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BIOCHEMICAL STUDIES OF THE EXCITABLE MEMBRANE OF *PARAMECIUM AURELIA*

I. $^{45}Ca^{2+}$ FLUXES ACROSS RESTING AND EXCITED MEMBRANE

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SUMMARY

The characteristics of Ca^{2+} transport across the excitable membrane of *Paramecium aurelia* were studied by measuring $^{45}Ca^{2+}$ influx and efflux. The intracellular concentration of free Ca^{2+} in resting *P. aurelia* was at least ten times less than the extracellular concentration. Ca^{2+} influx was easily measurable at 0 °C, but not at 23 °C. The influx of $^{45}Ca^{2+}$ was stimulated by the same conditions which cause membrane depolarization and ciliary reversal. Addition of Na^+ and K^+ (which stimulate ciliary reversal) resulted in a 10-fold increase in the rate of Ca^{2+} influx. An externally applied, pulsed, electric field (1–2 mA/cm^2 of electrode surface), caused the rate of Ca^{2+} influx to increase 3–5 times, with the extent of stimulation dependent on the current density and the pulse width. Ca^{2+} influx had the characteristics of a passive transport system and was associated with the chemically or electrically triggered Ca^{2+} "gating" mechanism, which has been studied electrophysiologically. In contrast, Ca^{2+} efflux appeared to be catalyzed by an active transport system. With cells previously loaded at 0 °C with $^{45}Ca^{2+}$, Ca^{2+} efflux was rapid at 23 °C, but did not occur at 0 °C. This active Ca^{2+} efflux mechanism is probably responsible for maintaining the low internal Ca^{2+} levels in unstimulated cells.

INTRODUCTION

The ciliate protozoan, *Paramecium aurelia*, has an excitable membrane which mediates the transfer of information from receptors for various stimuli to the numerous cilia located over the surface of the cell. *Paramecium* has proved to be an excellent organism with which to study the mechanisms associated with excitable membranes. This single-celled organism is large enough to allow the insertion of internal recording electrodes, and the electrophysiological studies of Eckert, Naitoh, Kinosita and their collaborators [1, 2] have established that the bounding membrane of these cells depolarizes in response to mechanical, chemical, or electrical stimuli. This depolarization is coupled to a reversal in the direction of ciliary beating, and consequently a reversal in swimming direction.

Electrophysiology indicates that a rapid influx of Ca^{2+} accompanies depolarization [3]. Studies of swimming behavior in cells made permeable to Ca^{2+} by mild treatment with detergent show that ciliary reversal results from an increased internal Ca^{2+} concentration [4]. Kung and his associates [5, 6] have selected mutants of *Paramecium* with altered swimming behavior, and have shown that many of the mutants have altered electrophysiological properties. Kung has thus begun to dissect, genetically, the components of an excitable membrane. We have begun biochemical studies of the excitable membrane of *Paramecium*, and in this paper we report direct measurements of Ca^{2+} fluxes across this membrane which confirm the deductions made from electrophysiological studies. Direct measurements of $^{45}Ca^{2+}$ fluxes associated with excitable systems have been made previously with squid axons and in barnacle muscle fibers [7–10].

EXPERIMENTAL PROCEDURE

Materials

$^{45}CaCl_2$ and [^{3}H]inulin were obtained from New England Nuclear. The 0.5 M HCl in which the $^{45}CaCl_2$ was dissolved was titrated to a phenol red endpoint with Tris base. RbCl was obtained from K and K Laboratories, and CsCl from Kawecki Berlyco Industries. Murexide was purchased from Sigma Chemical Co. All ions were used as the chloride salt unless otherwise noted.

Methods

Measurements of $^{45}Ca^{2+}$ uptake. Paramecium aurelia (syngen 4, stock 51 non-kappa bearing provided by Dr. C. Kung) was grown at 22–28 °C in cerophyl medium which was inoculated with *Aerobacter cloacae*, and supplemented with 1 mg/l of β-sitosterol [11]. The cells (2000–4000 cells/ml) were harvested at log phase by centrifugation at $150 \times g$ for 2–3 min in pear-shaped oil centrifuge tubes. The cells were transferred into Medium C: 10 % cerophyl media (v/v) (prepared without Na_2HPO_4), 1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 20 μM trisodium citrate, 20 μM Ca^{2+} and enough Tris (0.5–1.5 mM) to bring the pH to 7.0. Cells were washed once with Medium C, then allowed to equilibrate for 1 h in the same solution at 23 °C.

