Participation of Ca^{2+} in cessation of cytoplasmic streaming induced by membrane excitation in Characeae internodal cells

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CONTENTS

I. SAMMARY

The mechanism of the cessation of cytoplasmic streaming upon membrane excitation in Characeae internodal cells was investigated.

Cell fragments containing only cytoplasm were prepared by collecting the endoplasm at one cell end by centrifugation. In such cell fragments lacking the tonoplast, an action potential induced streaming cessation, indicating that an action potential at the plasmalemma alone is enough to stop the streaming.

The active rotation of chloroplasts passively flowing together with the endoplasm also stopped simultaneously with the streaming cessation upon excitation. The time lag or interval between the rotation cessation and the electrical stimulation for inducing the action potential increased with the distance of the chloroplasts from the cortex. The time lag was about 1 sec/15 μm, suggesting that an agent causing the rotation cessation is diffused throughout the endoplasm.

Using internodes whose tonoplast was removed by replacing the cell sap with EGTA-containing solution (tonoplast-free cells, Tazawa et al., 1976), we investigated the streaming rate with respect to the internal Ca^{2+} concentration. The rate was roughly identical to that of normal cells at a Ca^{2+} concentration of less than 10^{-7}M. It decreased with an increase in the internal Ca^{2+} concentration and was zero at 1 mM Ca^{2+}. The streaming once inhibited

completely by 1 mM Ca^{2+} was recovered partially by removing Ca^{2+} by EGTA immediately after the inhibition.

The effects of Ca^{2+} and other cations on chloroplast rotation in isolated cytoplasmic droplets of Chara were investigated by iontophoretically injecting them. Chloroplast rotation stopped immediately after Ca^{2+} injection and recovered with time, suggesting the existence of a Ca^{2+}-sequestering system in the cytoplasm. The Ca^{2+} concentration necessary for the stoppage was estimated to be 10^{-4}M. Sr^{2+} had the same effect as Ca^{2+}. Mn^{2+} and Cd^{2+} induced a gradual decrease in the rotation rate with low reversibility. K^{+} and Mg^{2+} had no effects. Ba^{2+} had effects sometimes similar to Ca^{2+} or Sr^{2+} and sometimes similar to Mn^{2+} or Cd^{2+}.

The above results, together with the fact that the cytoplasmic streaming in tonoplast-free cells does not stop upon excitation (Tazawa et al., 1976), lead us to conclude that a transient increase in the Ca^{2+} concentration in the cytoplasm directly stops the cytoplasmic streaming. Both Ca influxes across the resting and active membranes were roughly proportional to the external Ca^{2+} concentration, which did not affect the rate of streaming recovery. Based on these results, several possibilities for the increase in Ca^{2+} concentration in the cytoplasm causing streaming cessation were discussed.

II. INTRODUCTION

Rotational cytoplasmic streaming in Characeae cells results from the active shearing force generated at the interface between the flowing endoplasm and the stationary cortex (Kamiya and Kuroda, 1956; Kamiya, 1959). At the interface, fibrillar structures were found by light microscopy (Kamitsubo, 1966) and electron microscopy (Nagai and Rebhun, 1966). They are indispensable for streaming (Kamitsubo, 1972) and are composed of F-actin filaments (Palevitz et al., 1974; Williamson, 1974; Palevitz and Hepler, 1975; Kersey et al., 1976). Recently, data have accumulated which suggest that the factor existing in the endoplasm and responsible for the motive force generation with F-actin is myosin or myosin-like protein (Williamson, 1975; Chen and Kamiya, 1975; Kuroda and Kamiya, 1975; Tazawa et al., 1976; Kato and Tonomura, 1977; Shimmen, 1978; Nagai and Hayama). In spite of accumulation of data on the structural entities for the cytoplasmic streaming, information on the regulatory mechanism is scarce.

Cytoplasmic streaming in Characeae internodes stops temporarily at the moment of action potential generation (cf. Kamiya, 1959). The streaming cessation upon membrane excitation is not caused by an increase in the cytoplasmic viscosity but by the disappearance of the motive force (Tazawa and Kishimoto, 1968). The factor(s) leading to the loss of the motive force may play a key role in the regulatory mechanism of the motile system. Present work aimed at identification of the factor(s).

The present work deals with the following four subjects with respect to the streaming cessation upon membrane excitation.

(i) The action potential in Characeae internodes is generated not only at the plasmalemma but also at the tonoplast. (Findlay and Hope, 1964; Findlay, 1970; Kikuyama and Tazawa, 1976). Which action potential is responsible for the streaming cessation had not been known. The present work demonstrated that excitation of the plasmalemma alone is enough to halt the streaming.

(ii) The site of the motive force generation, the interface between the cortical gel and the flowing sol, is 4-6 μm from the plasmalemma. There should be an intermediary process which is triggered by the excitation of the plasmalemma and causes temporal inactivation of the motile system. We tried to determine whether the process involves a rapid electrical transmission or a slow chemical diffusion.

(iii) The fact that internal Ca^{2+} of more than 10^{-7}M inhibits the streaming (Williamson, 1975) suggests that Ca^{2+} is involved in the streaming cessation. The effect of Ca^{2+} on the motile system was investigated in detail using two experimental systems; (1) Using internodes whose tonoplast was removed by replacing the cell sap with an EGTA containing solution, the streaming rate was studied in respect to the internal Ca^{2+} concentrations, (2) The effects of Ca^{2+} and other cations on chloroplast rotation in isolated cytoplasmic droplets were studied by iontophoretically injecting them.

(iv) Since the above research demonstrated that Ca^{2+} reversibly inhibits the cytoplasmic streaming and chloroplast rotation, it may be concluded that the transient increase of the Ca^{2+} ions in the cytoplasm during excitation stops the streaming. If Ca^{2+} ions flowing in from the outside directly stop the streaming, significant increase in Ca influx upon excitation should occur. Thus ,we measured the ^{45}Ca influx across the resting and active membranes.

III. MATERIALS and METHODS

1. Cells

Internodal cells of Characeae, Chara australis, Chara corallina and Nitella axilliformis were used. Internodes isolated from adjacent cells were kept for more than one day in pond water or artificial pond water (APW) containing 0.1 mM each of KCl, NaCl and $CaCl_2$.

A cell fragment containing only cytoplasm was obtained as described by Kamiya and Kuroda (1956). Internodes of N. axilliformis were centrifuged at 110-130 x g for 10 min, then the centrifugal end where the endoplasm had accumulated was ligated with strips of polyester thread to obtain cell segments 0.5-1.5 mm long filled with endoplasm.

A cell fragment rich in endoplasm was prepared in the following manner. First, internodes of N. axilliformis were selected which were relatively rich in endoplasm and contained many rotating chloroplasts in the flowing endoplasm. An internode was partitioned into two by mounting it on a Plexiglass chamber having two pools (cf. Fig. 1). One pool was filled with APW and the other with cold APW (ca. 0°C). The cytoplasmic streaming in the cell part bathed in chilled APW slowed down causing accumulation of the flowing endoplasm in it. After about 15 minutes of treatment, the cell was divided into three fragments by ligation at two loci near the partition wall with polyester thread. By amputating the central fragment, we obtained two cell fragments, one rich in endoplasm and the other not.

2. Internal perfusion of cells

The method of preparing internodal cells lacking the tonoplast (tonoplast-free cells) by vacuolar perfusion was described elsewhere (Tazawa et al., 1976). Internodes of C. australis were internally perfused with an artificial 0Ca solution containing 5 mM EGTA (ethyleneglycol bis-β-aminoethyl ether N, N'-tetraacetic acid), 5 mM PIPES [Piperazine-N,N'-bis(2-ethanesulfonic acid)], 6 mM $MgCl_2$, 1 mM ATP, 290 mM sorbitol, 27 mM K^+ and 2 mM Na^+ (pH adjusted to 7). After internal perfusion, cells were ligated at both open ends with strips of polyester thread then bathed in APW. Loss of the tonoplast, which occurred within 10 minutes, was confirmed by microscopically observing the presence of endoplasm fragments in the original central vacuolar space. The internal Ca^{2+} concentration ($[Ca^{2+}]_i$) was estimated to be 1.3×10^{-8}M, under the assumption that total calcium in the protoplasm (3 mM, Tazawa et al., 1976) is free and therefore accessible to EGTA and also that the protoplasm volume is 1/10 the cell volume. The apparent association constant between EGTA and Ca^{2+} at pH 7 was taken as 4.83×10^6 M^{-1} (Jewell and Rüegg, 1966). To precisely control $[Ca^{2+}]_i$, tonoplast-free cells were perfused again with solutions of various Ca^{2+} concentrations. The solution with a $[Ca^{2+}]$ of 10^{-8}-10^{-5}M was prepared by adding $CaCl_2$ to the 0Ca solution. The solution with $[Ca^{2+}]$ above 10^{-4}M was prepared by adding $CaCl_2$ to the 0Ca solution from which EGTA had been removed.

3. Stopping time of cytoplasmic streaming and chloroplast rotation

Time interval between the electrical stimulation and cessations of the streaming and chloroplast rotation in the flowing endoplasm were measured in the following way. The endoplasm-enriched cell fragment was placed in a double chamber as showm in Fig. 1. Both pools were filled with APW. For optimum viewing conditions, the cytoplasmic streaming and chloroplast rotation were observed at loci where chloroplasts in the cortex were absent. At the moment the rotating chloroplast appeared in the observation area, electric stimulus was given to the cell through Ag-AgCl wire placed in the pools to induce an action potential. Immediately after the streaming cessation, we measured the distance between the stopped chloroplast and the outer surface of chloroplasts embedded in the cortex in the neibghoring area with the aid of the fine adjustment of the microscope. All the processes were recorded on video tape, National NV-71, with a video tape recorder (National Time Lapse VTR, NV-8030). A simultaneous flash with the electrical stimulus was given using a Nikon electronic flash device and recorded on the tape. The time intervals between the flash and the moments of the cessation of streaming and chloroplast rotation were determined with aid of slow motion reproduction.

4 Membrane potential

The membrane potential was measured by either the conventional microelectrode method or the external electrode method. The former was used to measure the membrane potential of the cell fragment containing only cytoplasm. Two glass microneedles were inserted into the cell fragment. The electric potential difference between one microelectrode and a reference electrode placed in the external medium (APW) was amplified and recorded on a pen-writing recorder. Using an electronic stimulator, MSE-3 (Nihon Kohden), electric current was applied to the cell between another microelectrode and a Ag-AgCl wire placed in the external medium to induce the action potential.

The external electrode method (cf. Shimmen et al., 1976; Hayama and Tazawa, 1978) was used when we investigated the relation between the external Ca^{2+} concentration ($[Ca^{2+}]_o$) and the recovery of the cytoplasmic streaming after its cessation induced by the action potential and also when we measured Ca influx. In the former case, an internode was partitioned into two as shown in Fig. 1. One pool was filled with 110 mM KCl solution and the other with APW which was made isotonic with the 110 mM KCl solution by adding sorbitol (isotonic APW). Cytoplasmic streaming was observed in the excitable cell part bathed in APW. The action potential was induced by applying electric current between the two chambers. The electric potential difference was measured between two electrodes placed in two different chambers and recorded with a pen-writing recorder.

5. Cytoplasmic droplets

The cytoplasmic droplets containing rotating chloroplasts were obtained in the following way. Internodes of C. australis or corallina containing many rotating chloroplasts in the following endoplasm were exposed to a centrifugal acceleration of 110-130 x g. Besides the accumulated endoplasm (Kamiya and Kuroda, 1956), many endoplasmic droplets were also observed at the centrifugal end, as shown in Fig. 2. The accumulated endoplasm moved back in the centripetal direction. After 10-20 min, the endoplasmic droplets dispersed rather homogeneously in the vacuole. They were then brought out onto a cover slip (Fig. 2) by means of internal perfusion (Tazawa, 1964; Tazawa et al., 1976). Thus, the bathing medium of the droplets became a mixture of the cell sap and the perfusion medium (mixing ratio, 1/5-1/20). The perfusion medium contained (i) 0.5 mM KCl, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 290 mM sorbitol, and 5 mM PIPES [Piperazine-N,N'-bis(2-ethanesulfonic acid)] (pH adjusted to 6.9 with NaOH) or (ii) 160 mM KCl, 5 mM $MgCl_2$ and 1 mM $CaCl_2$.

6. Ca^{2+} injection

Two glass microneedles were inserted into the droplets which contained 1-3 vigorously rotating chloroplasts and were 50-100 μm in diameter (Fig. 3). The microneedles were filled with 0.5 M salt solutions containing the monovalent or divalent cations which were to be injected iontophoretically and connected to Ag-AgCl electrodes through 3 M KCl solution. The salts used were KCl, $CaCl_2$,

$MgCl_2$, $SrCl_2$, $BaCl_2$, $MnCl_2$ and $CdCl_2$. Iontophoretical injection of cations was performed by applying an electric pulse between the two inserted electrodes using an electronic stimulator (Nihon Kohden, MSE-3). The duration of the pulse was fixed at 1 sec and its amplitude was recorded with a pen-writing recorder with the aid of an amplifier. The applied current was in the range of 1-100 nA. The concentration of the cation injected into the droplets was estimated from its volume and the amount of electric current based on the following assumptions. (i) The droplet is sphere. (ii) All electric current supplied from the microneedle to the endoplasm is carried by the cation in the solution, i.e., the transport number of the cation is 1. (iii) Cations injected diffuse rapidly in the droplet to become homogeneously distributed in a few seconds without binding with constituents in the droplet. (iv) The concentration of Ca^{2+} in the endoplasm, $[Ca^{2+}]_i$, is normally very low (less than 10^{-7} M, Williamson, 1975; Tazawa et al., 1976), therefore $[Ca^{2+}]_i$ just after the end of the injection can be estimated from the amount of Ca^{2+} injected. The propriety of the assumptions will be discussed later.

