Ca²⁺-DEPENDENT REGULATION OF BEAT FREQUENCY OF CILIA IN PARAMECIUM

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SUMMARY
Triton-extracted models of Paramecium cells prepared in the presence of Mg²⁺ and EGTA showed Ca²⁺ sensitivity not only in the direction of beat but also in the beat frequency of cilia. The beat frequency increased over a range of concentration of Ca²⁺ from 2 × 10⁻⁷ to 4 × 10⁻⁷ M, in which the model swam forwards. The frequency increased also above 10⁻⁶ M-Ca²⁺, in which the model swam backwards. The increase in frequency was inhibited by calmodulin antagonists. Intracellular injection of a Ca²⁺ buffer giving a free Ca²⁺ concentration of 2 × 10⁻⁷ to 5 × 10⁻⁷ M in intact cells induced an increase in the beat frequency. Therefore, it is very likely that the beat frequency of cilia is regulated by the intracellular concentration of Ca²⁺.

INTRODUCTION
Regulation of ciliary beating in Paramecium is performed in two ways; one by a change in the direction of the power stroke and the other by a change in the beat frequency. It has been established that reversal of the direction of the power stroke is induced by an increase in the intracellular concentration of Ca²⁺, which is associated with spikelike depolarization of the membrane (Eckert, 1972). The Triton-extracted model of Paramecium shows backward swimming when the concentration of Ca²⁺ attains 10⁻⁶ M (Naitoh & Kaneko, 1972). On the other hand, little is known about how the beat frequency is regulated.

The beat frequency is increased either by a membrane depolarization beyond 5 mV or by a hyperpolarization (Machemer, 1974; Brehm & Eckert, 1978). It has been found that the transfer of the cell to a high concentration of Ca²⁺ or a low concentration of K⁺ also induces an increase in beat frequency (Nakaoka et al. 1983). All of these conditions are expected to increase the influx of Ca²⁺. Actually, contraction of the cell body, especially in the posterior region, is often observed accompanying the increase in beat frequency. It therefore seems probable that the beat frequency is regulated by the intracellular concentration of Ca²⁺. However, the Triton-extracted model prepared by the usual method has never shown any notable change in beat frequency with a change in the Ca²⁺ concentration.

In this work, we have attempted to devise a new method of treatment with Triton, by use of which the model retains the sensitivity of the beat frequency to Ca²⁺. We have found that, if treated in the presence of Mg²⁺ and EGTA, the model shows a high frequency of beating at submicromolar concentrations of Ca²⁺.
**MATERIALS AND METHODS**

*Paramecium caudatum* syngen 3, mating type V, was cultured in a hay infusion inoculated with *Aerobacter aerogenes*. *Paramecium* cells were collected by a low-speed centrifugation and the loose pellet was dispersed for extraction with detergent.

Two kinds of media were used for extraction. One was almost identical with that used by Naitoh & Kaneko (1972). The composition of the medium was 20 mM-KCl, 10 mM-EDTA, 0.01 % Triton X-100 and 10 mM-Tris-maleate buffer (pH 7-0). After extraction for 30 min at 0°C, the cells were washed by suspension in 20 mM-KCl, 2 mM-EDTA and 10 mM-Tris-maleate buffer (pH 7-0), for 15 min at 0°C. The cells were further washed with a solution of 20 mM-KCl and 10 mM-Tris-maleate buffer (pH 7-0). The model prepared by this procedure is termed type I.

Another medium for extraction was identical to that used previously (Nakaoka & Toyotama, 1979). The composition was 20 mM-KCl, 5 mM-MgCl₂, 5 mM-EGTA, 0.008 % Triton X-100 and 10 mM-Tris-maleate buffer (pH 7-0). After extraction for 10-12 min at 25°C, the cells were washed by suspension in 20 mM-KCl, 5 mM-MgCl₂, 3 mM-EGTA and 10 mM-Tris-maleate buffer (pH 7-0) for 15 min at 0°C. The cells were further washed with a solution of 20 mM-KCl, 5 mM-MgCl₂ and 10 mM-Tris-maleate buffer (pH 7-0). The model prepared by this procedure is termed type II.