A 10 % cerophyl solution contained 10 μM Ca^{2+} as measured with a Perkin-Elmer 430 atomic absorption spectrophotometer. The Ca^{2+} concentration was also determined by a second method which measures primarily free Ca^{2+}; the increase in absorbance at 470 nm of a solution of murexide in the presence of Ca^{2+} was used as an indicator of the amount of free Ca^{2+} [12]. The absorbance of a 10 % cerophyl plus murexide solution was compared with a standard curve, and a correction was made for the amount of Na^+ and K^+ present in Medium C, since Na^+ and K^+ did decrease the absorbance at 470 nm. This method gave a free Ca^{2+} concentration of 10 μM Ca^{2+} in a 10 % cerophyl solution. Whenever sodium citrate was added, an equivalent amount of Ca^{2+} was added so that the free Ca^{2+} concentration remained unaltered. Initially, assays were done with a free Ca^{2+} concentration of 10 μM; later 25 μM was used. Na^+ and K^+ levels were determined with an IL model 430 flame photometer. The Na^+ concentration in Medium C was 90 μM and the K^+ level was 300 μM.

Cell densities of 5000–15 000 cells/ml were used in the Ca^{2+} uptake experiments. The cell suspensions were placed on ice 20 min prior to $^{45}Ca^{2+}$ addition and were kept at 0 °C during the experiment. Ca^{2+} uptake was measured in Medium C and was initiated by the addition of $^{45}Ca^{2+}$ to a final specific activity of $2 \cdot 10^3$–$4 \cdot 10^3$ cpm/nmol Ca^{2+} (as determined by liquid scintillation). 3-ml aliquots were passed over 8 μm pore size Millipore filters (at a rate of 3 ml/s), and the cells on the filter were washed with 10 ml of 1 mM HEPES/Tris buffer, pH 7.0, containing 1 mM unlabeled Ca^{2+} at room temperature. The relatively high Ca^{2+} concentration in the wash reduced the amount of $^{45}Ca^{2+}$ bound to the filter or the cell surface, but did not displace intracellular $^{45}Ca^{2+}$, since Ca^{2+} efflux is negligible over the 10 s required for washing (see Results). A centrifuge assay developed by Hansma [13] gave values similar to those obtained with this filter assay. Before use, all filters were soaked in the wash solution. The filters were counted either with a low background gas flow counter or by liquid scintillation in 5 ml of scintillation fluid [14]. The blank for these experiments (same procedure but without cells) accounted for 60–80 % of the zero time value. To determine cell mass per aliquot, cell numbers were routinely determined by direct count. 1000 cells contained 17-22 μg of protein.

The use of 8-μm pore size filters allows a fast filtration rate and prevents the buildup of a large pressure differential across the filter which might cause cell lysis. Filtration with and without negative pressure gave the same values for Ca^{2+} uptake.

The pH dependence of Ca^{2+} uptake was determined in two buffers, 1 mM Tris · HCl for pH values between 7.15 and 8.5, and 1 mM HEPES/Tris · HCl for pH values between 5.6 and 7.0. The concentration of the buffer was identical (except for HCl) at each pH. Cells were chilled and assayed immediately upon transfer into the new pH solution. Data obtained from the use of the two buffer systems were normalized at pH 7.0 in order to avoid complications from differing buffer effects.

Determination of the intracellular concentration of free Ca^{2+}. Cells were washed into 1 mM Ca^{2+}, 1 mM HEPES/Tris, pH 7.0, buffer and 100 μM trisodium citrate; $^{45}Ca^{2+}$ was added to a specific activity of 1000 cpm/nmol Ca^{2+} and the cells were allowed to equilibrate 8–24 h. Aliquots (3 ml) were filtered and washed as above. A second aliquot was treated in the same manner, and after the wash, cells were lysed on the filter with 4 ml of 50 % ethanol/buffer solution, followed by 2 ml of the wash solution. Blanks (no cells) were measured to determine $^{45}Ca^{2+}$ binding to the filters for each of the above treatments. As noted below, this method allows us to place an upper limit on internal free Ca^{2+}. The approximate intracellular volume was determined by treating the cell as an ellipsoid with a major axis of 140 μm and a minor axis of 50 μm. A value of $1.5 \cdot 10^{-7}$ ml/cell was calculated. To determine the surface area, the cell was approximated by a cylinder 140 μm long with a 40 μm diameter with 4500 cilia per cell, each with a surface area of 6–8 μm^2. The surface area by this calculation was 45 000 μm^2/cell.

Dark field microscopy. A dark field photomicrograph technique was used to observe the swimming behavior of the cells. An American Optical dissection microscope was used with the slide illuminated obliquely by two high intensity lamps. A Nikon Microflex photomicrographic attachment was used with a Polaroid camera. The electrical stimulation chamber consisted of a slide having a rectangular well $10.0 \times 13.5 \times 1.0$ mm. Two nichrome strips at opposite ends of the welll served as

electrodes and were separated by 1 cm. The chamber was filled with 0.1 ml of Medium C and 100–200 cells were added. The cells were allowed to equilibrate for 3 min prior to photography. Stimulation was provided by a variable pulse-width, square wave generator, and the camera shutter was opened immediately after stimulation began. All experiments were done at 24 °C and no attempt was made to control temperature during the stimulation.