The chloroplast rotation was recorded on 16-mm film, Fuji RP, Kodak Plus X or Double X by cine camera (Arriflex) or on video tape, National NV-71, by video tape recorder (Shibaden SV-700).

7. Ca influx

The influx of radioactive tracer, ^{45}Ca, across the

resting and active membranes was measured. ^{45}Ca, 16.8 mCi/mgCa, was supplied by New England Nuclear in the form of $CaCl_2$ solution. Internodes of C. australis were partitioned into three as shown in Fig. 4. Pools A, B and C were 1.0 cm in diameter and the distance between them was 1.5 cm. Pools A and C were filled with 110 mM KCl solution and Pool B with isotonic APW containing $^{45}Ca^{2+}$. The electric potential difference between pools A and B was measured and recorded on the pen-writing recorder. The action potential was generated every minute by applying electric current through the Ag-AgCl wire placed in each pool. After 15 min, the cell part in pool B was washed 10 times with cold isotonic APW. The cell was removed from the chamber and water on its surface was blotted with a piece of filter paper. After confirming the disappearance of turgor pressure, one cell end was amputated and the entire contents were squeezed out with a lucite blade. The amount of Ca influx (J_{in}) was calculated with the following equation:

$$J_{in} = C_i/Q \cdot A \cdot t$$

where C_i is the total activity of the cell content (cpm), A is the surface area exposed to ^{45}Ca, Q is the specific activity of the external medium (cpm/mol Ca^{2+}) and t is the duration of exposure.

All experiments were carried out at room temperature (20-25°C).

IV. RESULTS

1. Streaming cessation induced by the action potential of the plasmalemma

In Characeae cells, both plasmalemma and tonoplast generate action potential (Findlay and Hope, 1964; Findlay, 1970; Kikuyama and Tazawa, 1976). The ratio of $[Ca^{2+}]$ to $[Mg^{2+}]$ in the external medium influences both the form of action potential and the streaming cessation (Barry, 1968). Although this fact suggests that the action potential of the plasmalemma is responsible for the streaming cessation, decisive evidence is lacking. To clarify which membrane is responsible for the streaming cessation the action potential must be induced in a single membrane. For this we prepared a cell segment lacking its vacuole and containing only cytoplasm.

Active rotational streaming was observed in small cell fragments. The streaming was slow compared with that of normal cells, partly due to the countermovement of viscous endoplasm of either side of the indifferent line (cf. Kamiya and Kuroda, 1973) and partly to the very short length of the cell segments (Hayashi, 1952). An action potential similar to that of normal cells could be induced in such segments. At the moment of excitation, the streaming stopped completely and recovered gradually as in the case of normal cells.

Fig. 5 shows a typical example. The rate of streaming was 58 μm/ sec at 24°C. Fig. 5b shows the streaming cessation upon spontaneous generation of the action potential which

is illustrated in Fig. 5a. The half time of the streaming recovery was about 2 min. This fact clearly shows that the action potential of the plasmalemma alone is enough for streaming cessation.

2. Time relationship between plasmalemma excitation and rotation cessation of chloroplasts in the flowing endoplasm

The previous section showed that excitation of the plasmalemma alone is enough to stop the streaming. The motive force of cytoplasmic streaming is generated at the boundary between the streaming endoplasm and the stationary cortical gel (Kamiya and Kuroda, 1956; Kamiya, 1959). The boundary is located 4-6 μm from the plasmalemma. We suppose that the signal for the streaming cessation is generated at the plasmalemma upon excitation and transmitted through the stationary cortex to the site of the motive force generation.

In the flowing endoplasm, we sometimes observed chloroplast rotating on their own axes. The active shearing force between the chloroplast surface and the surrounding endoplasm is responsible for the rotation (Kuroda, 1964). The same mechanism for the cytoplasmic streaming probably also functions in this case, since the microfilament bundles are attached to chloroplasts (Kuroda, personal communication). The rotation also stopped at the moment the cytoplasmic streaming stopped on membrane excitation, and gradually recovered together with the streaming. This fact suggests that both types of movements are controlled by the same

regulatory mechanism and further that a sequence of events triggered by the excitation finally leads to the cessation of chloroplast rotation. Therefore, some signal to stop the rotation must be transmitted through the flowing endoplasm.

Is the signal transmitted electrically or via chemical diffusion in the streaming endoplasm? To answer this question, we investigated the effect of the excitation on rotation of chloroplasts located apart from the cortex using endoplasm-enriched cell fragments of N. axilliformis.

The passive flow of chloroplasts in the direction of the streaming stopped simultaneously with the cessation of the bulk streaming upon membrane excitation, even if the chloroplast was located apart from the cortex. However, the rotation of chloroplasts located about 20 μm from the cortex stopped about one second after the cessation of the passive movement. Sometimes, at the moment of action potential generation, the rotation of a chloroplast located about 50 μm from the cortex was not affected at all or slowed down only slightly, while the rotation of one just beneath the cortex stopped completely.

The data are summarized in Fig. 6, which shows the time interval (stopping time) between the start of electrical stimulation and the cessation of passive chloroplast movement (Fig. 6a) or active chloroplast rotation (Fig. 6b) in relation to the distance of the chloroplast from the cortex. The mean values of stopping times within the regions divided by dashed lines are shown with SEM by closed circles. The error of 2-3 μm for the distance is inevitable. Fig. 6a

clearly shows that the passive flow of chloroplasts stopped 0.4-0.6 sec after the start of stimulation irrespective of their distance from the cortex. This value agrees well with the stopping time of the streaming reported previously (Tazawa and Kishimoto, 1968). On the other hand, the stopping time of chloroplast rotation increased with the distance (Fig. 6b), suggesting that an agent which stops the rotation is transmitted by diffusion in the endoplasm. The velocity of transmission is estimated to be about 15 μm/sec.

3. Internal Ca^{2+} concentation and the streaming rate

Chara internodes lacking the tonoplast (tonoplast-free cells) were prepared by replacing the cell sap with an artificial medium containing a strong Ca^{2+} chelator EGTA (Williamson, 1975; Tazawa et al., 1976). The active cytoplasmic streaming observed in tonoplast-free cells does not stop upon membrane excitation (Tazawa et al., 1976). A straightforward interpretation of this fact is that Ca^{2+} released on excitation from the plasmalemma or other internal storage is trapped by EGTA and therefore unable to reach the site of the motive force generation. This idea conforms with the contention that diffusion of a chemical agent in the endoplasm is involved in stopping the chloroplast rotation. Furthermore, the possible participation of Ca^{2+} in regulating cytoplasmic streaming is supported by the statement that $[Ca^{2+}]_i$ above 10^{-7}M inhibited the streaming (Williamson, 1975). For the hypothesis asserting Ca^{2+} as a direct agent controlling

the streaming, it is essential to know in detail the quantitative relationship between $[Ca^{2+}]_i$ and the streaming rate.

Accurate control of $[Ca^{2+}]_i$ of tonoplast-free cells by replacing the cell sap with an EGTA-containing solution is difficult, since endogenous Ca^{2+} which is accessible to EGTA is unknown. The difficulty was overcome by perfusing tonoplast-free cells again with solutions of known Ca^{2+} concentrations as described in MATERIALS and METHOD.

After removal of the tonoplast by replacing the cell sap with 0Ca solution, most of the endoplasm originally lacated on the inner surface of the cortical gel dispersed into the original central vacuolar space and the remaining endoplasm streamed along the inner surface of the chloroplast layer at the rate (v) of 70-80 μm/sec. During the second perfusion with solutions of various Ca^{2+} concentrations with a volume about five times the cell volume, most of the endoplasm flowed out and the amount of streaming endoplasm became scarce. Microscopic observation of the streaming began about 5 min after teh beginning of the second perfusion. Movement of the endoplasm particles was saltatory. They sometimes stopped and then flowed smoothly. The streaming rate was determined with particles which showed smooth movement over a long distance. Measurement of v was completed within 5 min after the start of microscopic observation. The average speed of the fastest two particles was adopted as the streaming rate. Fig. 7 shows the

dependency of v on $[Ca^{2+}]_i$. Below 10^{-7}M, v was about 60 μm/sec which was slightly smaller than that after the first perfusion. The closed circle represents v obtained in the cell which was perfused with 0Ca solution twice. As this solution contained no Ca^{2+}, $[Ca^{2+}]_i$ was expected to be far less than 10^{-9}M. This fact shows that Ca^{2+} is not necessary for the streaming. At $[Ca^{2+}]_i$ above 10^{-6}M, v decreased with an increase in $[Ca^{2+}]_i$. No moving particles were observed at 10^{-3}M of $[Ca^{2+}]_i$. Between 5 and 10 min after the start of the observation, both the number of streaming particles and the streaming rate decreased significantly when $[Ca^{2+}]_i$ was 10^{-6}-10^{-4}M. These facts show that inhibition of v by high $[Ca^{2+}]_i$ should be less significant if v could be measured immediately after the second perfusion.

The streaming once inhibited completely by high $[Ca^{2+}]_i$ (1 mM) could, although not fully, be recovered only when the internal medium was replaced with 0Ca solution immediately after the inhibition. The degree of recovery was difficult to determine, since the endoplasmic granules after the third perfusion with 0 Ca solution did not move at a constant rate but saltatory. If the cell interior was exposed to high $[Ca^{2+}]_i$ for several minutes, the streaming could not be recovered by 0Ca solution. These results were obtained by observing moving granules in chloroplast-free "windowed area" (Kamitsubo, 1972) of the cell using the similar perfusion system developed by Tazawa (1968) (cf. also Williamson, 1975) under a Zeiss photomicroscope II with differential interference optics.

4. Ca^{2+} injection

The previous section showed that the cytoplasmic streaming in tonoplast-free cells slowed down with an increase in the internal Ca^{2+} concentration and completely stopped at 1 mM Ca^{2+}. However, the streaming once inhibited by high $[Ca^{2+}]_i$ could not be fully recovered even when Ca^{2+} ions were removed by introducing an EGTA containing solution into the cell immediately after the inhibition. This may have been partly due to the experimental system adopted. Namely, Ca^{2+} was introduced into the cell by internal perfusion, thus exposing the whole cell interior to Ca^{2+} for a relatively long period of time, which might have caused a secondary Ca^{2+} effect other than that on the motile system. Therefore, we tried to find the true effect of Ca^{2+} on the motile system by locally applying Ca^{2+} for the short period of time. As the experimental material, we selected cytoplasmic droplets of Chara containing vigorously rotating chloroplasts (Kamiya and Kuroda, 1957). Ca^{2+} and other cations were injected iontophoretically into such droplets.

4-1. Effects of Ca^{2+} injection on chloroplast rotation in endoplasmic droplets

The rate of chloroplat rotation in isolated endoplasmic droplets varied from a very low rate to 2 revolutions per second (rps). Variation of the rotation rate was previously reported in endoplasmic droplets of N. flexilis isolated by a different method (Kamiya and Kuroda, 1957). We selected

droplets containing one to three, sometimes more chloroplasts which rotated at a rate of about 0.5 rps or more. Two microneedles, one containing 0.5 M $CaCl_2$ solution and the other 0.5 M KCl or $MgCl_2$ solution, were inserted into the droplets. Insertion itself usually had no effect on the chloroplast rotation. Ca^{2+} was injected by transferring electric current with a duration of 1 sec from the microneedle containing Ca^{2+} (Ca^{2+}-side) to that containing K^+ or Mg^{2+} (K^+- or Mg^{2+}-side). When the intensity of the electric current was low, the chloroplast rotation was not affected at all. When it increased, the rotation slowed down or stopped completely immediately after its application then recovered gradually with time. On the other hand, a current of similar amplitude flowing from the K^+- or Mg^{2+}-side had no effect on the rotation. Typical example are shown in Fig. 8 and 9, in which the amplitude of the electric current and the rate of chloroplast rotation are both given. Fig. 8 shows that the rotation rate was not affected at all by a current of 1-3 nA from the Ca^{2+}-side. An increase in current intensity to ca. 7 nA caused an immediate decrease in the rotation rate to about 2/3 the original value. The inhibited rotation gradually recovered its initial rate. When a larger electric current of 13 and 18 nA was applied from the K^+-side, no responses were observed. However, chloroplast rotation stopped instantly when 24 nA was applied from the Ca^{2+}-side, then recovered completely in

about one minute. The rotation rate, which was not affected by 30 nA electrical current from the K^+-side, decreased to about 1/2 with 20 nA from the Ca^{2+}-side.

Fig. 9 shows that the rotating chlorplast did not respond to electric current of 9 to ca. 30 nA from the Mg^{2+}-side, while only ca. 1.5 nA (at the start of record) and ca. 7 nA (at the end of the record) from the Ca^{2+}-side inhibited rotation completely and reversibly.

