striated rootlet that can pull and rotate the basal body as suggested in part 1. This may support model R2.

R-R beating

The R- R beating indicates that the recovery stroke can be separated from and replace the effective stroke, and hence it can initiate without the preceding effective stroke. In the R-R beating, the recovery stroke replacing the effective stroke must be produced by the E- sliding, i.e. sliding by doublet no. 3 and neighbourings (Baba and Mogami, 1987), but by the program for sliding propagation in the recovery stroke. Thus, the R- R beating strongly suggests modulation of the internal activities without rotation of the whole apparatus, which is a basic assumption for model A. In model A, the modulation occurs gradually in the BPTR and rather instantaneously in the R-R beating.

Instability of beat period

Fluctuation of the ciliary beat periods does not seem to depending on interaction of neighboring cells, because it does not significantly change after isolation. Increases in fluctuation induced by dopamine in isolated cells suggest that dopamine can affect directly stability in switching of tubule sliding since fluctuation in the beat period in the presence of dopamine has been demonstrated to occur by inserting

pauses of more variable length between the effective and recovery strokes.

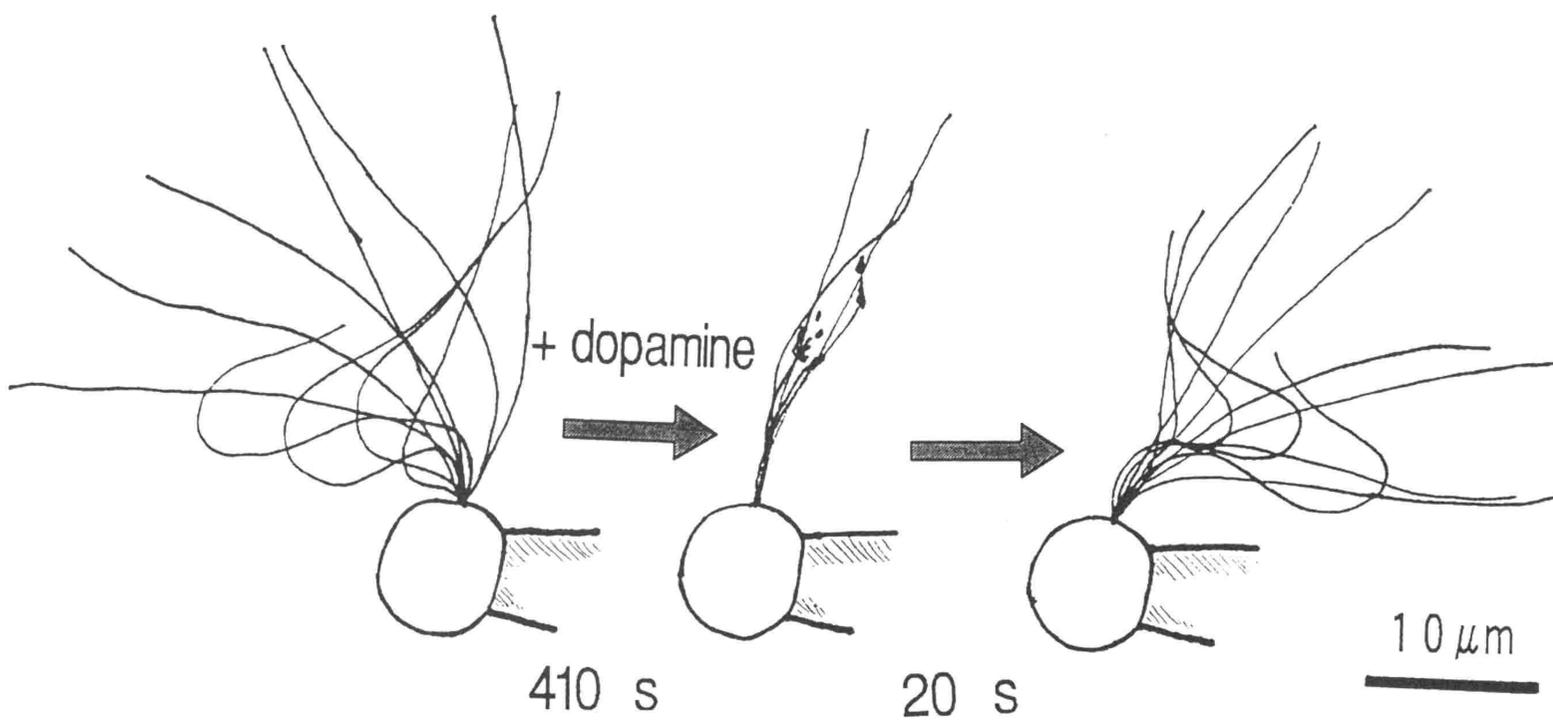


Fig. 2-1. Reconstructed beating forms from video recording of an isolated single ciliated cell of *Pseudocentrotus depressus* pluteus larva. A holding pipette and the cell body are also shown. The cilium shows the normal beating in dispersion medium (left). The beating forms were traced from successive video fields (60 fields/s). The lengths of time elapsing between each set of superimposed tracings are marked.