Electrical stimulation of Ca^{2+} influx. A rectangular glass chamber ($7.0 \times 1.5 \times 1.0$ cm) was placed in an ice bath during the experiment. Two opposite sides of the chamber were covered with platinum foil with a 1 cm separation of the two electrodes. Ca^{2+} uptake was measured as above, except that the cells were not placed in the chamber until immediately after the first time point. After 10 min of stimulation, the temperature was found to be 0–1 °C, even when the highest current densities were used. Electrically shocked cells appeared undamaged and regained motility after warming at the end of the experiment.

Ca^{2+} efflux. Cells were incubated in Medium C for 10 min at 0 °C. Then $^{45}Ca^{2+}$ was added to a specific activity of 10 000 cpm/nmol and the K^+ concentration was raised to 10 mM. The cells were left at 0 °C for 30 min. Efflux was initiated by transferring 2–3 ml of labeled cells into 100 ml of the same solution without $^{45}Ca^{2+}$ and 20-ml aliquots were filtered at timed intervals. Since a semi-log plot of efflux data was linear over the first 10 min, the initial rate of Ca^{2+} efflux was determined by taking the best linear fit to the data on a semi-log plot solving the equation: $\ln \text{cpm}_t/\text{cpm}_{t=0} = -kt$ for k using data from experimental points over 16 min. Rates are expressed for the first min of efflux.

EXPERIMENTAL RESULTS

(A) *Intracellular free Ca^{2+}.* Total cellular Ca^{2+} was determined by filtration and washing of the cells after equilibration with $^{45}Ca^{2+}$ at a known specific activity. A correction was made for bound Ca^{2+} by subtracting from total cellular Ca^{2+}, the amount of Ca^{2+} bound to the cells following lysis of the cells on the filter with 50 % ethanol and a short further washing with non-radioactive Ca^{2+}. Using this procedure, 40–50 % of the total Ca^{2+} was removed with the ethanol wash. At extracellular concentrations of 0.1, 0.5, 1.0, 5.0, and 10.0 mM Ca^{2+}, the intracellular concentrations of free Ca^{2+} were 30–50, 50–230, 30–380, 210, and 630 μM, respectively, while the total Ca^{2+} concentration was 0.11–0.15, 0.57–0.70, 0.93–1.10, 2.76 and 2.76 mM, respectively. This method certainly results in an overestimate of the intracellular concentration of free Ca^{2+}, since (a) loosely bound Ca^{2+} is probably removed during lysis and subsequent washing, (b) small organelles and soluble proteins which bind Ca^{2+} are not retained on the 8-μm filters, and (c) no correction was made for exchange of unlabeled and labeled Ca^{2+} after cell lysis. Our results therefore provide direct evidence that internal Ca^{2+} levels are lower than external levels, but underestimate the size of the concentration gradient.

(B) *Calcium uptake at 0 °C.* Little Ca^{2+} uptake was observed at 22 °C, but at 0 C° uptake of Ca^{2+} was measurable (Fig. 1). Results described below indicate that at 0 °C, the Ca^{2+} extrusion pump is inhibited more than the Ca^{2+} influx system.

(C) *K^+ and Na^+ stimulate Ca^{2+} influx.* The addition of 10 mM Na^+ or K^+ increased the rate of Ca^{2+} influx 5–20-fold (20 experiments) at 0 °C, but not at 22 °C

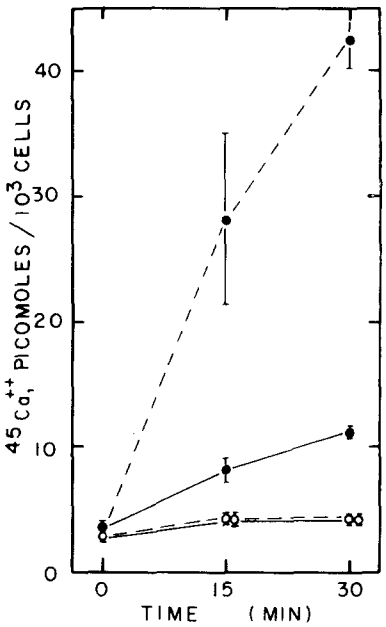

Fig. 1. Effects of Na^+ and temperature on $^{45}Ca^{2+}$ uptake. Ca^{2+} uptake was measured as described in Methods with (– – –) and without (—) the addition of 10 mM Na^+ at time zero. Uptake was measured at two temperatures, 22 °C (○) and at 0 °C (●); the error bars represent the range of two determinations.