The above results indicate clearly that not the electric current itself but iontophoretically injected Ca^{2+} caused the cessation of chloroplast rotation and that inhibition of the rotating chloroplasts by Ca^{2+} depended on the amount injected. Based on the four assumptions made in MATERIALS and METHOD, the $[Ca^{2+}]_i$ in the droplet of Fig.8 immediately after application of electric current of 7, 24 or 20 nA was calculated to be 0.4, 1.2 or 0.98 mM, respectively. Similarly, current of ca. 1.5 or 7 nA in Fig. 9 elevated $[Ca^{2+}]_i$ to ca. 0.1 or 0.4 mM, respectively.

Chloroplast rotation usually stopped 1-3 sec after the start of application of the electric current pulse and sometimes after more than 3 sec, especially when the chloroplast in larger droplets was rotating far from the tip of the Ca^{2+}-containing microneedle. As a typical example, a trace of one frame of cine film is shown in Fig. 10. The trace is of chloroplasts located near the focal plane containing the tip of the Ca^{2+}-containing microneedle immediately after the cessation of rotation. Two values are given for each chloroplast in the figure.

The first represents the distance of chloroplast from the tip of the Ca^{2+}-containing microneedle in μm and the second is the time between the start of the Ca^{2+} injection and the cessation of rotation in seconds (stopping time). The rate of decrease in the rotation velocity was larger in chlorplasts having a short stopping time than that in chloroplasts having a longer one. The facts are explainable by assuming that Ca^{2+} ions diffuse in the droplet to reach each chloroplast and affect its rotation.

Fig. 11 shows the relationship between $[Ca^{2+}]_i$ and the degree of inhibition which is classified into three levels: "stop","slowing down" and "no effect". Fig. 11 gives only the results obtained with C.corallina. In the experiment different amounts of Ca^{2+} were injected repeatedly into a droplet to get different degrees of response. Fig. 11 does not include the data of the experiment where the droplet showed always the same degree of response to repeated Ca^{2+} injection. The figure clearly shows that chloroplast rotation did not respond to Ca^{2+} less than about $10^{-3.5}$M but often responded to it above this concentration. In the case of C. australis, the rotating chloroplasts responded to Ca^{2+} of more than about 10^{-4}M.

4-2. Effects of other divalent cations on chloroplast rotation in endoplasmic droplets

After Ca^{2+} was shown to inhibit the chloroplast rotation reversibly, its specificity was investigated. As already shown in Fig. 9, Mg^{2+} had no effect. Sr^{2+}

of about 10^{-4}M inhibited the chloroplast rotation reversibly like Ca^{2+}. Mn^{2+} and Cd^{2+} had a different effect. A few seconds after their injection, the rate of chloroplast rotation gradually decreased with low reversibility. A typical example is shown in Fig. 12. The rotation rate decreased gradually with Cd^{2+} injection (middle part of the figure) and after about 1.5 min, was about 1/3 of that before the injection. Ba^{2+} had effects sometimes similar to Ca^{2+} or Sr^{2+} and sometimes Mn^{2+} or Cd^{2+}.

5. Ca influx

In view of the dependence of rates of cytoplasmic streaming and chloroplast rotation on $[Ca^{2+}]_i$, which is 10^{-7}M or less in the normal cytoplasm (Fig. 7, Williamson, 1975: Tazawa et al., 1976), $[Ca^{2+}]_i$ should increase enormously at the moment of the action potential generation, if Ca^{2+} is the factor causing streaming cessation. One possible origin of increased Ca^{2+} is the external Ca^{2+} which transiently invades the cell during membrane excitation. To check this, the following experiment was carried out.

The central part (1 cm long) of an internode of C. australis was exposed to solution containing $^{45}Ca^{2+}$ for 15 min (Fig.4). Fig. 13 shows the amount of Ca influx in relation to the external Ca^{2+} concentration, $[Ca^{2+}]_o$. In the resting membrane, the influx is proportional to $[Ca^{2+}]_o$ as shown by the open circles. At 0.13 mM $[Ca^{2+}]_o$, the influx (with SEM) was $4.0 \pm 0.9 \times 10^{-14}$ mol/cm^2/sec. This value coincides well with the $4.0 \pm 0.8 \times 10^{-14}$

mol/cm^2/sec obtained with N. translucens when the external medium contained 1.0 mM NaCl, 0.1 mM KCl and 0.1 mM $CaCl_2$ (Spanswick and Williams, 1965).

Hope and Findlay (1964) tried to detect the extra Ca influx upon membrane excitation. They induced action potentials in cells of C. corallina at 7-minute intervals and detected no extra uptake of activity. We induced the action potential at every minute. The result is given by the closed circles in Fig. 13, which clearly shows that Ca influx increased with the action potentials elicited 15 times during 15 minutes of exposure to $^{45}Ca^{2+}$. Assuming that the extra Ca influx occurs for 1 sec at around the peak of the action potential where the membrane conductance drastically increases, Ca influxes across the excited membrane for 10^{-5}, 10^{-4}, 10^{-3} and 10^{-2} M of $[Ca^{2+}]_o$ are calculated to be about 50, 90, 300 and 200 times those across the resting membrane for the respective $[Ca^{2+}]_o$.

Based on the data of Fig. 13, the increase in the cytoplasmic Ca^{2+} concentration, $[Ca^{2+}]_c$, upon membrane excitation was calculated under the following assumptions. (i) The cell diameter is 0.6 mm. (ii) The volume of the cytoplasm is 1/10 the cell volume (Tazawa et al., 1974). (iii) Ca^{2+} invading from the outside diffuses in the cytoplasm to become homogeneously without binding with the constituents in the cytoplasm. (iv) Ca^{2+} in the cytoplasm is normally very low. The results are given in Fig. 14, which clearly shows that the increase in $[Ca^{2+}]_c$ is roughly proportional to the increase in $[Ca^{2+}]_o$. At

1.3×10^{-5} M of $[Ca^{2+}]_o$, the increase in $[Ca^{2+}]_c$ is only 3×10^{-7}M/impulse, which is not enough to stop the streaming, judging from Fig. 7. At 10^{-2}M of $[Ca^{2+}]_o$, one action potential induced an increase in $[Ca^{2+}]_c$ amounting to 9×10^{-5}M which may induce the streaming cessation. Since the cytoplasmic streaming stops at a very low $[Ca^{2+}]_o$, it is suggested that the Ca influx itself does not directly cause the streaming cessation. The next experiment provided further evidence supporting this suggestion.

6. External Ca^{2+} and the streaming recovery rate

As shown in Fig. 5b, the streaming once stopped recovered its original rate within 5-10 min. If Ca^{2+} is the agent responsible for the streaming cessation, recovery should have been caused by a decrease of the $[Ca^{2+}]_c$ which had increased on excitation. This may be achieved either with an increased Ca efflux or by sequestering of Ca^{2+} in some cell organelle like the sarcoplasmic reticulum. The simplest assumption is that the recovery depends on the increment of $[Ca^{2+}]_i$ caused by excitation. This idea is supported by the fact that the streaming recovery is drastically delayed when the action potential is induced repeatedly before the start of streaming recovery (Kishimoto and Akabori, 1959). Since the increase of Ca influx is dependent on $[Ca^{2+}]_o$, the recovery time was also expected to be dependent on it, if the increment of $[Ca^{2+}]_c$ is caused mainly by an increase of Ca influx on excitation.

Since the recovery time is sensitive to temperature

(unpublished results), experiments were carried out at a constant room temperature (24 ± 1 °C). As a measure of recovery time, we adopted the half time of recovery ($T_{1/2}$), i.e., the time interval between the moment of streaming cessation or the generation of the action potential and the time when the streaming recovered to half its original rate. Fig. 15 shows clearly that the recovery time remained the same irrespective of $[Ca^{2+}]_o$. This fact suggests that the external Ca^{2+} does not have a direct influence on the mechanism of cytoplasmic streaming during excitation, though the Ca influx upon membrane excitation increases significantly with an increase in $[Ca^{2+}]_o$.

V. DISCUSSION

1. The membrane related to the streaming cessation

It was demonstrated that excitation of the plasmalemma alone is enough to stop the streaming using cell fragments containing only cytoplasm. This did not exclude the possibility that excitation of the tonoplast may also affect the streaming. However, the fact that the stopping time of chloroplast rotation is roughly proportional to the distance of the chloroplasts from the cortex suggests that the tonoplast action potential is not responsible for the streaming cessation. The rate of increase of the stopping time, which is about 1 sec/15 μm, does not contradict the idea of diffusional transmission of a substance from the cell surface to the inside.

2. Ca^{2+} injection

The effects of various cations on chloroplast rotation in cytoplasmic droplets were investigated by iontophoretically injecting them. They are classified into three groups according to the mode of inhibition. (i) Abrupt, reversible inhibition by Ca^{2+} and Sr^{2+}. (ii) Gradual, irreversible inhibition by Mn^{2+} and Cd^{2+}. (iii) No inhibition by K^{+} and Mg^{2+}.

The recovery of rotation after inhibiton by Ca^{2+} injection indicates that the Ca^{2+} concentration in the droplets decreased during the recovery phase and suggests that a mechanism which sequesters Ca^{2+} exists in the endoplasm. Candidates for such organelles are mitochondria (Vasington and Murphy, 1962) and endoplasmic reticulum

like the sarcoplasmic reticulum.

The increase in Ca^{2+} concentration in the droplets induced by Ca^{2+} injection was estimated by making four assumptions. (i) The droplet is a sphere. (ii) All electric current passing from the microneedle containing $CaCl_2$ solution to endoplasm is carried by Ca^{2+}, i.e., the transport number of Ca^{2+} is 1. (iii) Ca^{2+} ions injected diffuse homogeneously within the droplet in a few seconds without binding with constituents in the endoplasm. (iv) The amount of free Ca^{2+} in the droplet before injection of Ca^{2+} is negligible. The first assumption is not exactly correct, because the droplets are slightly flattened due to the influence of gravity (Kamiya and Kuroda, 1958). If the perpendicular section of the drop is an oval with the length of the minor axis being half of that the major one (r), the volume of such a drop is half the volume of the spherical drop (radius, r). Therefore, the error in estimating dropet volume does not lead to a serious error in Ca^{2+} concentration. As for the second assumption, it is difficult to determine the transport number of Ca^{2+}. However, it may be larger than that of Cl^- which is probably the main cytoplasmic anion carrying the electric current because the cytoplasmic Cl^- concentration is much lower (21 mM, Tazawa et al., 1974) than that of Ca^{2+} (500 mM in the microneedle). The third assumption is not correct because a Ca^{2+}-sequestering mechanism seems to operate in the cytoplasm. Therefore, the calculated values of $[Ca^{2+}]_i$ must be overestimated. After several injections into one

droplet, the intensity of the electric current for a Ca^{2+} injection to stop the rotation tends to increase, and globular clotting of endoplasm attached to the tip of the microneedle was often observed. These facts may indicate a change of the transport number of Ca^{2+} and/or the amount of diffusible Ca^{2+} near the microneedle tip.

The conclusion that Ca^{2+} of more than 10^{-4} M inhibits the chloroplast rotation agrees with the result obtained in tonoplast-free cells where the $[Ca^{2+}]_i$ was directly controlled by internal perfusion.

3. Participation of Ca^{2+} in streaming cessation

As shown in Fig. 7, the streaming was inhibited by increasing $[Ca^{2+}]_i$ above 10^{-6}M (cf. Williamson, 1975). The effect of Ca^{2+}, although not completely, was reversible. Ca^{2+} injected into the isolated cytoplasmic drops inhibits chloroplast rotation. This effect is completely reversible and specific to Ca^{2+}. Together with the information that the cytoplasmic streaming does not stop on excitation in tonoplast-free cells containing Ca^{2+}-chelator EGTA (Tazawa et al., 1976), these facts make it highly probable that the substance transmitted via diffusion during excitation from the plasmalemma to the site of motive force generation is Ca^{2+}.

Based on the results obtained in the present work, three explanations are possible for the cessation of cytoplasmic streaming upon excitation.

(i) Ca^{2+} is released from the plasmalemma upon excitation. It diffuses through the cortical gel layer and reaches the sol-gel interface, the site of motive force generation, to halt the streaming. It further diffuses in the endoplasm to reach the rotating chlroplasts. The transient increase in $[Ca^{2+}]_c$ is more than 10^{-4}M. The Ca^{2+} ions are pumped out of the cell or absorbed by a putative Ca^{2+} sequester in the cytoplasm and/or by the plasmalemma, resulting in the streaming recovery.

(ii) Ca^{2+} invading from the outside upon excitation directly stops the streaming and chloroplast rotation.

(iii) The excited plasmalemma generates some signal to induce Ca^{2+} release from putative internal Ca^{2+} storage. Ca^{2+} released induces cessation of cytoplasmic streaming and further diffuses in the endoplasm to stop the chloroplast rotation. The carrier of the signal can be Ca^{2+} itself flowing in from the outside or released from the plasmalemma. A Ca^{2+}-induced Ca^{2+} release is known in the sarcoplasmic reticulum of skinned muscle fibers (Endo et al., 1970).

As for the role of the external Ca^{2+}, Barry (1968) reported that the presence of free Ca^{2+} in the external medium is necessary for the streaming cessation upon excitation. On the other hand, Pickard (1972) found no correlation between the stopping time of the streaming cessation and the Ca influx estimated. Hayama and Tazawa (1978) suggested that external Ca^{2+} is not necessary for

the streaming cessation accompanying osmosis-induced membrane depolarization in Nitella flexilis.