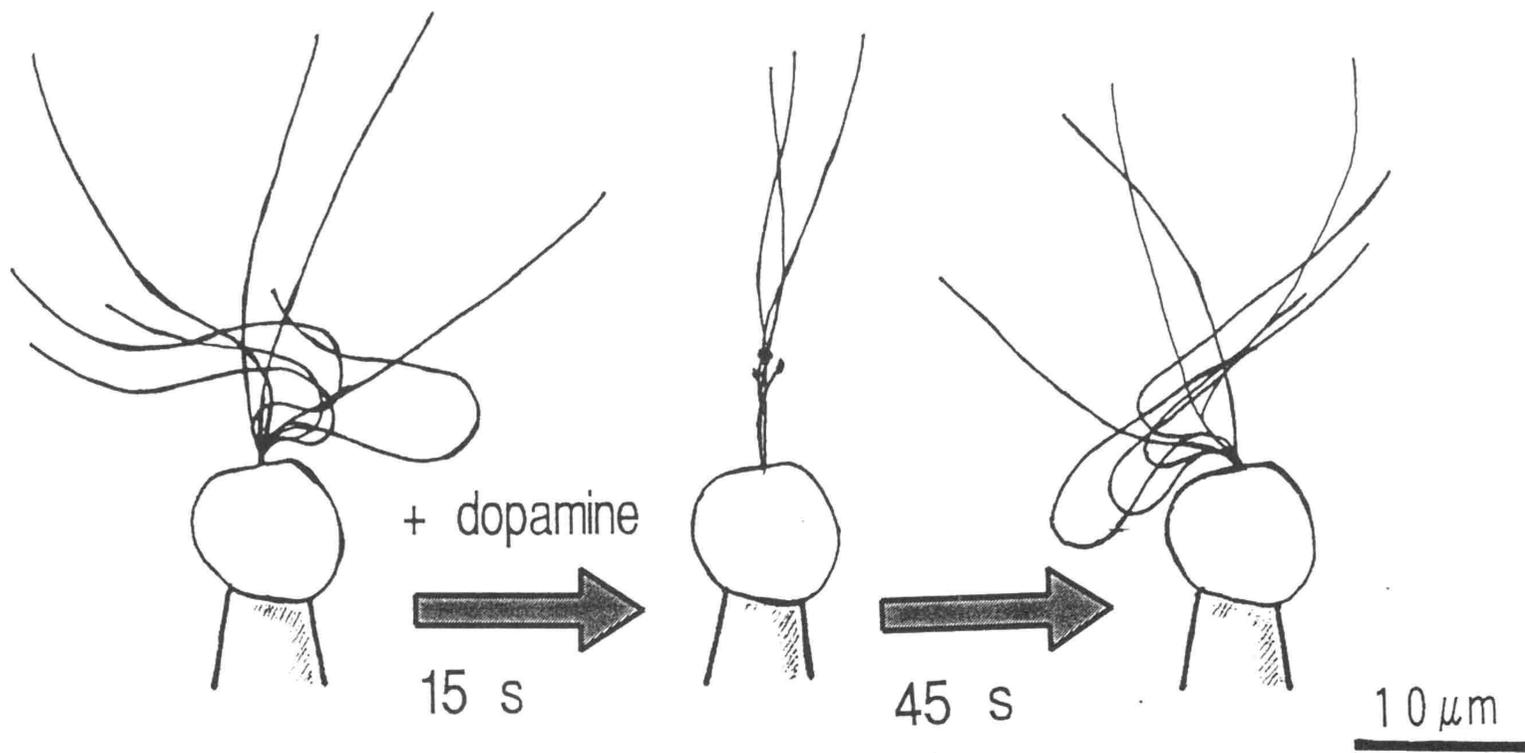


Fig. 2-2. Reconstructed beating forms from video recording of an isolated single ciliated cell of *Pseudocentrotus depressus* pluteus larva. The cilium shows the normal beating in Ca^{2+} -free ASW (left). After addition of 10^{-5}M dopamine, the cilium shows the BPTR (middle and right). Superimposed tracings are obtained from VTR as described in Fig.1.