The composition of the medium for reactivation of the models was usually 10 mM-KCl, 8 mM-MgCl₂, 20 mM-Tris-maleate buffer (pH 7-0), 4 mM-ATP and a Ca²⁺-EGTA buffer, which was composed of 1 mM-EGTA and various concentrations of CaCl₂ for control of free Ca below 3·3 × 10⁻⁷ M, or composed of 0·3 mM-CaCl₂ and various concentrations of EGTA for control of free Ca above 5 × 10⁻⁷ M. Apparent association constants of EGTA with Ca²⁺ and Mg²⁺ were assumed to be 1·3 × 10⁻⁴ M⁻¹ and 40 M⁻¹, respectively (Portzehl, Coldwell & Ruegg, 1964). The constants of ATP with Ca²⁺ and Mg²⁺ were assumed to be 5 × 10³ M⁻¹ and 11 × 10⁴ M⁻¹, respectively (Nanninga, 1961).

In the reactivation experiment, a drop of a concentrated suspension of each of the models was added to about 1 ml of the reactivation medium and the mixture was stirred gently for a few seconds. At 3-20 min after mixing, measurements of the swimming velocity and the beat frequency of cilia were made at room temperature (20-22°C).

Swimming velocities of reactivated models and native cells were obtained by averaging the end-to-end distances of 20-30 tracks in photographs. The beat frequency of cilia was measured by projecting the image of a model or cell fixed to a glass plate onto a screen in front of a photomultiplier tube in such a way that the image of the oral groove fell on a pinhole in the screen. The beating frequency was then determined electronically from the modulation frequency of the photomultiplier output current (Naitoh & Kaneko, 1973).

For micro-injection, live cells were suspended in a medium containing 8 mM-KCl, 0.25 mM-CaCl₂ and 1 mM-Tris-HCl buffer (pH 7.2). Glass microelectrodes with a diameter of about 1 μm were back-filled with solutions containing 20 mM-EGTA, 50 mM-piperazine-N,N'-bis-diethanesulphonic acid (Pipes) buffer (pH 7.0) and 0, 4 or 8 mM-CaCl₂. After insertion into live cells, volumes of solution corresponding to about 1/20 of the cell’s volume of 5·6 × 10⁻⁷ ml (Fortner, 1925) were injected by pressure.

**RESULTS**

*Relation between the beat frequency and the ATP concentration*

After extraction with Triton X-100, the model was reactivated by transfer to solutions containing various concentrations of ATP. The beat frequency of cilia increased with increasing concentration of ATP, as shown in Fig. 1. In the type I model, the frequency increased and attained a maximum level at about 2 mM of ATP. Further increase in the concentration of ATP had no effect on the frequency, at either 10⁻⁸ M or 1·9 × 10⁻⁷ M of Ca²⁺. In the type II model, at 10⁻⁸ M-Ca²⁺ the frequency was identical to that of the type I model over the whole range of ATP concentrations.
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Fig. 1. Beat frequency of cilia of type I model (■) and type II model (○, ●, △) as a function of ATP concentration. Concentrations of KCl (10 mM), MgCl₂ (8 mM) and Tris-maleate, pH 7-0 (20 mM) were kept constant throughout. Ca²⁺ concentration was either 10⁻⁸ M (○), 1-9 × 10⁻⁷ M (●, ■) or 1-2 × 10⁻⁶ M (△). Bars represent standard deviations.

At 1-9 × 10⁻⁷ M and 1-2 × 10⁻⁶ M-Ca²⁺, however, the frequency of the type II model continued to increase above 2 mM-ATP, so that its frequency became much higher than that of the type I model at high concentrations of ATP.

Changes in swimming velocity and beat frequency with concentration of Ca²⁺

The swimming velocity and beat frequency of cilia of the Triton-extracted models were measured at various concentrations of Ca²⁺ in the presence of 4 mM-ATP. As shown in Fig. 2, the swimming velocity of the type I model did not change with increases in the concentration of Ca²⁺ until reversal of the swimming direction began to occur at around 7 × 10⁻⁷ M-Ca²⁺. On the other hand, the swimming velocity of the type II model began to increase at around 5 × 10⁻⁸ M-Ca²⁺ and attained a maximum value around 2 × 10⁻⁷ M-Ca²⁺. With further increase in the Ca²⁺ concentration, the velocity decreased and reversal of the swimming direction occurred similarly to that in the type I model. The velocity of backward swimming at 10⁻⁶ M-Ca²⁺ was greater in the type II than in the type I model.