The present work showed that the increase in $[Ca^{2+}]_c$ due to Ca influx during an action potentail was 3 x 10^{-7}M/ impulse when $[Ca^{2+}]_o$ was 10^{-5}M. Such an increase in $[Ca^{2+}]_c$ estimated under several assumptions is not enough to halt the streaming (Fig. 7), although the streaming stops at this concentration of $[Ca^{2+}]_o$. In the estimation of Ca influx due to action potentials, it was tacitly postulated that the Ca efflux is very low and is not affected by $[Ca^{2+}]_c$. If it is assumed that the Ca efflux increases proportional to $[Ca^{2+}]_c$, the Ca efflux from the repeatedly stimulated cells should be larger than that from the cell in the resting state. If this Ca efflux in the former cells is so high that the most of Ca^{2+} ions transported from the outside during excitation are extruded, the actual influx should be far larger than the measured one and may halt the streaming. However, the hypothesis on the origin of the Ca^{2+} causing streaming cessation upon excitation must explain at least the following two facts. (1) The rate of the streaming recovery was independent of $[Ca^{2+}]_o$ (Fig. 15) although the increase in $[Ca^{2+}]_c$ caused by an increase in Ca influx during an action potential at 10^{-2}M of $[Ca^{2+}]_o$ was estimated to be more than 100 times the increase found at 10^{-5}M (Fig. 14). (2) The streaming recovery delays drastically when several action potentials are elicited repeatedly before the start of the streaming recovery (Kishimoto and Akabori, 1959). The idea that the external Ca^{2+} invading during excitation directly stops the streaming can well explain the fact (2)

but not the fact (1). If the fact (1) is explained by the presence of Ca^{2+} extruding pump which works in proportion to the level of $[Ca^{2+}]_c$, it is difficult to explain the fact (2). Thus, these speculations make the hypothesis of external origin of the Ca^{2+} less probable.

Although the time course of Ca influx during the action potential is unknown, we assume that most of the extra Ca influx upon excitation occurs during a limited period (less than 1 sec), i.e., at the peak of the action potential where the membrane conductance drastically increases (Findlay and Hope, 1964). At $[Ca^{2+}]_o$ of 10^{-3} and 10^{-2}M, the increase of $[Ca^{2+}]_c$ upon excitation was estimated to be 4×10^{-5} and 9×10^{-5}M, respectively (Fig. 14). Such an increase of $[Ca^{2+}]_c$ can inhibit or eventually almost stop the streaming, judging from the dependence of v on $[Ca^{2+}]_i$ shown in Fig. 7. However, these values of $[Ca^{2+}]_c$ may be overestimated, if it is assumed that a mechanism sequestering Ca^{2+} is always operating in the endoplasm. The existance of such a mechanism was suggested in the previous section.

4. Comparison of roles of Ca^{2+} in cytoplasmic streaming in Characeae cells and other cytoplasmic movements

If the motive force of the cytoplasmic streaming in Characeae is generated by a mechanism similar to the actomyosin system in muscle cells, the streaming and the cessation can be considered as a repetition of processes corresponding to "contraction" and "relaxation", respectively.

Ca^{2+} is not necessary for the motive force generation in the cytoplasmic streaming and Ca^{2+} of relatively high concentration rather inhibits the cytoplasmic streaming and chloroplast rotation. These two facts markedly contrast with the stimulating role of Ca^{2+} in muscle contraction (Ebashi and Endo, 1968). Also cytoplasmic movement of an demembranated amoeba, Chaos carolinensis, is under the control of Ca^{2+} (Taylor et al., 1973). The cytoplasm relaxes at $[Ca^{2+}]$ less than 10^{-7} M and contracts at 10^{-6}M. When $[Ca^{2+}]$ is 7×10^{-7} M, the cytoplasm streams and forms pseudopods. In Physarum plasmodium, the demembranated cytoplasmic mass actively moves forming numerous moving lobes in an EGTA-containing solution(Kuroda). When Ca^{2+} ions are locally applied to the moving mass, a contraction center is formed at the application site pulling the surrounding cytoplasm. Therefore, the effect of Ca^{2+} on the actomyosin system in Characeae cells seems to differ from that of Ca^{2+} on the actomyosin system in muscle, amoeba and Phsarum plasmdium which leads us to conclude that the motile system in Characeae might be controlled by a different regulatory mechanism.

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VIII. Legends

Fig. 1

Experimental setup for measuring the membrane potential externally. An internode of Characeae (Cha) was mounted on a plexiglass chamber having two pools, A and B. Pool A was filled with 110 mM KCl solution and pool B with APW made isotonic to 110 mM KCl solution by adding sorbitol. The electric potential difference between A and B was amplified and recorded. The action potential was induced by electrical stimulation applied through Ag-AgCl wire placed in both pools.

Fig. 2

Preparation of cytoplasmic droplets. a; an internode. b; accumulation of the endoplasm and formation of droplets by longitudinal centrifugation of the cell. Some droplets contain rotating chloroplasts. c; back streaming of the accumulated endoplasm and dispersion of droplets after centrifugal treatment. d; isolation of droplets by internal perfusion.

Fig. 3

Iontophoretical injection of Ca^{2+} into the cytoplasmic droplets containing rotating chloroplasts. Two microneedles were inserted into each droplet. One contained 0.5 M $CaCl_2$ solution and the other 0.5 M KCl solution. Electric current was applied between the two microneedles.

Fig. 4

Experimental setup for externally measuring membrane potential simultaneously with ^{45}Ca influx measurements. An internode of Chara (Cha) was partitioned into three. The diameter of each pool was 1.0 cm and the distance between them was 1.5 cm. Pools A and C were filled with 110 mM KCl solution and pool B with APW isotonic to 110 mM KCl and containing ^{45}Ca. The electric potential difference between A and B was amplified and recorded. Electrical stimulus was applied through Ag-AgCl wire placed in the pools to induce an action potential.

Fig. 5

A spontaneously generated action potential (a) and the streaming cessation (b) observed in a tonoplast-free segment filled with only cytoplasm. The arrow in b indicates the moment of the action potential generation . The membrane potential just before the action potential generation was -125 mV.

Fig. 6

Stopping time, i.e., the time interval between the electrical stimulus and the cessation of the passive (streaming, a) and the active (rotation, b) chloroplast movements with respect to the distance of the chloroplast from the cortex. The mean values of the stopping times within the regions divided by dashed lines are shown with SEM by closed circles. The points falling on the dashed lines were averaged together with those of the group to their left.

Fig. 7

Streaming rate in relation to the internal Ca^{2+} concentration. The rate was measured after internal perfusion of tonoplast-free cells with solutions of known Ca^{2+} concentrations. The closed circle shows the rate of cells which were perfused twice with a solution containing no Ca^{2+}.

Fig. 8

Inhibition of chloroplast rotation by iontophoretically injected Ca^{2+} (lower figure). Two microneedles were inserted into the cytoplasmic drop. One contained 0.5 M $CaCl_2$ solution and the other 0.5 M KCl solution. The upper figure is the record of the electric current applied. Downward bars represent current flowing from the microneedle containing $CaCl_2$ to that containing KCl and upward bars represent current flowing in the opposite direction.

Fig. 9

Inhibition of chloroplast rotaiton by iontophoretically injected Ca^{2+}. One glass needle contained 0.5 M $CaCl_2$ solution and the other 0.5 M $MgCl_2$ solution. The upper figure is the record of the electric current applied. Downward bars show current flowing from the microneedle containing $CaCl_2$ to that containing $MgCl_2$ and upward bars show current flowing in the opposite direction.

Fig. 10

Stopping time of chloroplast rotation, i.e., time interval between Ca^{2+} injection and rotation cessation in relation to the distance of the chloroplast from the tip of the $CaCl_2$-containing glass needle. Two values are given for each chloroplast, one showing the distance in μm and the other, in brackets, the stopping time in sec.

Fig. 11

Degree of inhibition of chloroplast rotation with respect to the calculated concentration of Ca^{2+} injected into cytoplasmic droplets.

Fig. 12

Gradual decrease in rotation rate caused by iontophoretical Cd^{2+} injection (lower figure). One glass needle contained 0.5 M $MnCl_2$ solution and the other 0.5 M $CdCl_2$ solution. The upper figure is the record of the electric current. Downward bars represent Cd^{2+} injection.

Fig. 13

Ca influx across the resting membrane (open circles) and its increase due to repeated action potentials (closed circles) with respect to the external Ca^{2+} concentration. The duration of exposure to $^{45}Ca^{2+}$ was 15 min during which the action potential was elicited 15 times.

Fig. 14

Increase in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) upon membrane excitation as a function of the external Ca^{2+} concentration ($[Ca^{2+}]_o$). The values were calculated from the data in Fig. 13 based on several assumptions (See the text).

Fig. 15

Relative rate of the streaming recovery with respect to the external Ca^{2+} concentration ($[Ca^{2+}]_o$). As a measure, the half time of recovery was adopted. The ratio of the half time at each $[Ca^{2+}]_o$ to that at 10^{-5} M of $[Ca^{2+}]_o$ is shown.