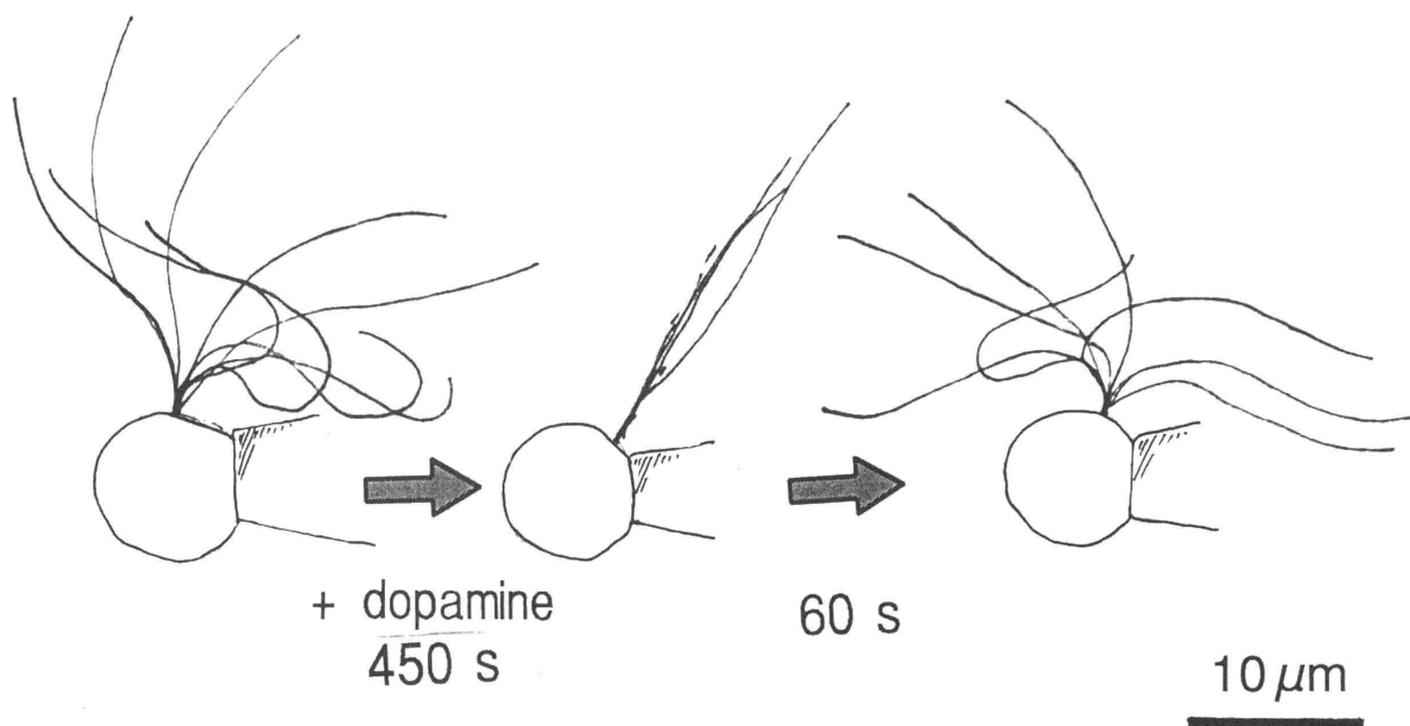


Fig. 2-3. Reconstructed beating forms from video recording of an isolated single ciliated cell of *Pseudocentrotus depressus* pluteus larva. The cilium shows the normal beating in ASW (left). After addition of 10^{-5} M dopamine, the cilium shows the BPTR (middle and right). Superimposed tracings are obtained from VTR as described in Fig.1.