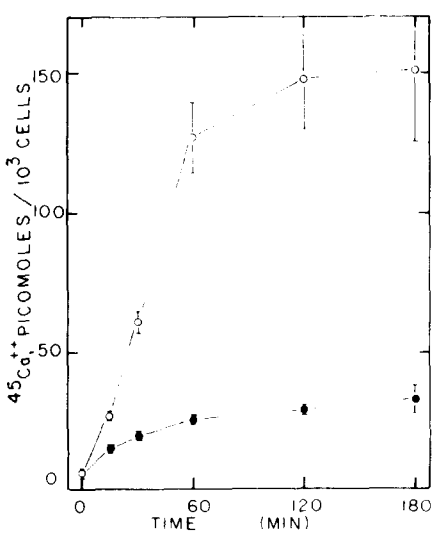

Fig. 2. Extent of $^{45}Ca^{2+}$ uptake. Uptake was measured at 0 °C, as described in Methods. At time zero, 10 mM Na^+ was added to one sample (○); solid circles (●) represent uptake by unstimulated cells. Each point represents the average of two determinations.

(Fig. 1). The unstimulated rate of Ca^{2+} influx ranged from 0.3 to 1.0 pmol/10^3 cells per min in 20 experiments, while K^+-stimulated rates ranged from 3 to 10 pmol/10^3 cells per min. No Na^+- or K^+-triggered Ca^{2+} influx was observed at 10 °C. After obtaining most of the data reported here, we discovered the rate of Ca^{2+} influx was several times faster (10 pmol/10^3 cells per min) when 20 mM citrate was included in the assay buffer. Stimulation of Ca^{2+} influx by Na^+ or K^+ was not due to osmotic imbalances, since the addition of 20 mM sucrose had no effect on Ca^{2+} influx.

In the presence of 10 mM K^+, $^{45}Ca^{2+}$ uptake slowed after 1 h, and equilibration was complete in 3 h, with a final intracellular total Ca^{2+} concentration of 1 mM (Fig. 2). The fact that the intracellular concentration was higher than the external level may reflect extensive internal Ca^{2+} binding. Uptake of Ca^{2+} in the absence of stimulation was not complete after 3 h.

(D) *Effects of divalent cations on Ca^{2+} influx.* The initial rate of $^{45}Ca^{2+}$ uptake increased with increasing concentrations of external Ca^{2+}, but above 20–50 μM up to 1 mM Ca^{2+}, no further increase in the rate of K^+-stimulated Ca^{2+} influx was observed (Fig. 3). At higher levels of external K^+ (15 and 30 mM), no further stimulation of Ca^{2+} influx occurred. Hence, maximal stimulation occurred at 10 mM K^+ regardless of Ca^{2+} concentration. In the unstimulated case, the influx rate increased slowly above 20–50 μM, reaching a rate of 2.0–2.5 pmol/10^3 cells per min at 1 mM Ca^{2+}.

The divalent cations Mg^{2+}, Sr^{2+}, Ba^{2+} (all at 5 mM), and Ni^{2+}, Mn^{2+} and Co^{2+} (all at 200 μM) strongly inhibited Ca^{2+} influx (Table I). At high concentrations (10 mM) of Ba^{2+}, detached cilia were observed. At a lower concentration (1 mM), Ba^{2+} still inhibited K^+-stimulated Ca^{2+} influx (Fig. 4a) but no free cilia or visible

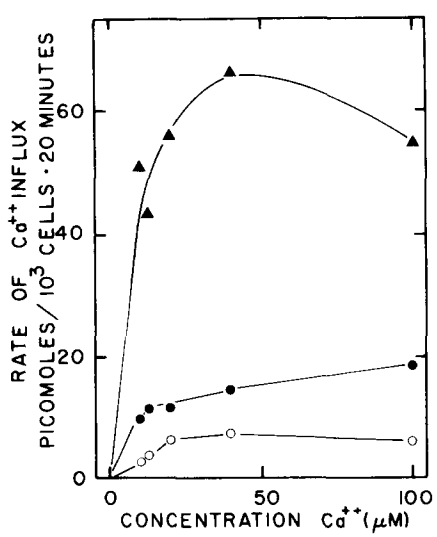

Fig. 3. Effects of Ca^{2+} on the rate of Ca^{2+} uptake. Ca^{2+} was added at time zero to the desired concentration and Ca^{2+} influx was measured at 0 °C as described in Methods. Cells were equilibrated for 30 min in 10 μM Ca^{2+} in Medium C prior to beginning the experiment. K^+ was also added at time zero to stimulate Ca^{2+} uptake: 3 mM K^+ (●—●), 10 mM K^+ (▲—▲), and the unstimulated control (○—○).

TABLE I

THE EFFECTS OF DIVALENT CATIONS ON THE RATE OF Ca^{2+} INFLUX

Ca^{2+} influx was measured as described in Methods and the ions were added at time zero.