The difference between the beat frequency of type I and type II models is shown
in Fig. 3. In the type I model, the beat frequency did not depend on the concentration of Ca\(^{2+}\). In the type II model, the frequency increased with increasing concentrations of Ca\(^{2+}\) above 5 \times 10^{-8} \text{M}, attaining a maximum at around 2 \times 10^{-7} \text{M}. Then it

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Fig. 2. Swimming velocity of type I model (□) and type II model (○) as a function of Ca\(^{2+}\) concentration in the reactivating medium. Composition of the reactivating media were as described in Materials and Methods. Bars represent standard deviations.

Fig. 3. Beat frequency of cilia of type I model (□) and type II model (○) as a function of Ca\(^{2+}\) concentration. Composition of the reactivating media were as described in Materials and Methods. Bars represent standard deviations.
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decreased to the value of the type I model at around $6 \times 10^{-7}$ M. With further increases in the concentration of Ca$^{2+}$, the direction of the power stroke was reversed and the frequency increased again, attaining a second maximum at $2 \times 10^{-6}$ M-Ca$^{2+}$.

Thus, the type II model has Ca$^{2+}$ sensitivity, which results in increase in the beat frequency of cilia at around $2 \times 10^{-7}$ M and above $10^{-6}$ M-Ca$^{2+}$.

Effect of calmodulin antagonists on the beat frequency

It has recently been reported that Paramecium cells have calmodulin localized along the entire length of their cilia and basal bodies (Maihle et al. 1981). Therefore, the effects of calmodulin antagonists such as trifluoperadin (TFP) and 5-chloro-1-naphthalenesulphone amide (W-7) on the beat frequency of the models were examined. Both TFP and W-7 reduced the frequency of the type II model to the value of the type I model over the whole range of Ca$^{2+}$ concentration. As shown in Fig. 4, at $1.9 \times 10^{-7}$ M-Ca$^{2+}$, the concentrations of TFP and W-7 necessary for half-inhibition of the increase in beat frequency were $6 \times 10^{-6}$ M and $4.5 \times 10^{-5}$ M, respectively. At $1.2 \times 10^{-6}$ M-Ca$^{2+}$ where the beating direction was reversed, the concentration for half-inhibition was less. These antagonists had no effect on the beat

![Fig. 4. Effects of TFP (○, ●) and W-7 (△, ▲) on the beat frequency of type II model. Concentrations of ATP (4 mM), KCl (10 mM), MgCl$_2$ (8 mM) and Tris-maleate, pH 7.0 (20 mM) were kept constant. Ca$^{2+}$ concentration was either $1.9 \times 10^{-7}$ M (○, △) or $1.2 \times 10^{-6}$ M (●, ▲). Bars represent standard deviations.](image)
frequency of the type I model. Ciliary reversal induced with $1 \times 10^{-6}$ M-Ca$^{2+}$ was not inhibited by these antagonists in either type of model.

**Intracellular injection of Ca$^{2+}$ into live cells**

In order to confirm the effect of Ca$^{2+}$ concentration on the beat frequency of cilia, Ca$^{2+}$ buffers were injected into live cells. Upon injection of a buffer giving $2 \times 10^{-7}$ M free Ca$^{2+}$, the beat frequency increased to about twice that before injection, as shown in Table 1. A few minutes after injection, the frequency decreased to the original value. A similar increase in the frequency was observed with a buffer giving $5 \times 10^{-7}$ M-Ca$^{2+}$. However, at $8 \times 10^{-7}$ M-Ca$^{2+}$, the reversal of beating was induced but the increase in frequency was not produced.

**Contractions of live and extracted cells**

In the live *Paramecium* cell, contraction and shortening of the cell shape were observed when the swimming velocity in the forward direction was increased. For example, when the swimming velocity had a resting value of 0.9 mm/s in a solution containing 8 mM-KCl and 0.25 mM-CaCl$_2$, the mean cell length was $230 \pm 22 \mu$m (mean ± s.d.; number, $n=110$). When the swimming velocity was increased to 2.4 mm/s in a solution containing 0.5 mM-KCl and 0.25 mM-CaCl$_2$, the mean cell length was reduced to $190 \pm 19 \mu$m ($n=130$).