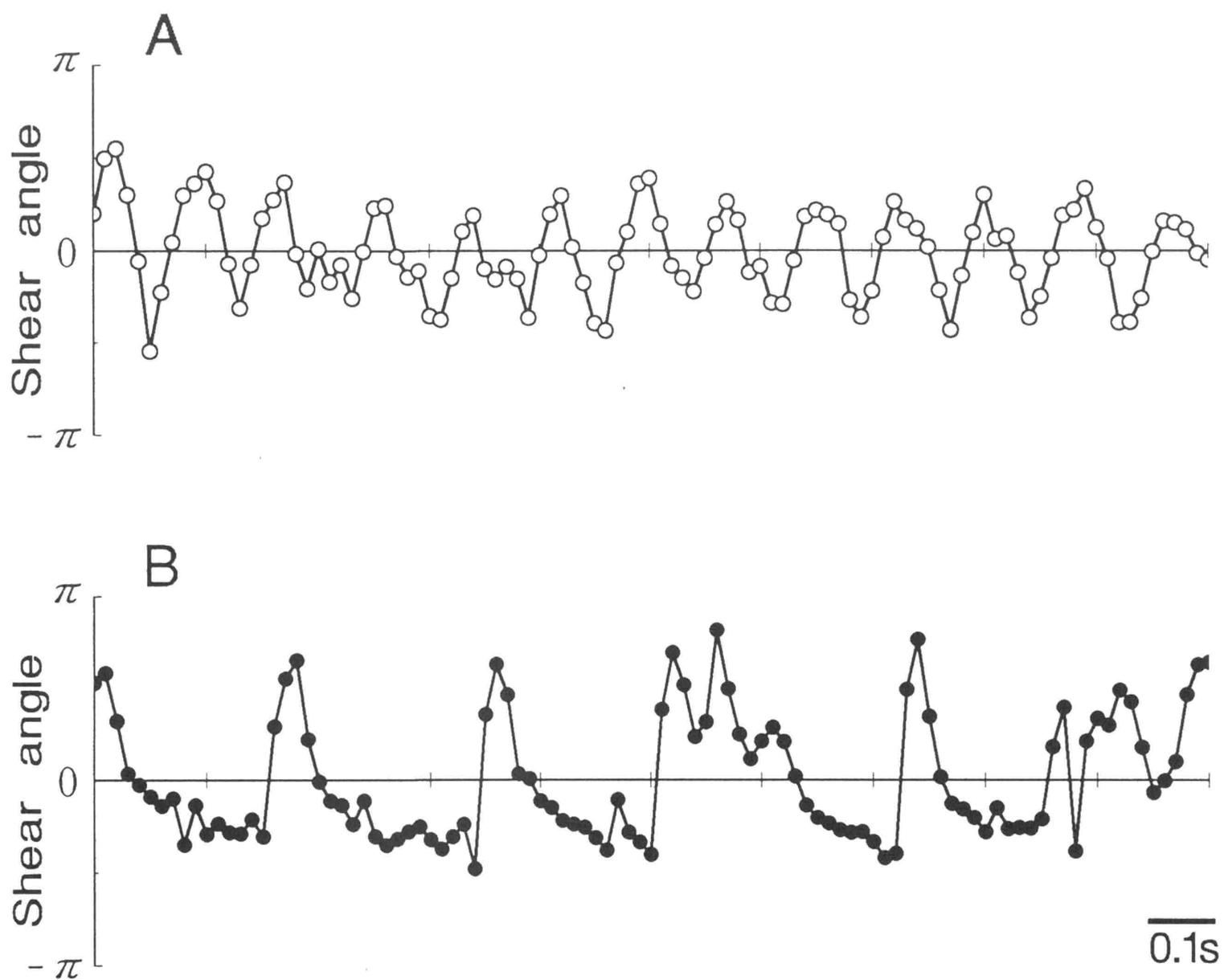


Fig. 2-4. Time course of shear angle at the proximal portion of a single cilium of an isolated cell of *Pseudocentrotus depressus* pluteus larva. (A) Normal beating in Ca^{2+} -free ASW. (B) Turned beating. The tendency of alteration of shear angle by dopamine was the same as in intact larvae (to see Fig. 5 of part 1).