Fig. 1

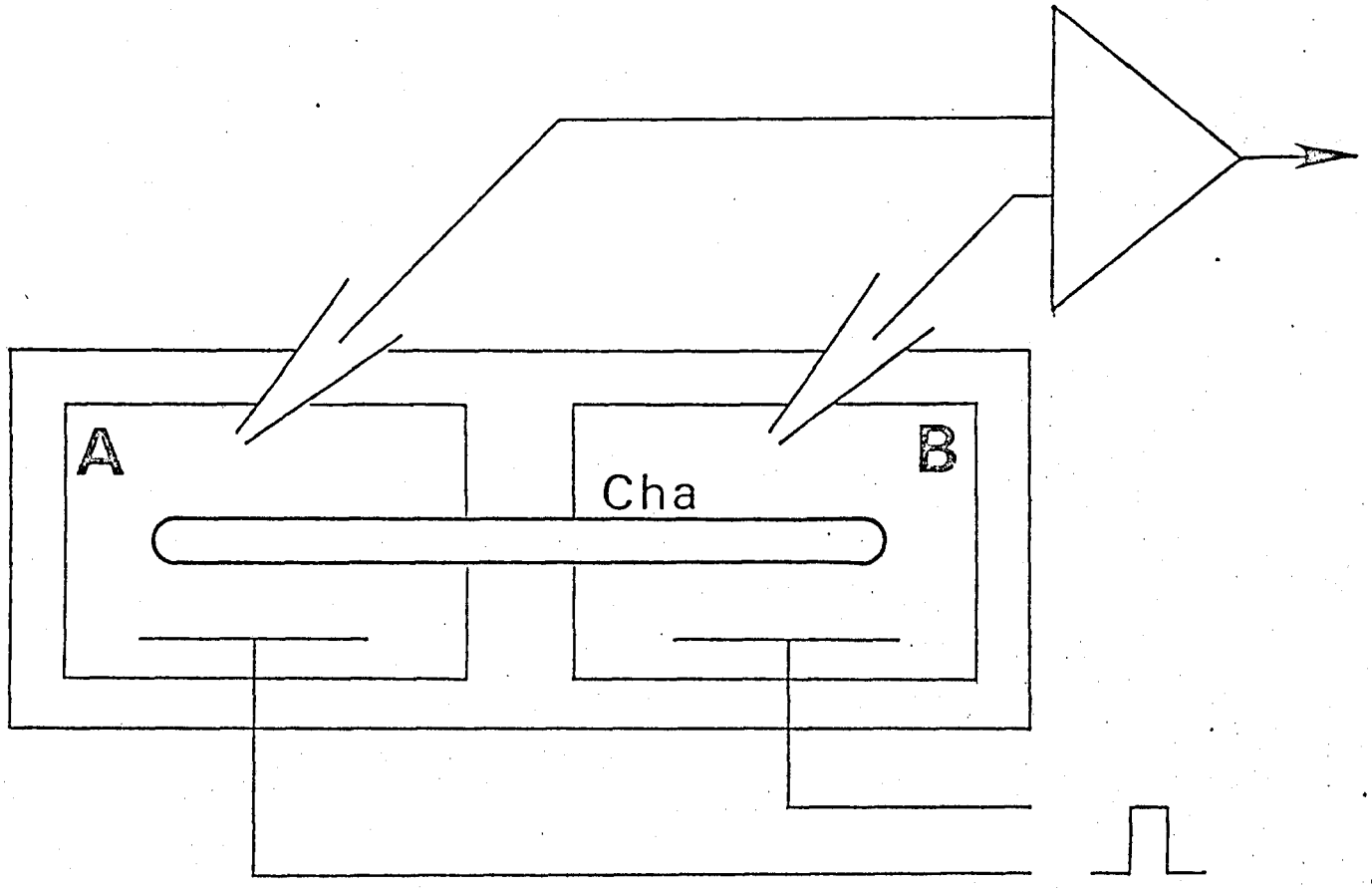

Fig. 2

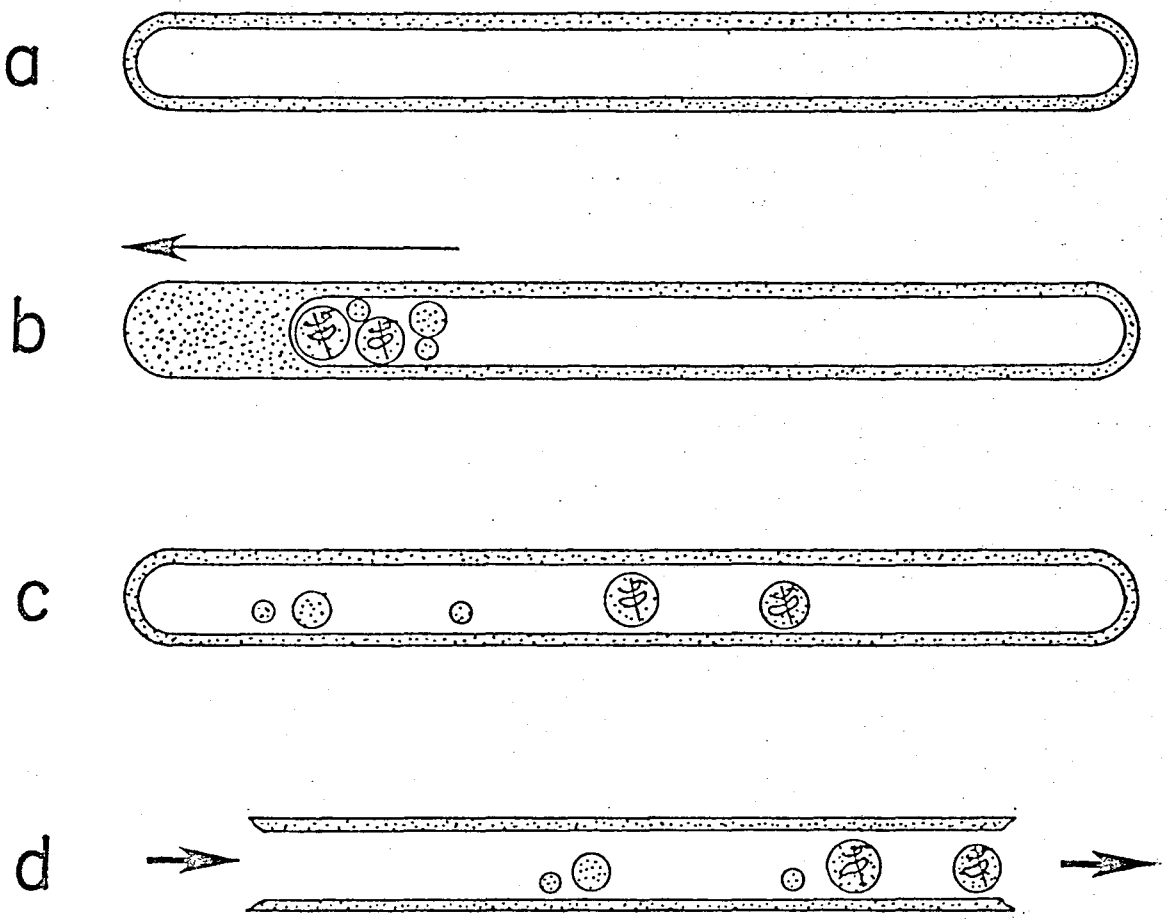

Fig 3

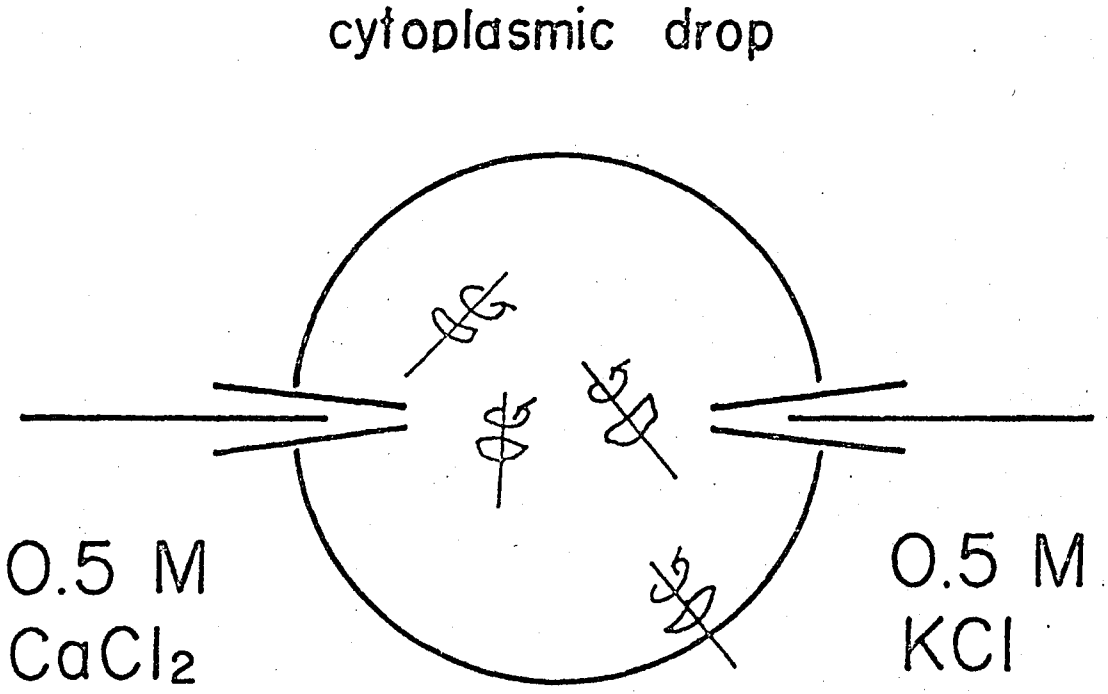

Fig.4

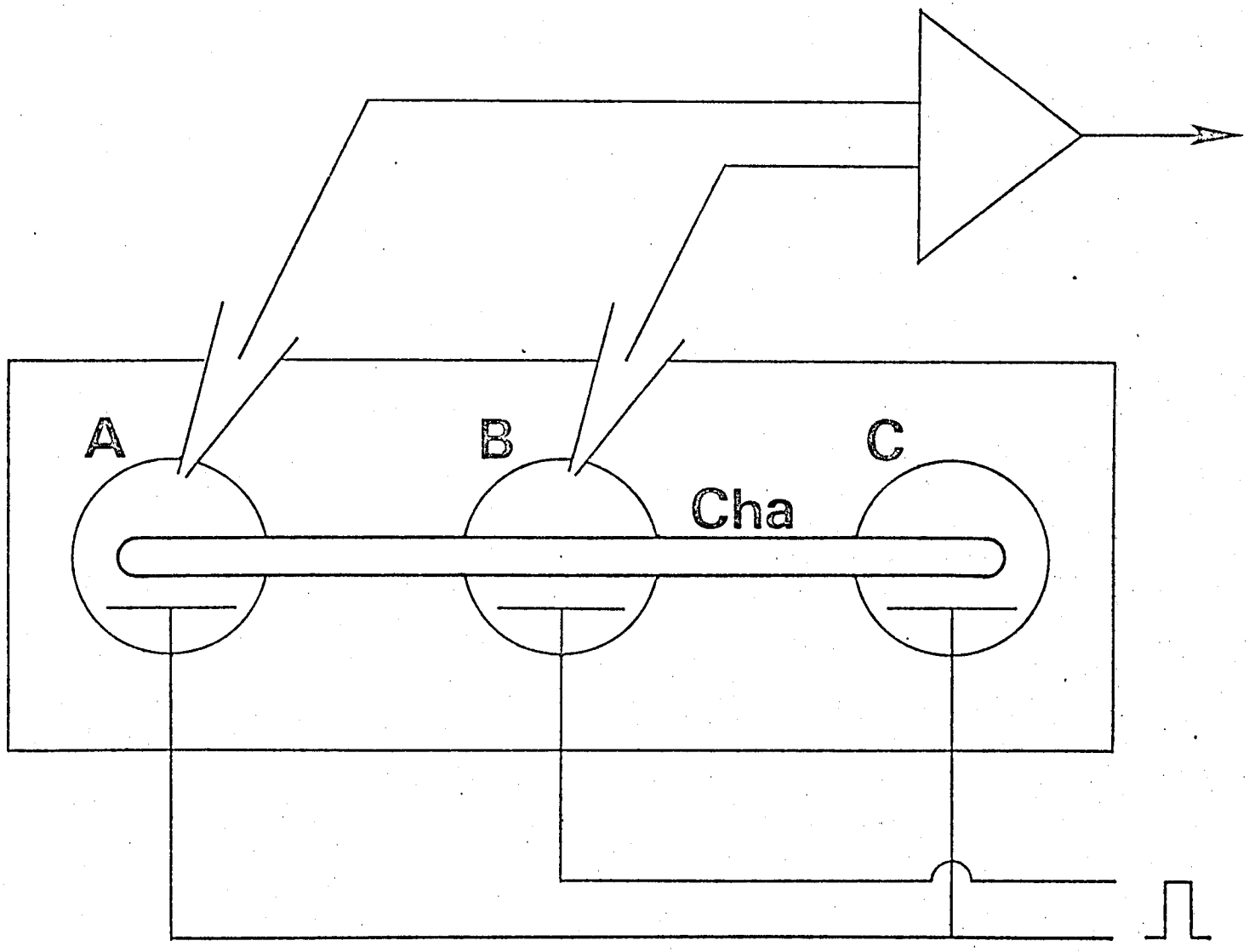

Fig. 5a

