

AN ALL-OR-NOTHING RISE IN CYTOSOLIC $[Ca^{2+}]$ IN *VORTICELLA* SP.

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Summary

The peritrich ciliate *Vorticella* sp. exhibits cellular contraction of an all-or-nothing type in response to a mechanical stimulus. Many authors have suggested that the contraction may be controlled by the cytosolic level of Ca^{2+} , since glycerol-extracted *Vorticella* contracts when Ca^{2+} is added to the external solution. However, no direct evidence for the increase in cytosolic $[Ca^{2+}]$ has yet been obtained in living *Vorticella*.

In the present study, by injecting a fluorescent Ca^{2+} indicator into living *Vorticella* and monitoring the cytosolic $[Ca^{2+}]$ with a confocal microscope, we have demonstrated

that a mechanical stimulus evoked an all-or-nothing rise in cytosolic $[Ca^{2+}]$ (Ca^{2+} 'spike'). The onset of the Ca^{2+} spike was similar in its time course to that of cellular contraction. Since the Ca^{2+} spike was recorded in a Ca^{2+} -deprived solution containing 1 mmol l^{-1} EGTA, we concluded that release of Ca^{2+} from intracellular Ca^{2+} storage site(s) is responsible for the Ca^{2+} spike.

Key words: *Vorticella* sp., contraction, Ca^{2+} transient, Ca^{2+} concentration.

Introduction

The peritrich ciliate *Vorticella* sp. exhibits contraction of an all-or-nothing type involving shrinkage of the cell body and coiling of the stalk in response to mechanical stimulation (Sugi, 1960). As early as 1958, Hoffmann-Berling found that the stalk of glycerol-extracted *Vorticella* contracted when Ca^{2+} was added to the external solution and relaxed when Ca^{2+} was removed (see also Amos, 1971). Many authors later examined the Ca^{2+} -dependent contraction in glycerol-extracted *Vorticella* in detail and reported that the degree of contraction depended on the Ca^{2+} concentration ($[Ca^{2+}]$) in the reactivation medium (Townes and Brown, 1965; Weis-Fogh and Amos, 1972; Routledge *et al.* 1975; Ochiai *et al.* 1979). A Ca^{2+} -binding protein, spasmin, was extracted from contractile elements of *Zoothamnium geniculatum*, a close relative of *Vorticella* (Amos *et al.* 1975; Ochiai *et al.* 1988). These findings suggest that an increase in cytosolic $[Ca^{2+}]$ may activate the contractile system of *Vorticella* (Amos *et al.* 1976).

Several authors assumed that the Ca^{2+} required to activate the contractile system is released from intracellular Ca^{2+} storage site(s) in *Vorticella*, for living *Vorticella* can contract even in Ca^{2+} -deprived media containing EGTA (Allen, 1973a; Katoh and Naitoh, 1992, 1994). Electron microscopic studies of vorticellid ciliates showed that membranous tubules (tubular endoplasmic reticulum) are present in the spasmoneme, which is responsible for coiling of the stalk, and in the myoneme,

which is responsible for shrinkage of the cell body. These studies also showed that endoplasmic reticulum is present around the myoneme (Carasso and Favard, 1966; Amos, 1972; Allen, 1973a,b). Moreover, Carasso and Favard (1966) demonstrated the presence of Ca^{2+} in the tubules and endoplasmic reticulum using cytochemical methods. These membranous systems are candidates for the Ca^{2+} storage sites.

Recently, cytosolic $[Ca^{2+}]$ has been monitored in various cells using AM esters of fluorescent Ca^{2+} indicators, which permeate the cell membrane of higher animals. However, in protozoan cells, it is difficult to measure cytosolic Ca^{2+} levels, because their cell membrane is less permeable to the AM ester. In some protozoans other than *Vorticella*, researchers have detected an increase in cytosolic $[Ca^{2+}]$; for example, in *Spirostomum ambiguum* (Ettienne, 1970) and in *Didinium nasutum* (Pernberg and Machemer, 1995). However, no one has observed a rise in cytosolic $[Ca^{2+}]$ in living *Vorticella*, in which it has been well established, in glycerol-extracted cell models, that the contractile system is activated by Ca^{2+} .