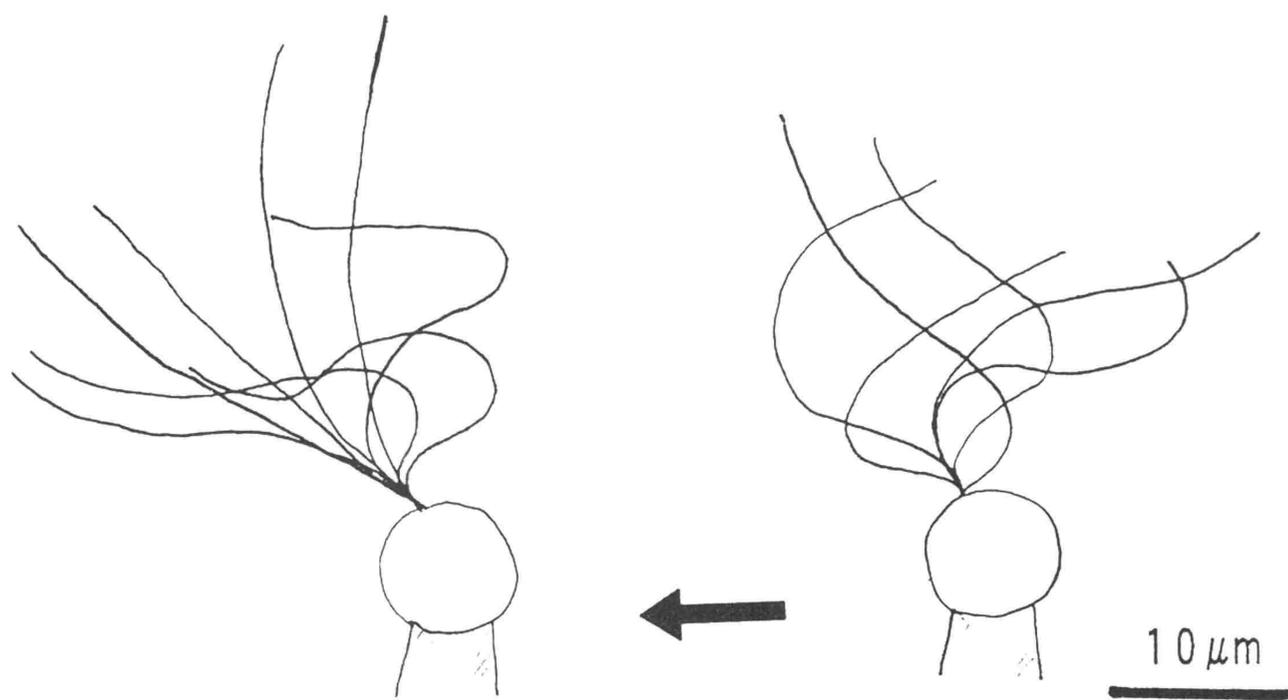


Fig. 2-5. Reversed beating in response to mechanical stimulation by suction with a pipette in an isolated cell of *Pseudocentrotus depressus* pluteus larva. The cell shows the reversed beating when held in dispersion medium which contains 2mmol^{-1} EDTA and no Ca^{2+} (right) and then eventually restores the normal beating (left).