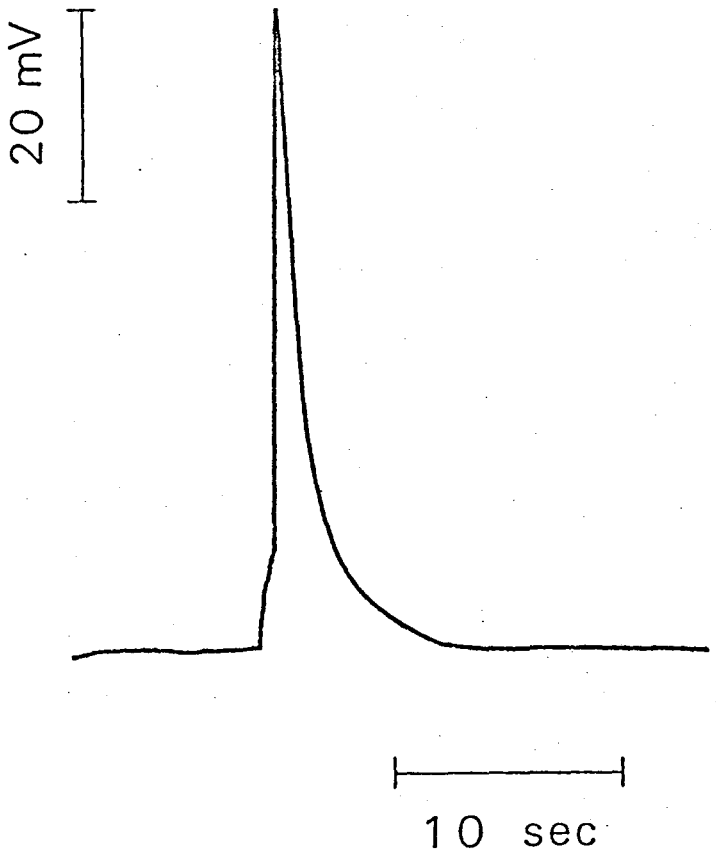

Fig. 5b

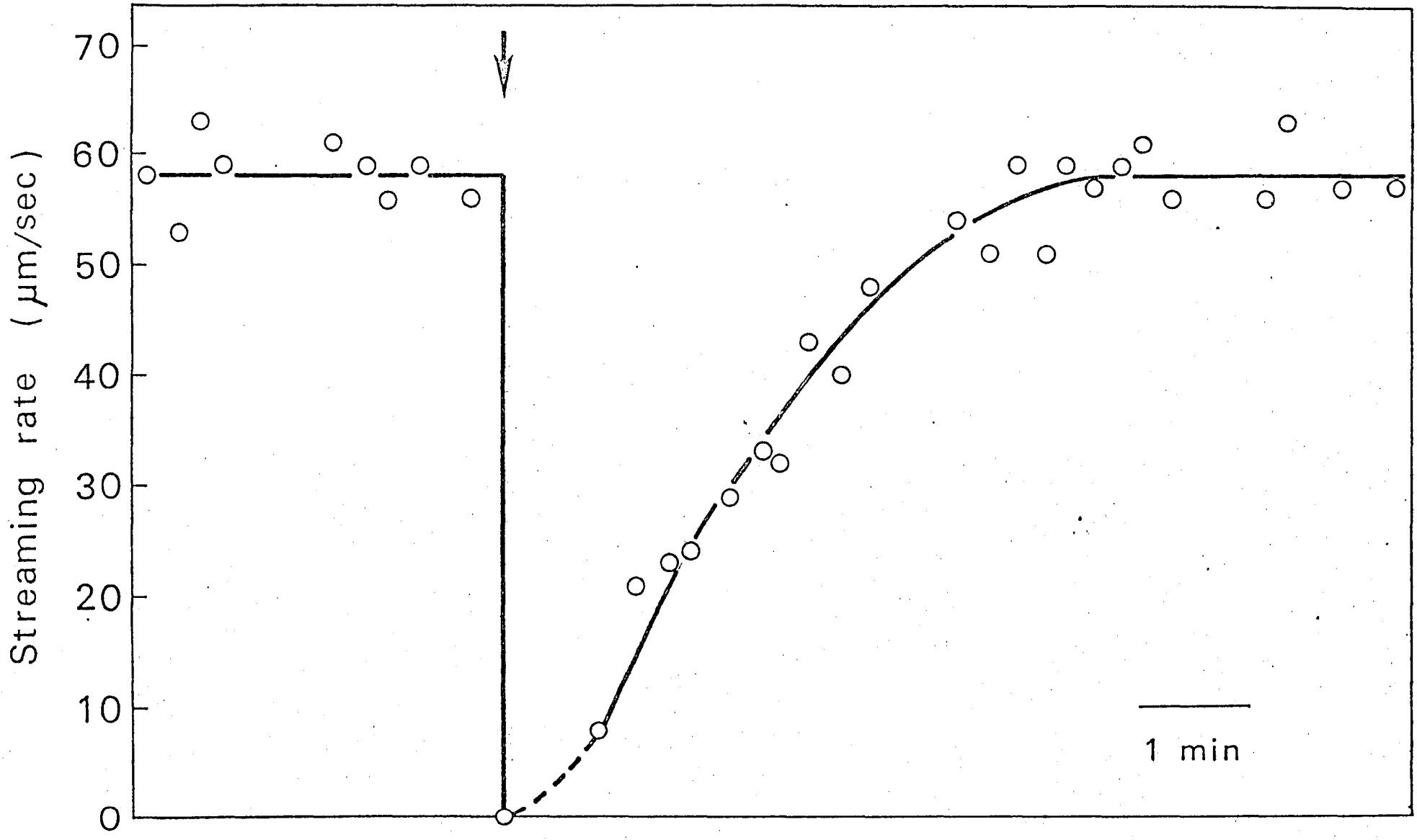

Fig. 6a

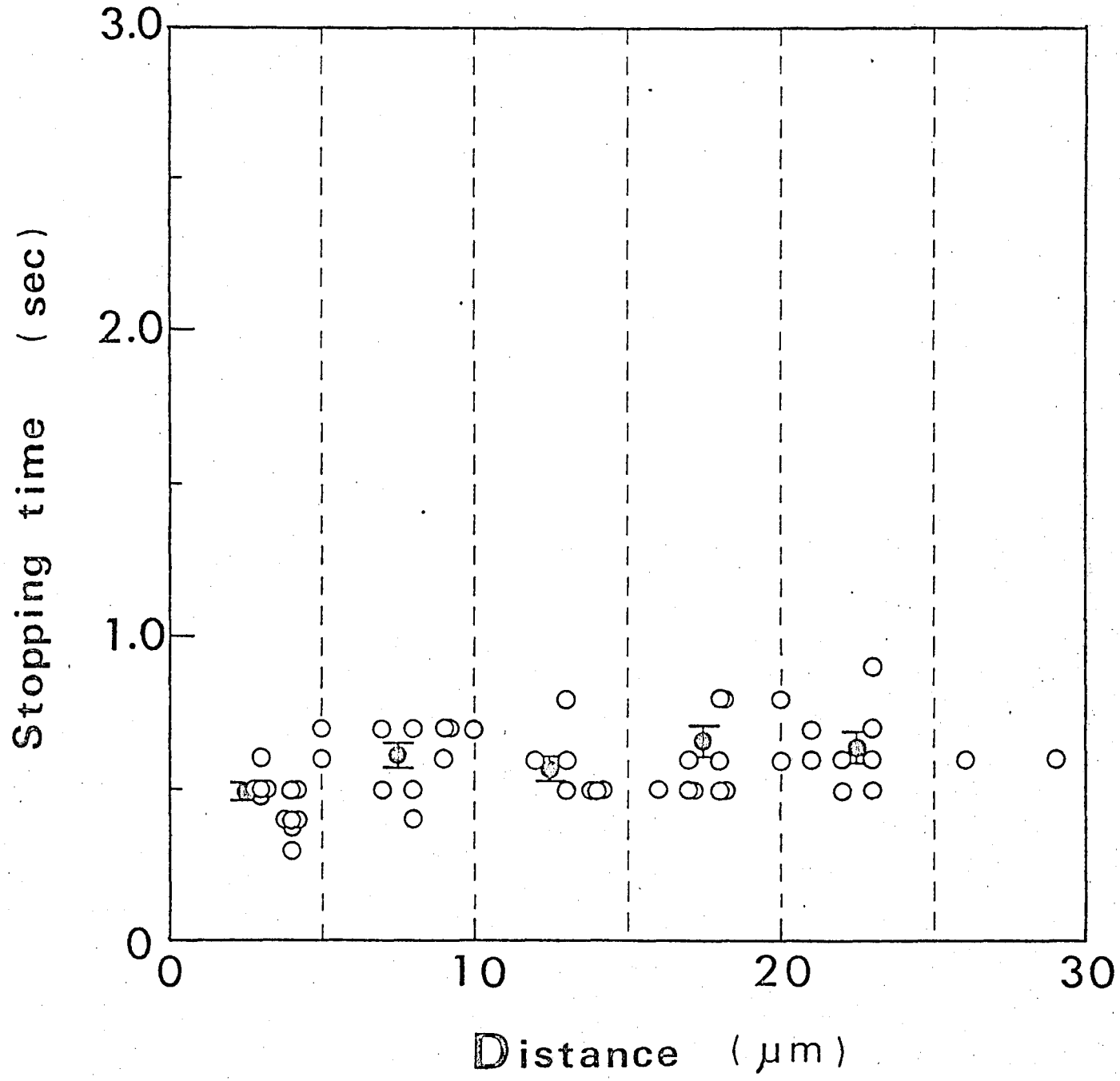

Fig. 6b

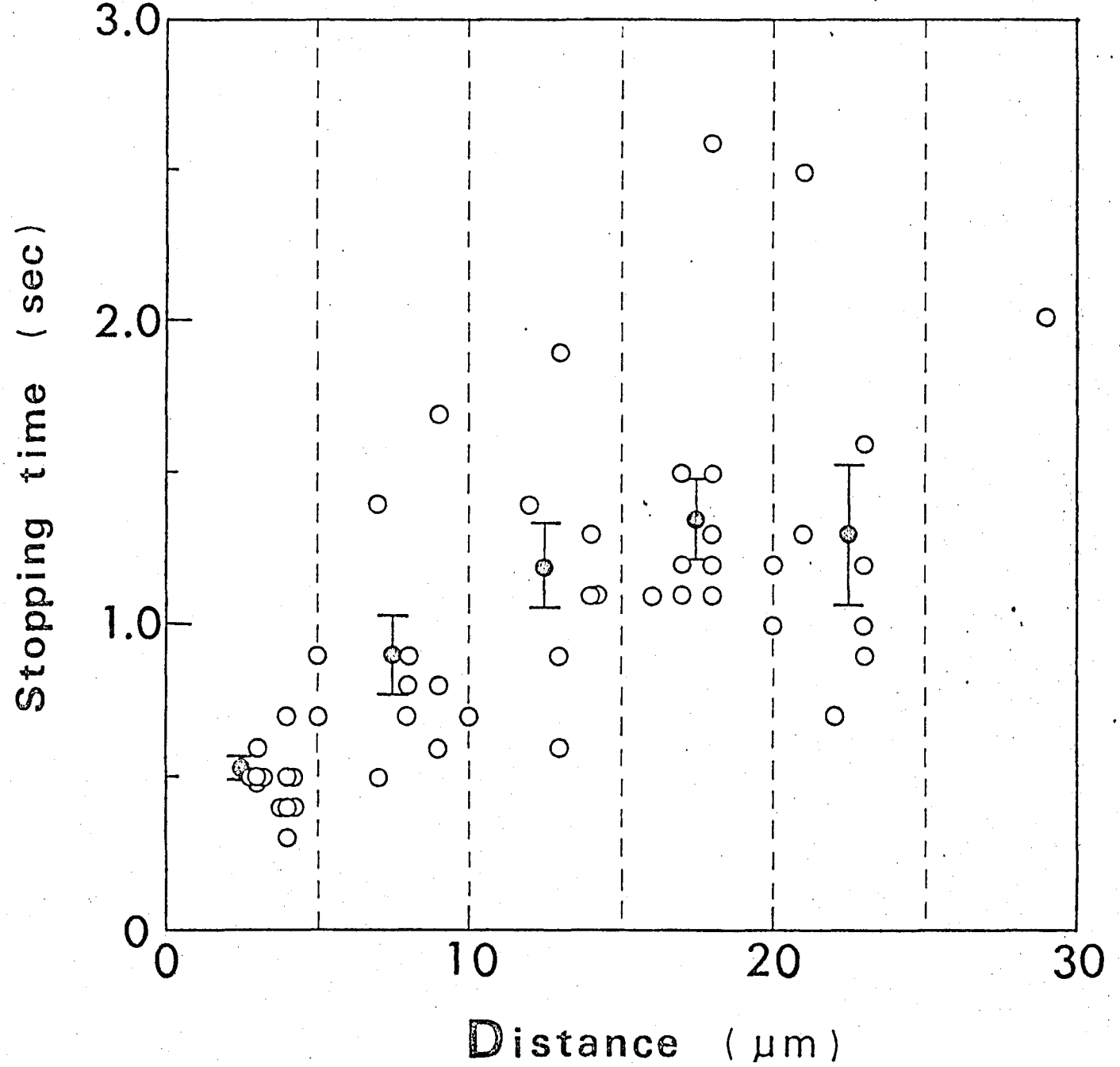

Fig. 7