The primary objective of the research described in this paper was to detect a rise in the cytosolic $[Ca^{2+}]$ of living *Vorticella*. Under a confocal microscope, we observed the cytosolic $[Ca^{2+}]$ of living *Vorticella* loaded with a fluorescent Ca^{2+} indicator and demonstrated an all-or-nothing rise in cytosolic $[Ca^{2+}]$ (Ca^{2+} spike). Some of these results have been presented verbally elsewhere (Katoh, 1995; Katoh and Kikuyama, 1996).

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Materials and methods

Specimens of *Vorticella* sp. were grown at 20 °C on a glass slide in a bacterized saline solution (final concentrations 0.1 mmol l⁻¹ KCl, 0.09 mmol l⁻¹ CaCl₂ and 0.1 mmol l⁻¹ MgSO₄) containing 0.1% (w/w) dehydrated cereal leaves (Sigma). Specimens were washed with a standard saline solution containing 1 mmol l⁻¹ KCl, 1 mmol l⁻¹ CaCl₂ and 10 mmol l⁻¹ Tris-maleate buffer (pH 7.0) and then immersed in a Ca²⁺-deprived solution for approximately 10 min prior to each experiment. The Ca²⁺-deprived solution was a mixture of 1 mmol l⁻¹ KCl, 1 mmol l⁻¹ EGTA and 10 mmol l⁻¹ Tris-maleate buffer (pH 7.0).

In order to monitor the cytosolic [Ca²⁺] of *Vorticella*, a Calcium Green solution was injected into the cell body according to the method of Hiramoto (1974). The distal end of the stalk of a specimen was first detached from its substratum (a glass slide) and the cell body was fixed at the tip of a suction pipette (approximately 5 μm in inner diameter) by lowering the hydrostatic pressure inside the pipette. The tip of a microneedle (about 1 μm in inner diameter) for injection was then inserted into the cell body. The Calcium Green solution contained 1 μmol l⁻¹ Calcium Green-1 dextran (*M_r* 10 000, Molecular Probes, Inc., Eugene, USA), 150 mmol l⁻¹ KCl and 1 mmol l⁻¹ Pipes (pH 7.0, adjusted using KOH). The amount of solution injected into the cell body was 0.7–1 pl, which corresponded to 2–3% of the volume of the cell body. Thus, the final concentration of Calcium Green-1 dextran contained in the cytoplasm was approximately 20–30 nmol l⁻¹.

A mechanical stimulus was applied to the cell body with a microneedle. The microneedle was driven perpendicularly by a piezoelectric transducer according to the method of Katoh and Naitoh (1992) (see also Naitoh and Eckert, 1969). The amplitude of the motion was represented as the amplitude of a rectangular voltage pulse (2–10 V) applied to the piezoelectric transducer attached to the microneedle (Fig. 1). The tip of the microneedle moved approximately 0.15 μm in response to a

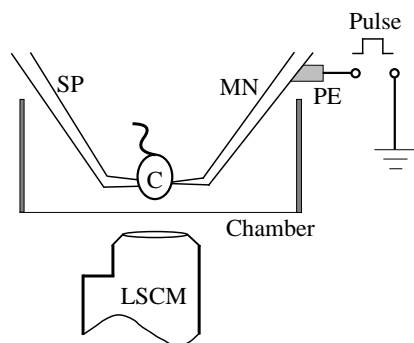


Fig. 1. Schematic view of the experimental arrangement. A cell of *Vorticella* sp. (C) detached from the substratum was fixed with a suction pipette (SP) in an experimental chamber. A mechanical stimulus was applied to the cell body by a microneedle (MN) driven by a piezoelectric transducer (PE) according to the method of Katoh and Naitoh (1992). LSCM, laser scanning confocal microscope.

rectangular electrical pulse of 5 V. The duration of the electrical pulse was constant (15 ms).