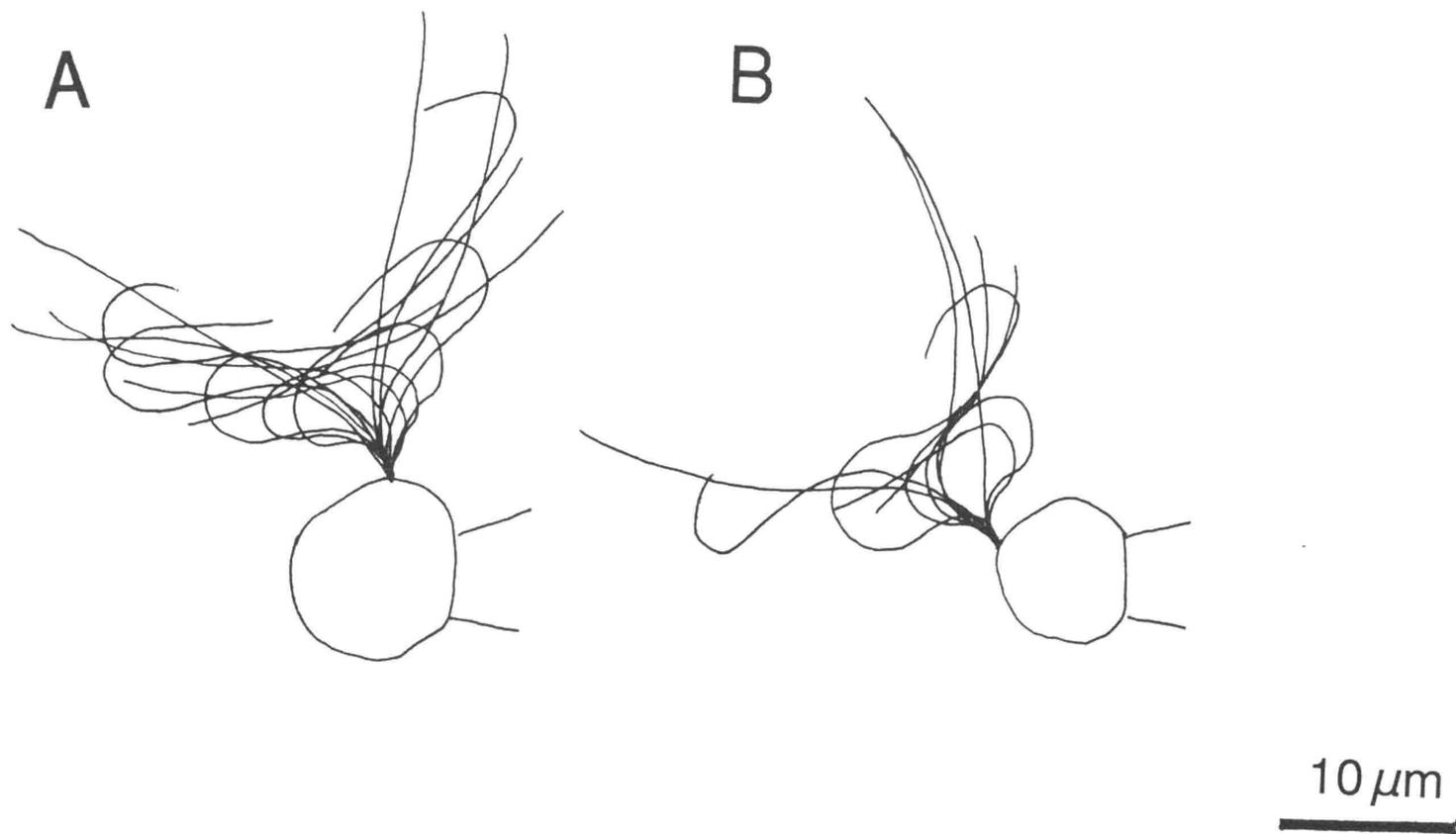


Fig. 2-6. R-R beating in isolated cells of (A) *Pseudocentrotus depressus* in dispersion medium and (B) *Hemicentrotus pulcherrimus* in Ca^{2+} -free ASW. The cilium beats in either direction with forms of the recovery stroke.