Ion	Concentration	Rate of Ca^{2+} influx (pmol/10^3 cells per 15 min)
None	–	20
Mg^{2+}	5 mM	2
Sr^{2+}	5 mM	1
Ba^{2+}	5 mM	1
Ni^{2+}	200 μM	4
Mn^{2+}	200 μM	7
Co^{2+}	200 μM	2

changes in the cell's shape were noted, Cells exposed to concentrations of Ba^{2+} higher than 1 mM quickly became immotile, and it was necessary to determine whether the added Ba^{2+} was simply toxic to the cells. When cells were exposed to 5 mM Ba^{2+} for 15 min, then washed out of Ba^{2+} and assayed for Ca^{2+} influx, no difference was found between Ba^{2+}-treated and untreated cells (Fig. 4b); thus the effect of Ba^{2+} was reversible. The decrease in the amount of $^{45}Ca^{2+}$ in cells after treatment with Ba^{2+} was a result of losing some of the cells during the washing procedure.

(E) *Effects of monovalent cations on Ca^{2+} influx.* To determine whether ciliary reversal at 23 °C and $^{45}Ca^{2+}$ influx at 0 °C responded to the same stimuli, we measured the duration of ciliary reversal and the rate of Ca^{2+} influx under various conditions. The rate of Ca^{2+} influx increased with increasing K^+ concentrations, up to a maximum at 10 mM K^+ (Fig. 5); K^+ concentrations above 10 mM were

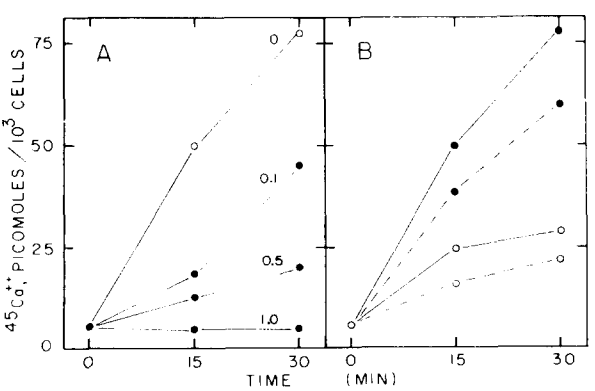

Fig. 4 (A) Inhibition of K^+-stimulated Ca^{2+} influx by Ba^{2+}. All assays were in the presence of 10 mM K^+ at 0 °C, as described in Methods. Both Ba^{2+} and K^+ were added at time zero, with Ba^{2+} concentrations of 0, 0.1, 0.5, and 1.0 mM. (B) Reversibility of Ba^{2+} inhibition of Ca^{2+} influx. Cells were placed on ice in Medium C for 5 min, then Ba^{2+} was added to 5 mM and the cells chilled for a further 15 min. The cells were washed twice into chilled Ba^{2+}-free Medium C and assayed for Ca^{2+} influx. The broken line (– – –) represents Ba^{2+}-treated cells, and the solid line (—), untreated cells; ● — ●, with 10 mM K^+; ○ — ○, no added K^+.

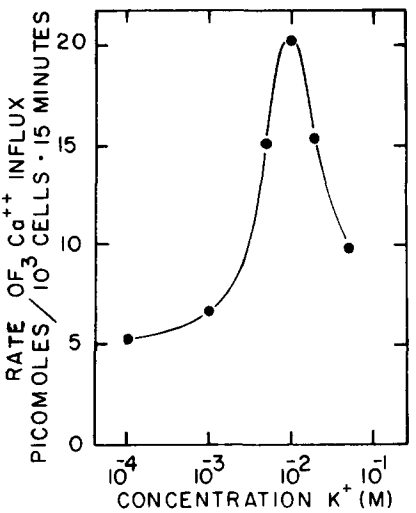

Fig. 5. The effects of K^+ concentrations on $^{45}Ca^{2+}$ uptake. K^+ was added at time zero, and the rate of $^{45}Ca^{2+}$ uptake at 0 °C was taken as the difference between the amount of Ca^{2+} taken up at 15 and 0 min. Each point represents the average of two determinations.

inhibitory. The length of continuous ciliary reversal following the addition of various concentrations of K^+ in Medium C was measured by the method of Naitoh [15], and was also found to be maximal at 10–20 mM, while higher concentrations of K^+ decreased the response.

The effects of several monovalent cations on the rate of Ca^{2+} influx corresponded with their effects on duration of ciliary reversal (Table II).

(F) *pH dependence of Ca^{2+} influx.* K^+-triggered Ca^{2+} influx was not observed below pH 6.0–6.5, and was maximal at 7.5–8.0. The unstimulated rate of Ca^{2+} influx was lowest at pH 7.0–8.0 and increased outside of this range.