An inverted microscope (TMD, Nikon, Tokyo) equipped with a laser confocal scanner unit (Insight Plus-IQ, Meridian Instruments Far East, Inc., Tokyo) was used with excitation at a wavelength of 488 nm (Fig. 1). The magnified image of the specimen was recorded with a video recorder (BR-S811, Victor, Tokyo) through a CCD camera (HCC-600M, Flouel, Tokyo). The recorded images were transferred to a computer (Apple Macintosh 7100) and analyzed. Relative cytosolic [Ca²⁺] was expressed as F/F_0 , where F is the intensity of fluorescence and F_0 is the mean value of F of the cell body immediately before stimulation. All experiments were performed at room temperature (20–23 °C).

Results

Transient rise in cytosolic [Ca²⁺]

Before the experiments, the fluorescent Ca²⁺ indicator (Calcium Green-1 dextran) was injected into a specimen bathed in a Ca²⁺-deprived solution. Although the injection of the Ca²⁺ indicator often caused a contraction of *Vorticella*, the specimen soon relaxed and resumed ciliary beating within 1 min. All experiments were carried out on specimens in this state.

We observed relative cytosolic [Ca²⁺] (F/F_0) in a cell body of *Vorticella* before, during and after applying a mechanical stimulus to the cell body. The mechanical stimulus evoked a transient rise in cytosolic [Ca²⁺]. Fig. 2 shows representative images of this transient rise. The cytosolic [Ca²⁺] increased rapidly and then slowly returned to the resting level.

To determine the precise time course of the Ca²⁺ transient, [Ca²⁺] in the cell body was plotted against time (Fig. 3). When a mechanical stimulus was applied to the cell body, cytosolic [Ca²⁺] increased rapidly and then gradually returned to the initial level. The latency between the stimulus and the onset of the Ca²⁺ transient was 43 ± 10 ms (mean ± S.E.M., $N=12$) and the rise time of the Ca²⁺ transient was 92 ± 10 ms (mean ± S.E.M., $N=12$).

Relationship between the Ca²⁺ spike and contraction of the cell body

To determine the temporal relationship between the Ca²⁺ transient and contraction of the cell body, we compared the onset of the Ca²⁺ transient with that of contraction in the same specimen injected with Calcium Green. First, under a light microscope, we recorded the contraction evoked by a mechanical stimulus. We then switched the optical pathway to a laser scanning confocal microscope and recorded the Ca²⁺ transient evoked by mechanical stimulus in the same cell. Since the cell body of *Vorticella* was fixed with a suction pipette and a microneedle, the degree of contraction was smaller than that in normal specimens attached to a substratum by the stalk (see Fig. 4B). We compared the time course of the small contraction with that of the Ca²⁺ transient.