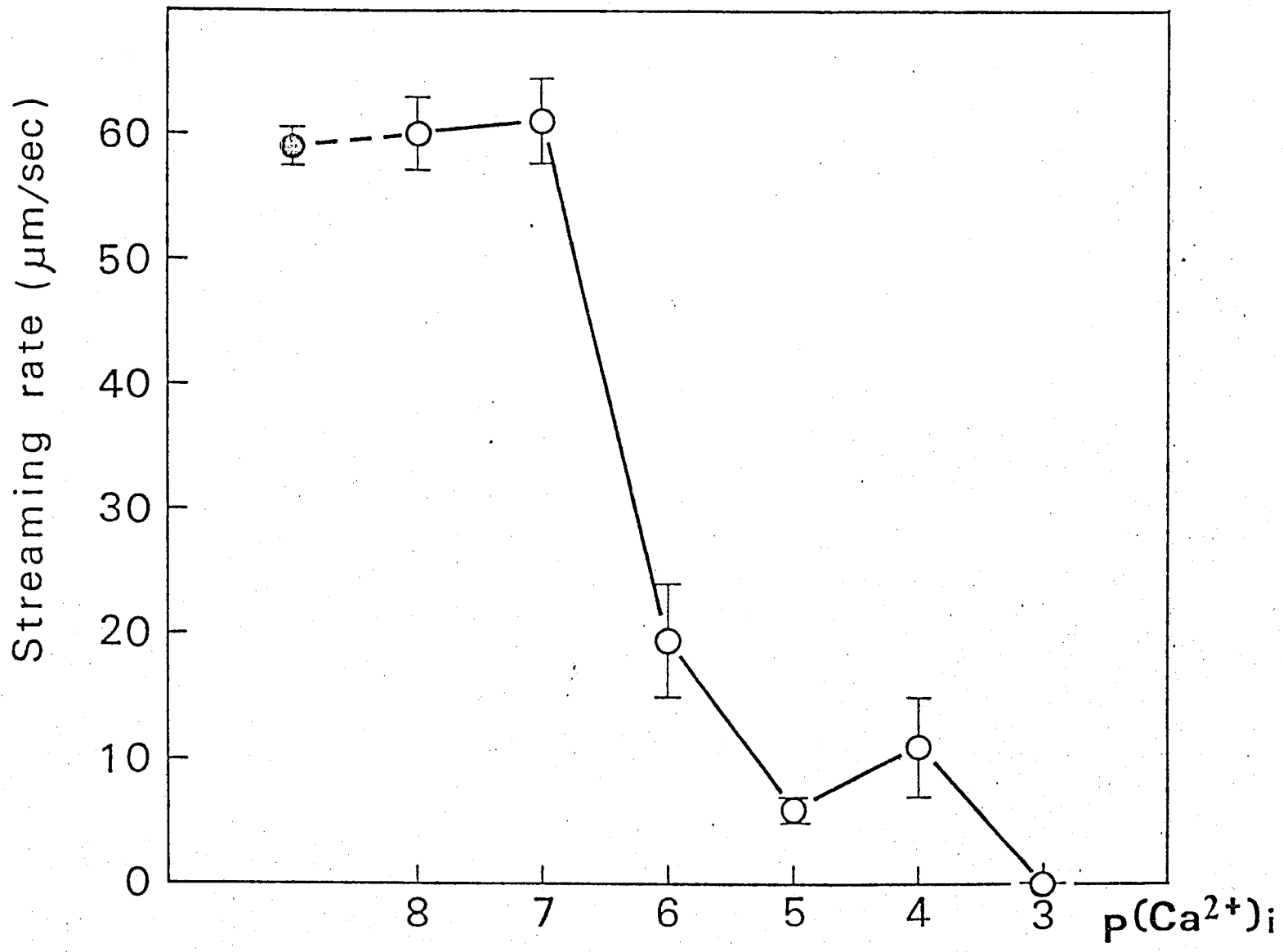

Fig. 8

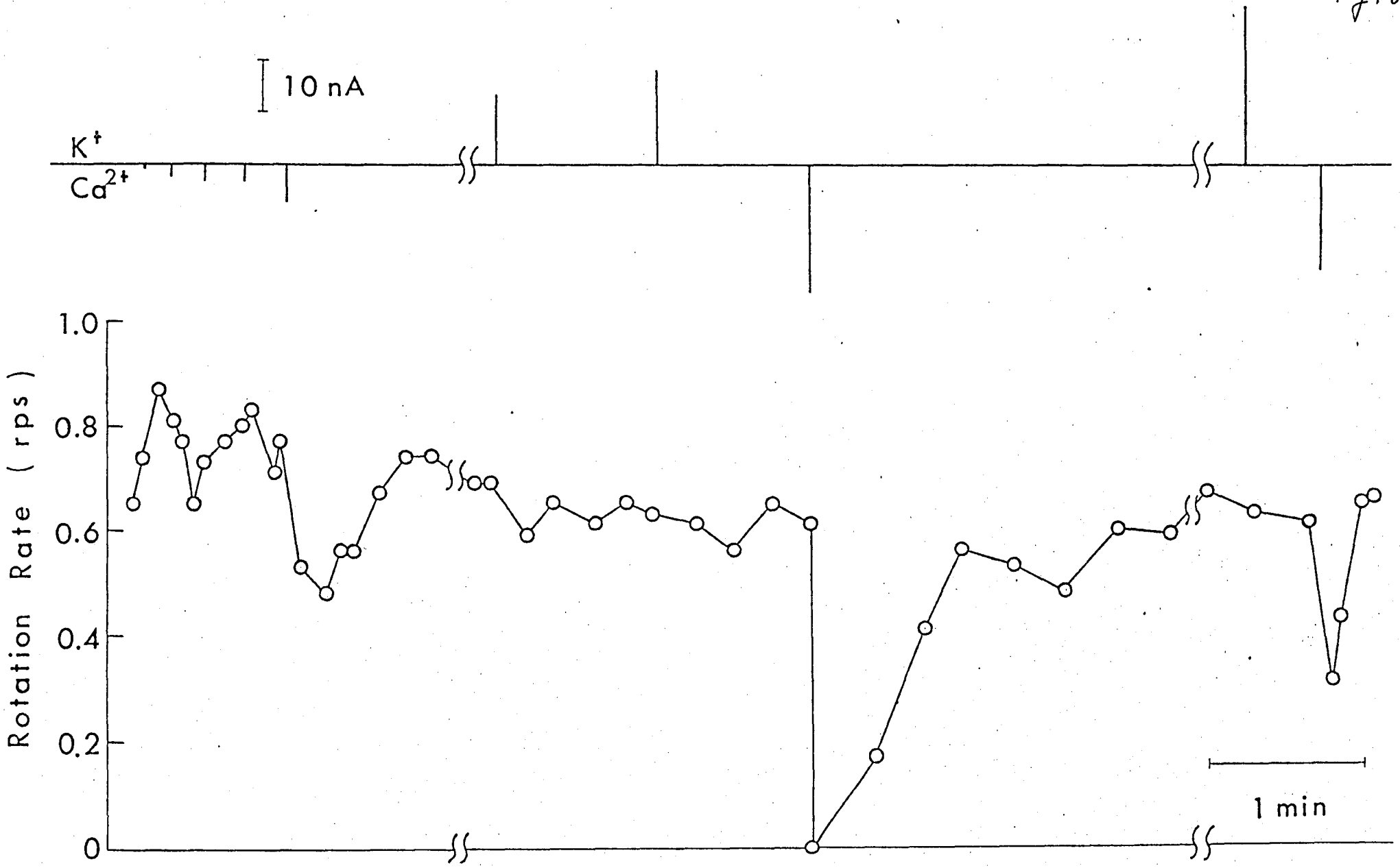

Fig. 9

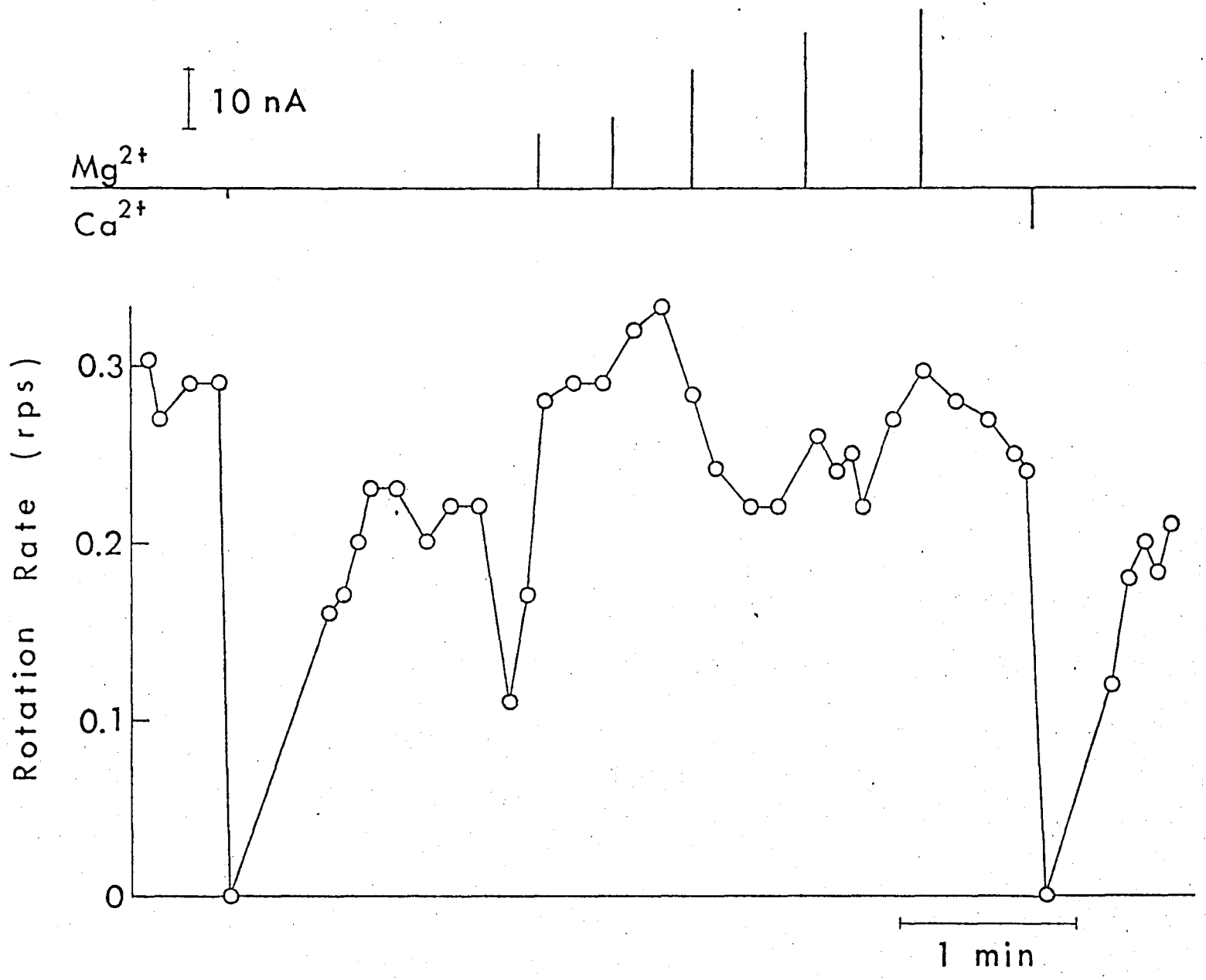

Fig. 10

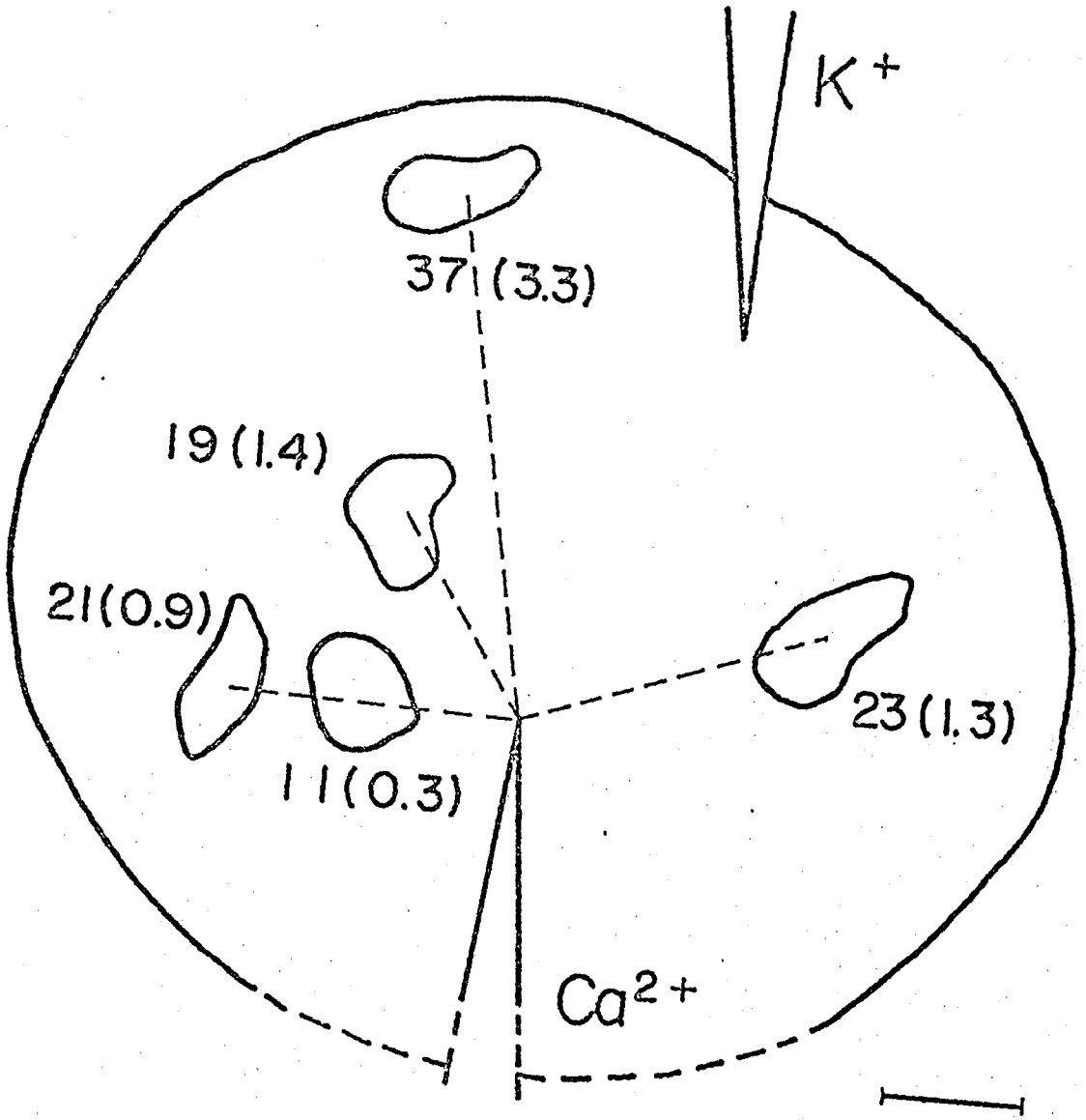

Fig. 11