(G) *Electrical stimulation of Ca^{2+} influx.* Time exposure photographs of cells viewed by dark-field microscopy illustrated the effects of pulsed current of

TABLE II

THE EFFECTS OF MONOVALENT CATIONS ON THE RATE OF Ca^{2+} INFLUX

Ca^{2+} influx was measured as described in Methods and the ions were added at time zero. All ions were added at 10 mM.

Ion	Rate of Ca^{2+} influx (pmol/10^3 cells per 15 min)	Duration of ciliary reversal (s)
None	20	0
Cs^+	30	0
Li^+	30	20
Na^+	39	30
Rb^+	48	50
K^+	58	55

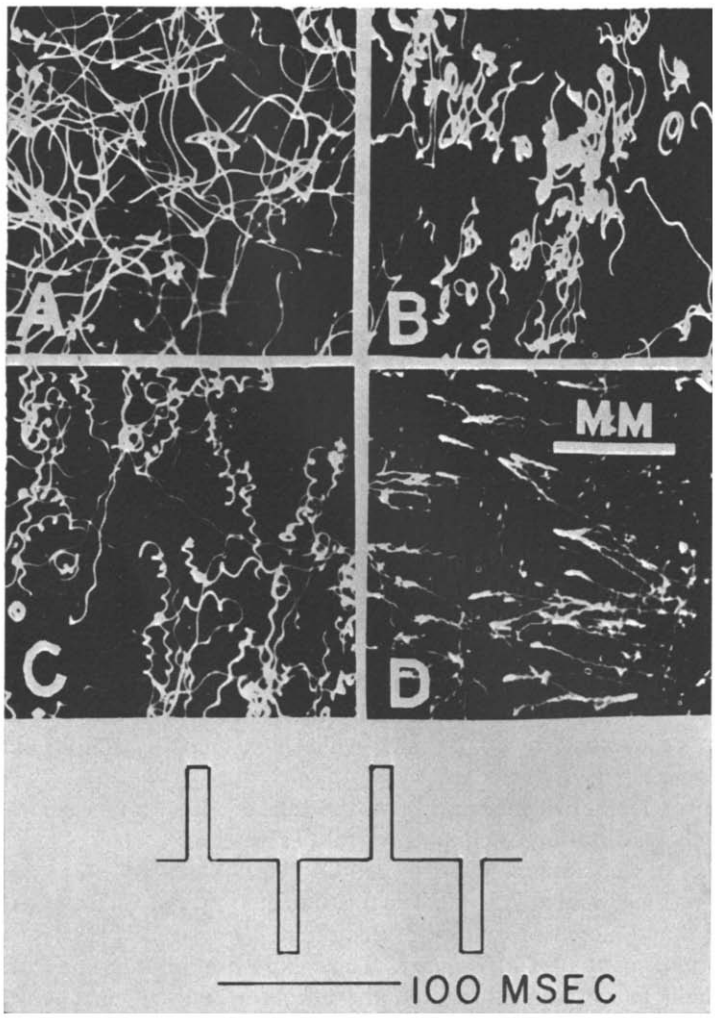

Fig. 6. Dark-field photomicrographs illustrating the effects of electrical pulses of varying current density on the behavior of wild type cells. Each track represents the path of one cell during the application of a series of square voltage pulses. The film was exposed for 5 s. The stimulus consisted of a 10 Hz pulse train, where each pulse had a duration of 10 ms. The current density for photographs A–D was respectively, 0, 1.2, 2.4 and 11.50 mA/cm^2, while the corresponding voltage was, 0, 2.5, 5.0 and 25 V/cm, respectively. The direction of current flow is in the same direction as the time calibration bar. The electrodes were positioned 1.0 cm apart. The cells were preequilibrated and stimulated in Medium C. The bar in the photograph represents 1 mm.

alternating polarity on swimming behavior (Fig. 6). As current was applied, an increasing number of sharp bends were found in the paths, indicating a ciliary reversal response to each current pulse. At the highest current density shown, continuous ciliary reversal was observed. This was indicated by the short thick lines with a higher frequency of spiralling (for a further discussion of the interpretation of dark-field micrographs of *Paramecium* behavior, see ref. 5). The response of cells to increasing pulse widths at a constant current density was similar to that observed for increasing the current density.