Fig. 4 shows representative examples of contraction and of

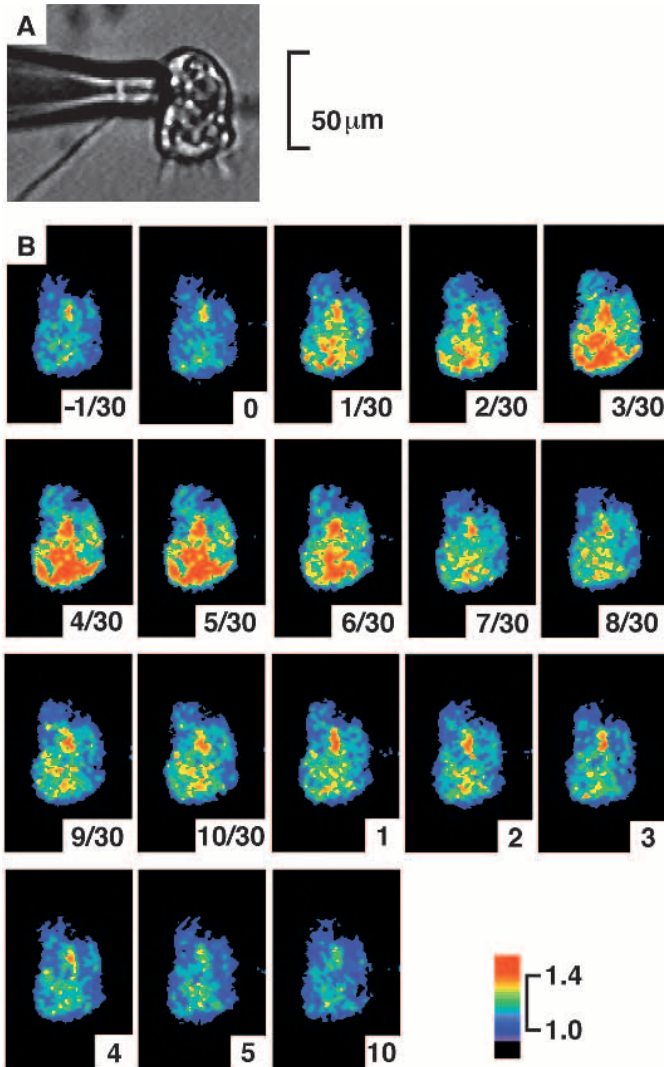


Fig. 2. Cytosolic $[Ca^{2+}]$ before, during and after mechanical stimulation. (A) A cell of *Vorticella* sp. used for the measurements. (B) Cytosolic $[Ca^{2+}]$ expressed as pseudo-colour images. Cytosolic $[Ca^{2+}]$ is expressed as a relative value, F/F_0 , where F is the intensity of fluorescence of the Ca^{2+} indicator and F_0 is the mean value of F immediately before stimulation. Each number in each image shows the time (in s) after application of the mechanical stimulus to the cell body. The image with the number $-1/30$ is just before stimulation. At time 0, a mechanical stimulus was applied to the cell body with a microneedle. The microneedle was moved perpendicularly (less than $0.15\mu m$) against the cell surface by a piezoelectric transducer driven by an electrical pulse (amplitude 5 V; duration 15 ms). The displacement of the microneedle was so small that no movement of the microneedle was detected in the video images. A result typical of 12 specimens.

the Ca^{2+} transient in Calcium-Green-injected *Vorticella*. Both were evoked by mechanical stimuli with the same intensity (3 V). Contraction occurred one frame after the stimulation in Fig. 4B, and the Ca^{2+} transient also occurred one frame after the stimulation in Fig. 4A. The onset of contraction was similar in its time course to that of the Ca^{2+} transient. Moreover, the threshold intensity of the mechanical stimulus required to

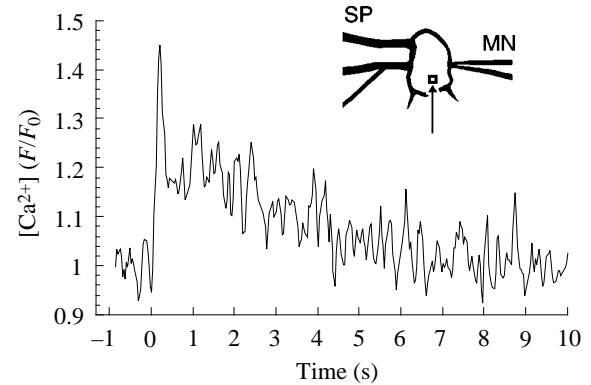


Fig. 3. Time course of the transient rise in cytosolic $[Ca^{2+}]$. At time 0, a mechanical stimulus was applied to a *Vorticella* cell. Cytosolic $[Ca^{2+}]$ is defined in the same way as in Fig. 2. A result typical of 12 specimens. Inset, the position within the cell of the area in which cytosolic $[Ca^{2+}]$ was measured (shown with an arrow). SP, suction pipette; MN, microneedle. Stimulus intensity, 5 V.

evoke contraction (2.9 ± 0.2 V, $N=5$) was similar to that required to evoke the Ca^{2+} transient (3.2 ± 0.2 V, $N=5$).