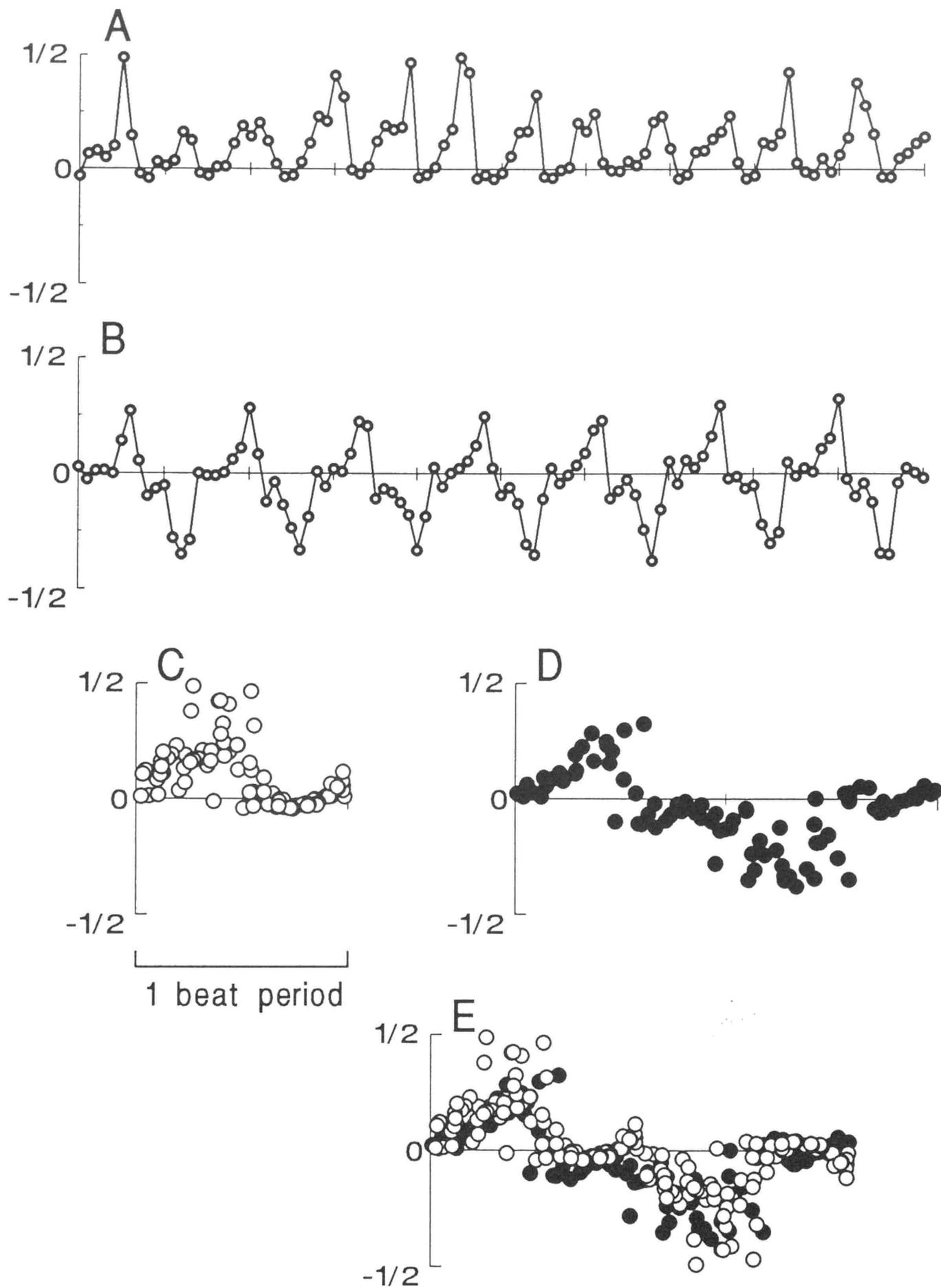


Fig. 2-7. Time course of curvature at the middle of the axoneme ($10\ \mu\text{m}$ from the base) of *Pseudocentrotus depressus* pluteus larvae in dispersion medium. (A) Normal beating. (B) R-R beating. (C) Superimposition of the curvature after normalization of the time between zero-crossing points in A for the beat period. (D) Normalized superimposition for B. (E) C extended with inversion of itself and D showing that the plots are now closely superimposed.