In the extracted type II model, shortening and contraction of the cell, especially in the posterior region, was induced by increasing Ca$^{2+}$ in the reactivating medium. The length of the extracted cells was measured as a function of Ca$^{2+}$ in the reactivating medium. As shown in Fig. 5, contraction was induced when the concentration of Ca$^{2+}$ exceeded $10^{-7}$ M, which was almost the same as the concentration needed to increase the beat frequency. The type I model, however, showed no contraction in these concentrations of Ca$^{2+}$.

**Discussion**

In the type II model, which is extracted in the presence of Mg$^{2+}$, the beat frequency of cilia increases when the Ca$^{2+}$ concentration in the reactivating medium exceeds...
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Fig. 5. Effect of Ca$^{2+}$ concentration on the cell length of type I model (□) and type II model (○). Composition of the reactivating media were as described in Materials and Methods. Each point represents the mean obtained from measurements of 20–25 cells. Bars represent standard deviations.

10$^{-7}$ M. At the same time, the shape of the model is changed to a contracted one which is similar to that of the live cell accelerating the forward swimming velocity. These results suggest that the intracellular concentration of Ca$^{2+}$ is kept below 10$^{-7}$ M in the resting state, in which the swimming velocity and beat frequency are low and cell shape is normal. For an increase in beat frequency, the intracellular concentration of Ca$^{2+}$ must rise above 10$^{-7}$ M. Conditions that produce an increase in beat frequency in living cells are those that promote Ca$^{2+}$ influx. For example, a current stimulus that hyperpolarizes the membrane must increase the motive force for cation influx, including Ca$^{2+}$. Increase in Ca$^{2+}$ or decrease in K$^+$ concentration in the external medium, which induces an increase in swimming velocity, increases the motive force for Ca$^{2+}$ influx (Nakaoka et al. 1983).

At 6 × 10$^{-7}$ M-Ca$^{2+}$, which is slightly lower than that inducing ciliary reversal, the beat frequency of the type II model decreases to a minimum value. Machemer (1974) reported that a decrease in normal beat frequency resembling inactivation was induced with a small depolarization of the membrane and that the cilia were inactivated in the course of transition from reversed to forward beating. Such a decrease in beat frequency in the type II model may correspond to ciliary inactivation in the living cell.

Above 10$^{-6}$ M-Ca$^{2+}$, both the frequency increase and ciliary reversal are induced in the type II model. In the living cell, depolarization of the membrane inducing ciliary reversal always accompanies the frequency increase and an intracellular injection of EGTA inhibits both the ciliary reversal and the frequency increase (Brehm & Eckert, 1978). The present results with the type II model resemble the Ca$^{2+}$ sensitivity in the beat frequency of living cells.

On the other hand, the type I model showed no Ca$^{2+}$ sensitivity in the beat
frequency. This difference between type I and II models stems from the conditions of the detergent extraction. Some factors that contribute to the Ca\(^{2+}\) sensitivity of beat frequency have been broken down or extracted in the type I model. Calmodulin is probably included in these factors, because Ca\(^{2+}\) sensitivity of ciliary frequency is lost with calmodulin antagonists. In order to confirm further the regulatory role of calmodulin, it would be desirable to reconstitute the Ca\(^{2+}\) sensitivity of the beat frequency of the type I model. However, attempts at such reconstitution have not yet been successful.

The type II model shows contraction of the cell body, particularly of the posterior end, upon increasing the concentration of Ca\(^{2+}\) above 10\(^{-7}\)M. In this model, the increase in the beat frequency of cilia and the contraction occurred almost in parallel. On the other hand, the type I model did not show contraction over the whole range of concentrations of Ca\(^{2+}\). The increase in the beat frequency of cilia and the contraction of the cell body therefore seemed to be regulated by a common mechanism. However, the increase in the frequency and the contraction have not been found to be directly coupled with each other. Calmodulin antagonists, which inhibited the increase in beat frequency by Ca\(^{2+}\) in the type II model, had no effect on the contraction of the cell body. The beat frequency of the type II model had a minimum value at around 6 \times 10^{-7}\text{M}\cdot\text{Ca}^{2+}, at which the contraction normally occurred (circles in Figs 3, 5).

In order to clarify the mechanism by which swimming velocity is regulated by the concentration of Ca\(^{2+}\), it would be valuable to compare the structure and properties of the two types of model. It is also important to confirm that Ca\(^{2+}\) actually regulates the swimming velocity of intact cells, by direct measurement of the Ca\(^{2+}\) concentration in the cell.

REFERENCES


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