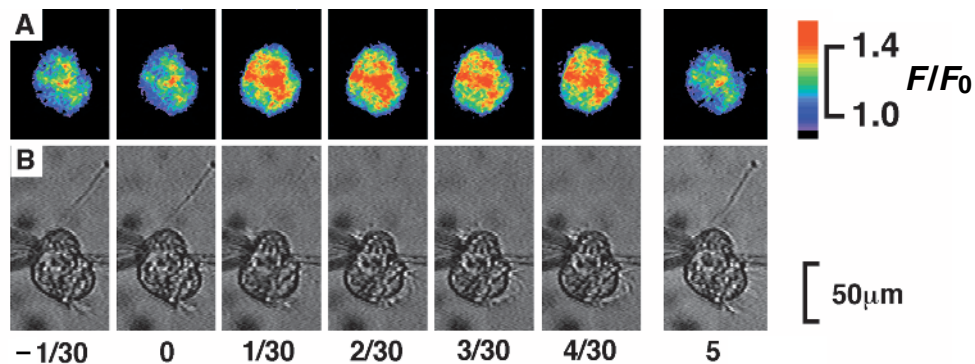
Relationship between stimulus intensity and cytosolic $[Ca^{2+}]$

To examine the relationship between the intensities of mechanical stimuli and cytosolic $[Ca^{2+}]$, we applied mechanical stimuli with different intensities to a *Vorticella* cell and measured cytosolic $[Ca^{2+}]$. Fig. 5 shows representative examples of responses of cytosolic $[Ca^{2+}]$ to mechanical stimuli of different intensities. The Ca^{2+} images shown in Fig. 5 were recorded from the same specimen. No rise in cytosolic $[Ca^{2+}]$ was evoked when the intensity of the mechanical stimulus was lower than 2 V (Fig. 5B). When the intensity was 3 V or more, a transient rise in cytosolic $[Ca^{2+}]$ was evoked (Fig. 5C,D). The spatial pattern of the rise in cytosolic $[Ca^{2+}]$ was similar irrespective of the intensity of the mechanical stimulus provided that the intensity was over the threshold level (3 V, in Fig. 5) for evoking the Ca^{2+} transient (compare Fig. 5C with Fig. 5D).

To determine the effect of the intensity of the mechanical stimulus on the time course of the Ca^{2+} transients, cytosolic $[Ca^{2+}]$ was plotted against time (Fig. 6). A rise in cytosolic $[Ca^{2+}]$ was observed when the intensity of the mechanical stimulus was 3 V or more, as was expected from the Ca^{2+} images in Fig. 5. The form of the Ca^{2+} transient, a fast rising phase followed by a slow falling phase, was consistent irrespective of the intensity of the mechanical stimulus (compare Fig. 6B with Fig. 6C).

In the next series of experiments, the relationship between the peak level of the Ca^{2+} transient and the intensity of the mechanical stimulus was examined more precisely (Fig. 7). The peak level was constant irrespective of the intensity of the mechanical stimulus provided that the intensity was above a certain threshold level (3 V). This clearly shows that the Ca^{2+} transient is of an all-or-nothing type.

Fig. 4. Comparison between the time of onset of the Ca^{2+} transient and of contraction in the same specimen. Times after applying the mechanical stimulus are labelled (in s) under each image. At time 0, a mechanical stimulus was applied to a *Vorticella* cell. Cytosolic $[\text{Ca}^{2+}]$ is defined in the same way as in Fig. 2. (A) Cytosolic $[\text{Ca}^{2+}]$; (B) cellular contraction. A result typical of five specimens.