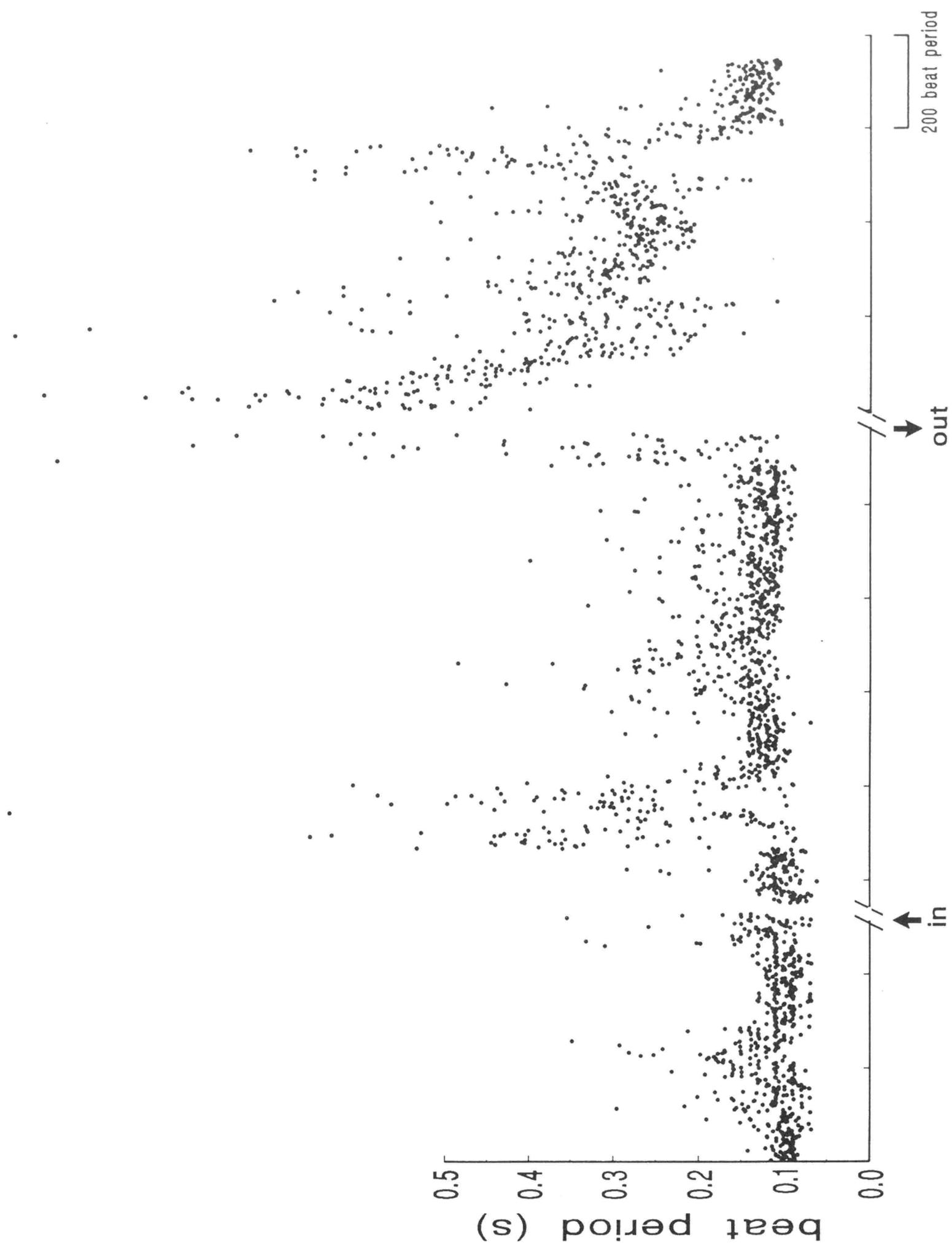


Fig. 2-8. Plots of successive beat periods of an isolated single cilium of *Pseudocentrotus depressus*. 10^{-5} M dopamine was applied between two arrows in Ca^{2+} -free ASW. Beat periods beyond 1 s are not shown. The cilium stops beating at the end of the effective stroke at longest 1.2 min after addition of dopamine. It gradually restored the normal state after removal of dopamine.