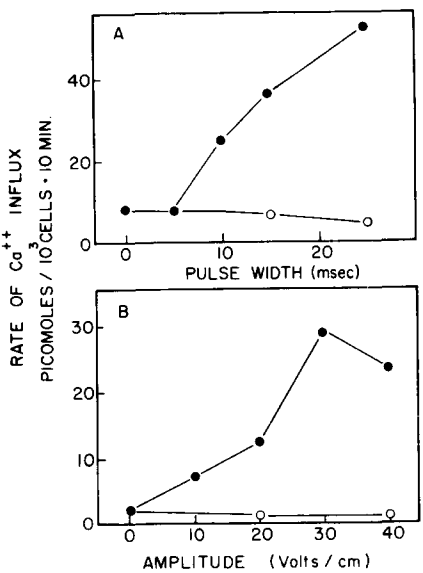

Fig. 7. The effect of an externally applied, pulsed electric field on the rate of Ca^{2+} influx. In both cases, the closed circles represent cells exposed to the electric field, while the open circles represent uptake by unstimulated cells assayed for Ca^{2+} influx in a solution which had been pretreated with the electric field as described in Methods. Under these conditions, with a potential of 10 V/cm, current density was 0.7 mA/cm^2. (A) The effect of pulse width on the rate of Ca^{2+} influx. The current was pulsed at a frequency of 10 Hz, at a potential of 20 V/cm. (B) The effect of electrical potential on the rate of Ca^{2+} influx. The current was pulsed at a frequency of 10 Hz at a pulse width of 10 ms.

Electrical stimulation with pulses of increasing current density increased the rate of Ca^{2+} influx to a level approximately half that obtained by stimulation with 10 mM K^+ (Fig. 7). Alternating polarity current of a low density at the electrode surfaces is not effective in causing electrolysis of the solution [16] and no gas evolution was ever noted at the electrode surface. Nonetheless, to be sure that the apparent electrical stimulation of Ca^{2+} uptake was not an artifact resulting from electrode breakdown or chlorine production and subsequent chemical reaction with cells, we "stimulated" solutions without cells under conditions identical to those used in Fig. 7, then transferred fresh cells into the "stimulated" solution and assayed for Ca^{2+} influx. Previous exposure of the solution to varying current densities had no effect on the rate of Ca^{2+} influx (Fig. 7).

(H) *Other factors which influence* $^{45}Ca^{2+}$ *influx.* Since Paramecia ingest their food by phagocytosis, the contribution of this form of uptake was examined. There was a very small uptake of [^{3}H]inulin (assumed to be representative of phagocytosis) by Paramecia in the assay solution at room temperature; however, the kinetics of this uptake was identical with the absorption of [^{3}H]inulin by cells which had been killed by a short heat shock (100 °C for 30 s; after this treatment cells are still discernible as whole bodies). This suggested that the slow rate of [^{3}H]inulin uptake was a binding process and not active phagocytosis or pinocytosis. Presumably, *Paramecium* does not phagocytose in the bacteria-free conditions of the assay solution. Pinocytosis has never been observed in *Paramecium* [17]. Phagocytosis is most

certainly an energy-requiring process and should be considerably slowed at 0 °C. For these reasons, we do not believe that phagocytosis makes a major contribution to Ca^{2+} influx.

We found that the stability of *Paramecium* in low Ca^{2+} solutions or solutions which contain only Ca^{2+} was dependent on the heavy metal content of the solution and was dramatically improved upon addition of Ca-EDTA or calcium citrate. Cell survival in low Ca^{2+} solutions was poor and the Ca^{2+} influx assay was not reproducible in the absence of a chelating agent. This probably accounts for the increased rate of Ca^{2+} influx in the presence of calcium citrate or Ca-EDTA. Cells consistently regained normal motility upon warming after measurement of Ca^{2+} influx.

The growth stage of the cells affected the ability of monovalent cations to stimulate Ca^{2+} influx. Stimulation was maximal shortly after transfer into fresh medium and during log phase growth. Stationary phase cells showed little response to the addition of monovalent cations (at 10 mM). This was found to be due partially to the higher concentration of K^+ required for maximal stimulation. The duration of ciliary reversal during the various phases of cell growth also was found to parallel the ability to show K^+-stimulated Ca^{2+} influx.

(I) *Ca^{2+} efflux.* Cells which were loaded with $^{45}Ca^{2+}$ at 0 °C took up enough Ca^{2+} to allow efflux studies. Ca^{2+} efflux at room temperature occurred at an initial rate of 15–25 pmol $Ca^{2+}/10^3$ cells per min and the half time for total efflux was 4–6 min (range of six determinations) (Fig. 8). Ca^{2+} efflux was almost completely inhibited at 0 °C, and was not significantly affected by the presence of either 10 mM Ca^{2+} or 10 mM K^+ in the assay medium during efflux. When the energy poisons NaN_3 (2 mM) or NaCN (2 mM) were present during the last 20 min of loading and during efflux, cellular ATP levels were reduced by 95 % (Browning, J. L., unpublished