Discussion

Many authors have reported that glycerol-extracted *Vorticella* contracts when it is bathed in a solution containing Ca^{2+} (Hoffmann-Berling, 1958; Townes and Brown, 1965; Weis-Fogh and Amos, 1972; Ochiai *et al.* 1979). They have assumed that an increase in cytosolic $[\text{Ca}^{2+}]$ is required to activate the contractile system of living *Vorticella*. However, no one has yet succeeded in recording an actual rise in cytosolic $[\text{Ca}^{2+}]$ in living *Vorticella*, probably because of the difficulty of loading a Ca^{2+} indicator into a protozoan cell. In the present experiments, we succeeded in injecting a Ca^{2+} indicator (Calcium Green-1 dextran) directly into living *Vorticella* and observing a transient rise in cytosolic $[\text{Ca}^{2+}]$ in response to mechanical stimulation. The rise in cytosolic $[\text{Ca}^{2+}]$ was expressed as a series of Ca^{2+} images (see Fig. 2). A plot of cytosolic $[\text{Ca}^{2+}]$ against time clearly shows the time course of the Ca^{2+} transient in living *Vorticella* (Fig. 3). The Ca^{2+} transient will be called a Ca^{2+} spike hereafter.

The relationship between the intensity of the mechanical

stimulus and the peak value of the Ca^{2+} spike reveals that the Ca^{2+} spike of *Vorticella* is of an all-or-nothing type (Figs 5, 6, 7). This is the first report of an all-or-nothing rise in cytosolic $[\text{Ca}^{2+}]$ (Ca^{2+} spike) in contractile protozoa. Using a giant contractile ciliate *Spirostomum ambiguum*, Etienne (1970) observed a small increase in light emission from a Ca^{2+} -sensitive photoprotein, aequorin, which had been injected into the cell. However, it is not known whether the Ca^{2+} transient of *Spirostomum* is all-or-nothing in type. Moreover, the time course of the Ca^{2+} transient of *Spirostomum* was very different from that of *Vorticella*. The Ca^{2+} transient of *Spirostomum* never resembles a Ca^{2+} spike.

To examine the causal relationship between the Ca^{2+} spike and contraction of the cell body in *Vorticella*, we compared the time course of the onset of the Ca^{2+} spike with that of the contraction in the same specimen. As shown in Fig. 4, the time course of the onset of the Ca^{2+} spike was similar to that of contraction. Moreover, the threshold intensity of the mechanical stimulus required to evoke a Ca^{2+} spike was similar