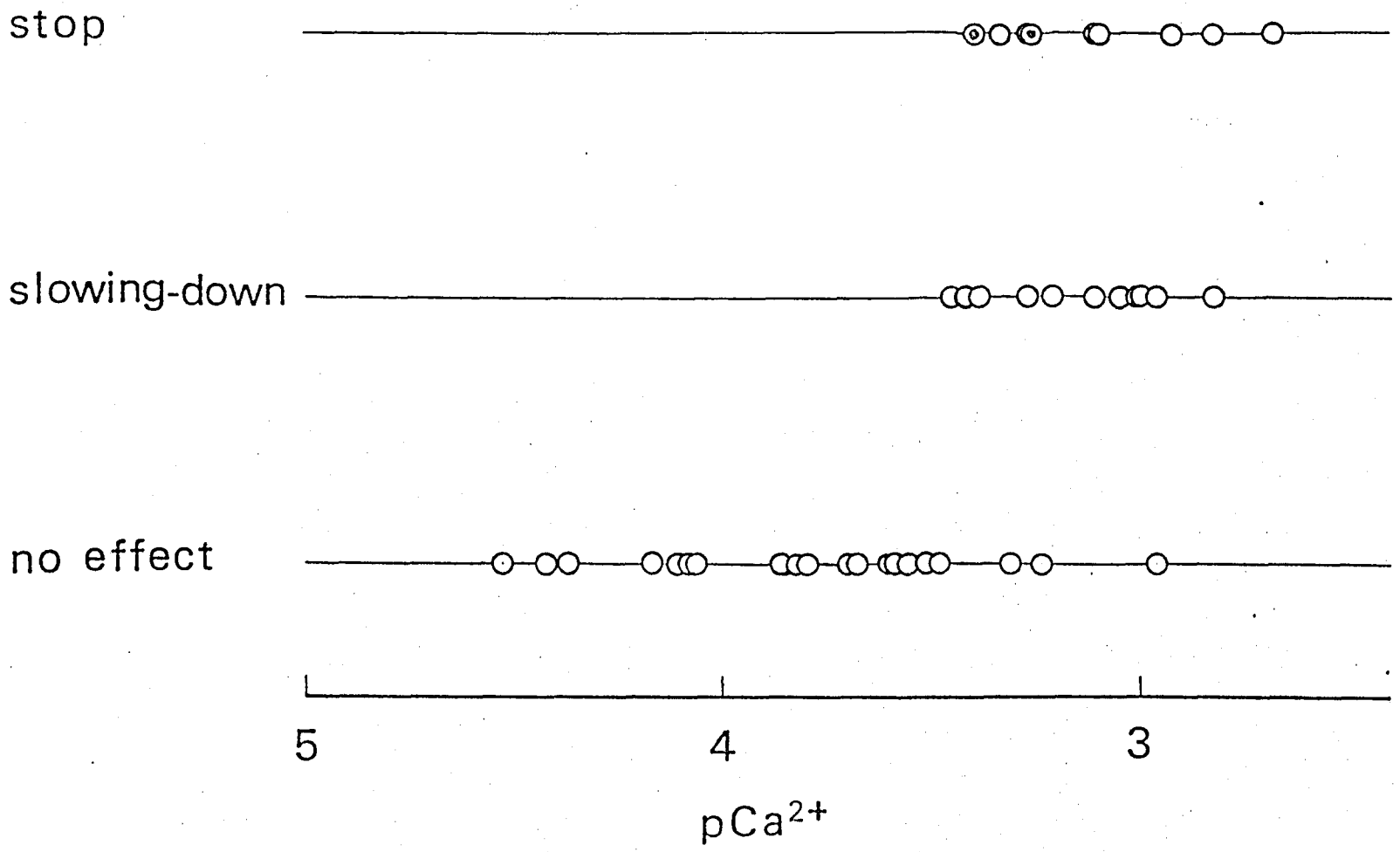

Fig. 12

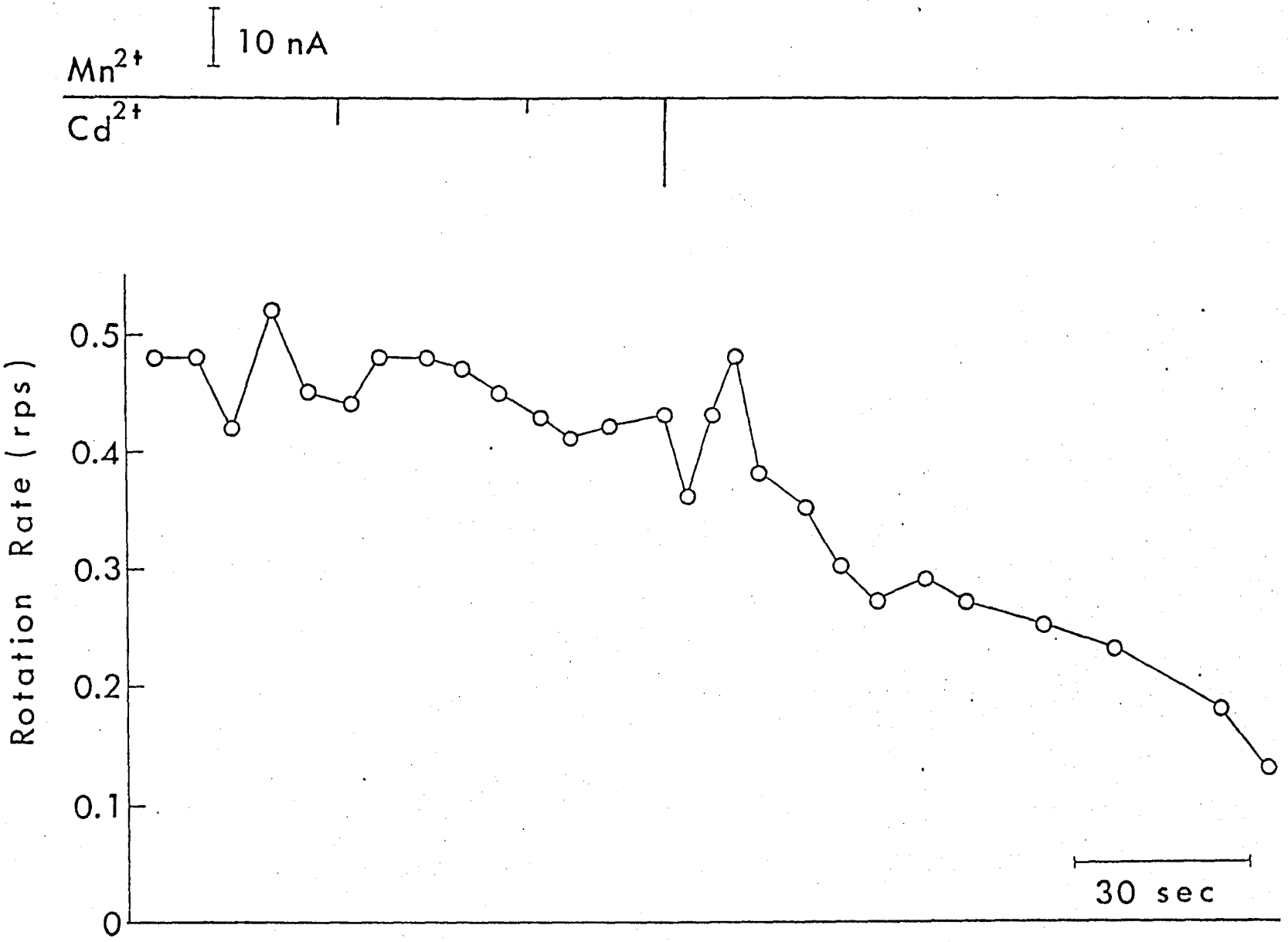

Fig. 13

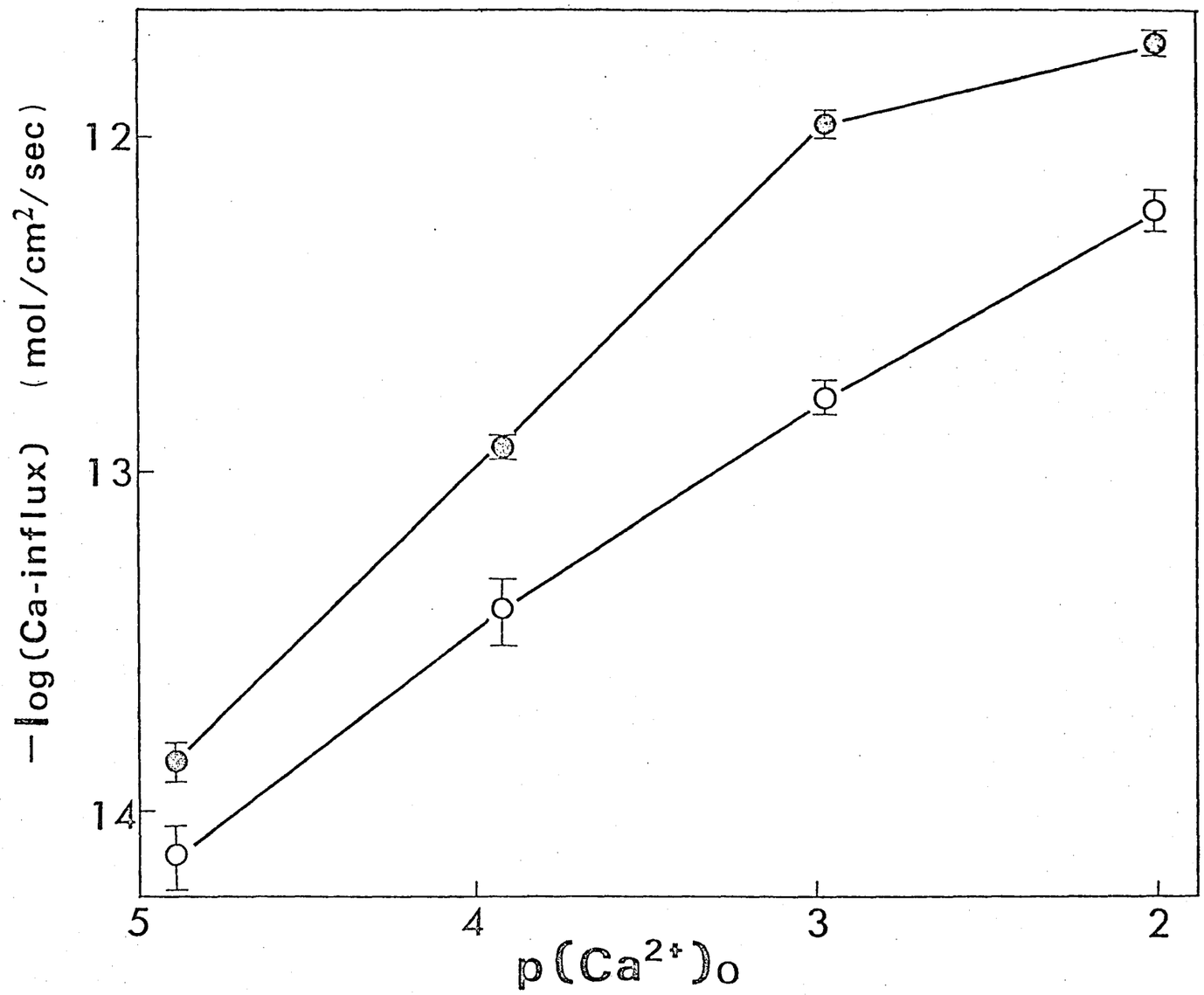

Fig. 14

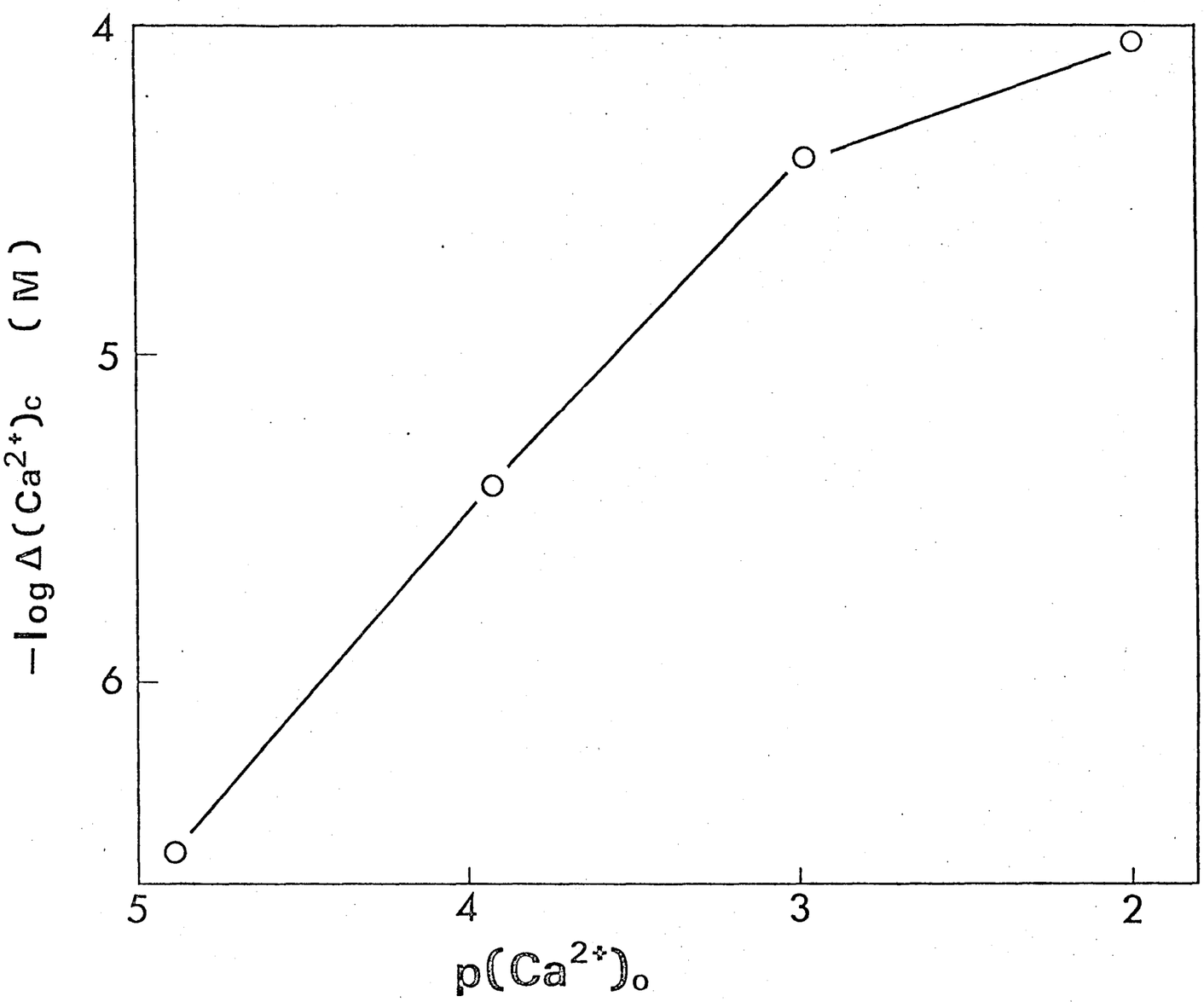

Fig. 15