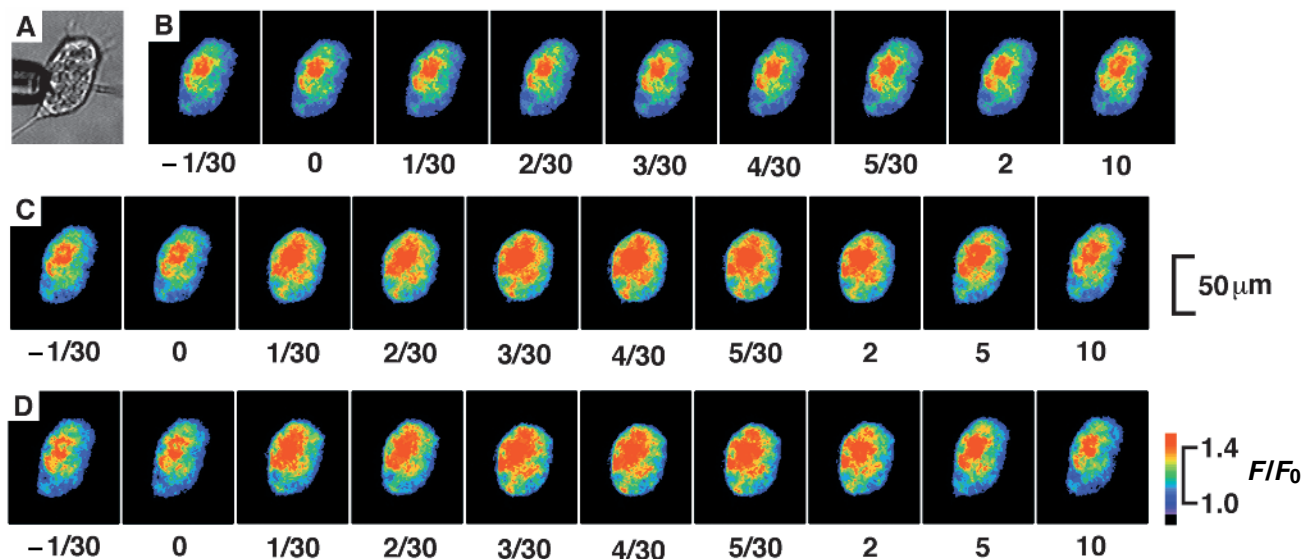


Fig. 5. Cytosolic $[\text{Ca}^{2+}]$ after mechanical stimuli of different intensities. All the data were recorded from the same specimen. (A) The *Vorticella* cell used for the measurement; (B–D) Ca^{2+} images after mechanical stimuli of different intensities. The intensity was 2 V in B, 3 V in C and 10 V in D. Times after applying the mechanical stimuli are labelled (in s) under each image. At time 0, mechanical stimuli were applied to a *Vorticella* cell. Cytosolic $[\text{Ca}^{2+}]$ is defined in the same way as in Fig. 2. A result typical of five specimens.

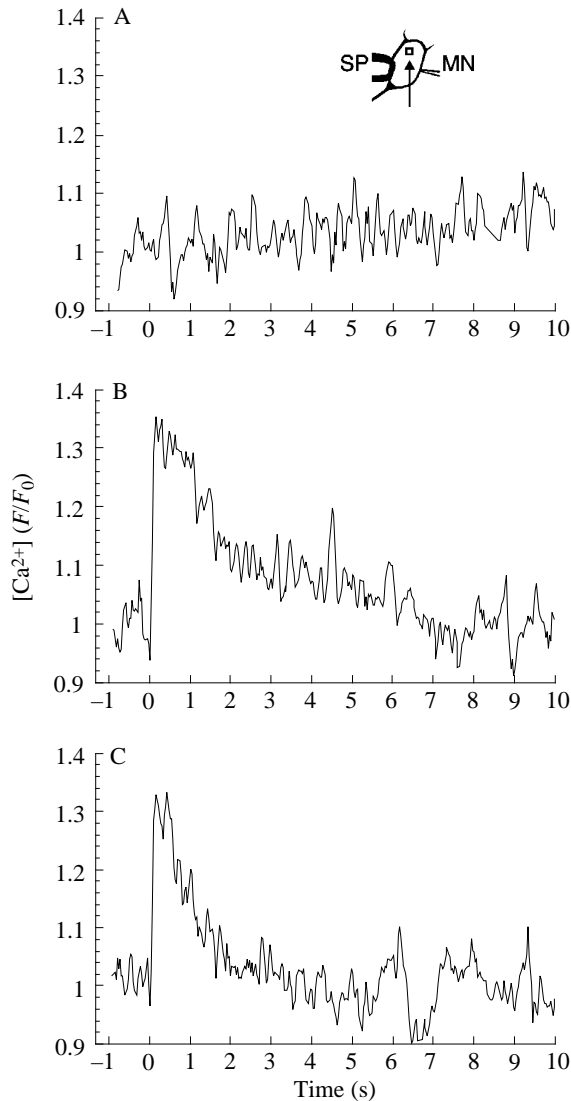


Fig. 6. Time course of Ca²⁺ transients evoked by mechanical stimuli of different intensities. At time 0, mechanical stimuli were applied to a *Vorticella* cell. These data were recorded from the same specimen. The intensities of mechanical stimuli were 2 V in A, 3 V in B and 10 V in C. Cytosolic [Ca²⁺] is defined in the same way as in Fig. 2. A result typical of five specimens. Inset, the position of the area of the cell in which cytosolic [Ca²⁺] was measured (shown with an arrow). SP, suction pipette; MN, microneedle.

to that required to evoke cellular contraction. These results support the idea that the Ca²⁺ ‘spike’ is responsible for the contraction of *Vorticella*.

Baylor and Hollingworth (1988) reported that the time courses of Ca²⁺ transients were prolonged by the fluorescent Ca²⁺ indicator loaded into the cell because the fluorescent Ca²⁺ indicator acts, more or less, like a Ca²⁺ buffer. This may be the reason why the time courses of both the Ca²⁺ spike and the contraction in the Calcium-Green-injected *Vorticella* were more prolonged than in normal *Vorticella*.

It is well known that contraction of *Vorticella* occurs even in the absence of extracellular Ca²⁺ (Allen 1973a,b; Katoh

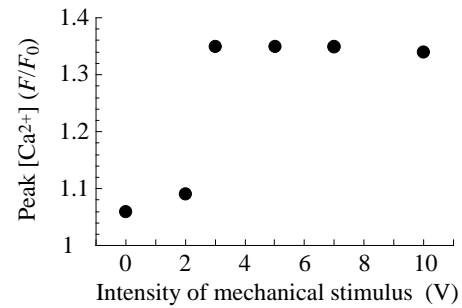


Fig. 7. The relationship between the intensity of the mechanical stimulus and the peak value of the Ca²⁺ spike. All these data were recorded from one specimen. An intensity of zero is the resting state in the absence of mechanical stimulation. Cytosolic [Ca²⁺] is defined in the same way as in Fig. 2. A result typically characterizes the responses of five specimens.

and Naitoh, 1992, 1994). In the present experiment, the Ca²⁺ spike was recorded in the absence of extracellular Ca²⁺. These results show that Ca²⁺ required to evoke the contraction and the Ca²⁺ spike is released from some intracellular Ca²⁺ storage site(s). Katoh and Naitoh (1992, 1994) suggested that regenerative liberation of Ca²⁺ from intracellular storage sites is involved in the increase in cytosolic [Ca²⁺] that evokes all-or-nothing-type contraction. The regenerative liberation of Ca²⁺ may be responsible for the all-or-nothing-type Ca²⁺ spike in *Vorticella*. Moreton and Amos (1979) proposed that an influx of Ca²⁺ from an external solution into the cell, accompanied by a Ca²⁺ action potential, might be responsible for the rise in the cytosolic [Ca²⁺] in *Zoothamnium geniculatum*, a close relative of *Vorticella*. However, the mechanism mobilizing Ca²⁺ for evoking contraction in *Z. geniculatum* may be different from that in *Vorticella*.

Several authors have reported that endoplasmic reticulum and/or membranous tubules (tubular ER) are present near the myoneme, which is responsible for the contraction of the cell body (Carasso and Favard, 1966; Amos, 1972; Allen, 1973a,b). Ca²⁺ was detected in the endoplasmic reticulum and membranous tubules (tubular ER) (Carasso and Favard, 1966). Moreover, Katoh and Naitoh (1994) reported that drugs that affect the liberation of Ca²⁺ from the sarcoplasmic reticulum of skeletal muscle fibres (Endo, 1977) also induce or inhibit contraction in living *Vorticella*. Thus, these membranous systems are strong candidates for Ca²⁺ storage site(s).

In the present paper, we describe a Ca²⁺ spike of all-or-nothing type in living *Vorticella*. We show that release of Ca²⁺ from intracellular storage site(s) is responsible for generating the Ca²⁺ spike. *Vorticella* may utilize intracellular Ca²⁺ storage sites to control cytosolic [Ca²⁺] in a way similar to sarcoplasmic reticulum in muscle cells, although the contractile element of *Vorticella* is very different from that of muscle cells. Our findings are important for understanding the evolution of the Ca²⁺ mobilization mechanisms used to evoke cellular contraction in the animal kingdom.

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