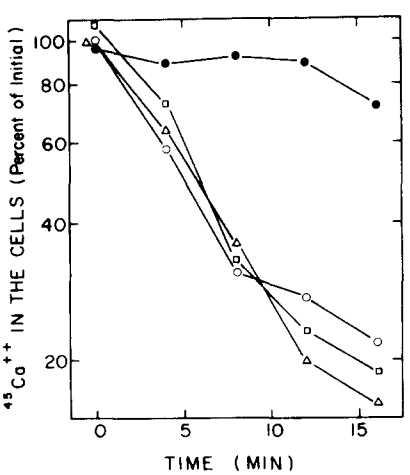

Fig. 8. The effect of temperature and energy poisons on Ca^{2+} efflux. Cells were loaded with $^{45}Ca^{2+}$, and efflux was measured as described in Methods, at 23 °C (○—○), 0 °C (●—●), 23 °C with 2 mM NaN_3 (□—□), and 23 °C with 2 mM NaCN (△—△). Cells were exposed to the energy poisons for 20 min prior to assaying for Ca^{2+} efflux. At time zero, 10 % of the $^{45}Ca^{2+}$ in cells remained associated with cells even after a 30 s treatment at 100 °C.

observations), but neither energy poison inhibited Ca^{2+} efflux measurably (Fig. 8).

Ca^{2+} in the pool from which efflux was measured was assumed to be labeled at the same specific activity as Ca^{2+} in the loading solution, for these reasons: (1) Since little $^{45}Ca^{2+}$ influx is observed at room temperature, this Ca^{2+} pool must be small and Ca^{2+} in this pool does not readily exchange with Ca^{2+} from other pools. (2) When cells are labeled at 0 °C with $^{45}Ca^{2+}$, and efflux is measured, at least 90 % of the $^{45}Ca^{2+}$ is quickly removed; indicating that the Ca^{2+} pool labeled at 0 °C does not readily exchange with other Ca^{2+} pools.

DISCUSSION

Our direct measurement of $^{45}Ca^{2+}$ fluxes support the following model developed from the electrophysiological and behavioral studies of Naitoh, Eckert and coworkers [1–4], for the regulation of swimming behavior in *Paramecium*. A low internal Ca^{2+} level is normally maintained by an active Ca^{2+} extrusion pump. Chemical or electrical stimulation causes membrane depolarization and the opening of a Ca^{2+} gate, through which Ca^{2+} rushes into the cell. The elevated internal Ca^{2+} concentration triggers ciliary reversal, which continues until the Ca^{2+} pump has reduced internal Ca^{2+} to the pre-stimulation level. Using $^{45}Ca^{2+}$, we have measured directly the activity of the influx mechanism and of the extrusion pump.

Properties of the Ca^{2+} efflux system. Internal Ca^{2+} is at least 10-fold lower than external, and very probably much lower, since our experimental bias almost certainly leads to overestimation of the internal free Ca^{2+} concentration. An indirect measurement of the internal Ca^{2+} level in the region of the ciliary reversal apparatus was obtained by Naitoh [18]. He showed that in cells made freely permeable to small ions by a mild detergent treatment, the cilia reversed their beating direction when the internal Ca^{2+} concentration was raised from 10^{-8} to 10^{-6} M. This result indicates that in the "resting" state the internal Ca^{2+} concentration must be below 10^{-6} M. Thus, at room temperature the Ca^{2+} extrusion mechanism is very effective, a conclusion which is consistent with the fact that ciliary reversal in response to mechanical stimulus is of very short duration (less than 1 s).

We have shown by direct measurement that Ca^{2+} efflux is strongly inhibited at low temperature (Fig. 8), and this fact has allowed us to measure at 0 °C Ca^{2+} influx, which is apparently less temperature dependent. Although the direct extrusion of Ca^{2+} is likely to be an active process which requires metabolic energy, we have not been able to inhibit efflux by poisoning cells with cyanide or azide, which drastically reduced ATP levels. Paramecia may have a Ca^{2+} exchange system analogous to the ATP-independent ($Na^+ + Ca^{2+}$)-exchange systems found in squid axons and barnacle muscle [7, 8, 10]. This model would account for the lack of any direct effect of energy poisons on Ca^{2+} efflux.

Properties of the Ca^{2+} influx system. Ca^{2+} influx at 0 C° had the properties expected for a gated pore, not those of an active transport process. The direction of flux was down the concentration gradient, the rate of flux was altered by ionic or electrical stimulation, and the process occurred at 0 °C. Ion flux through the Na^+ channel of neurons is also less dependent on temperature ($Q_{10} = 1.1$–1.4) than typical enzymatic reactions [19]. In the resting state, the amount of Ca^{2+} influx should equal the amount of Ca^{2+} efflux. The initial rate of Ca^{2+} efflux could not be

compared with the resting Ca^{2+} influx rate, since abnormally high levels of Ca^{2+} were in the cell at the beginning of the efflux assay. The specificity of the influx mechanism was relatively low for both the transported species and the stimulating species. Both Ba^{2+} and Sr^{2+} inhibited Ca^{2+} influx when added in large excess over Ca^{2+}; and, although we have not determined whether they actually enter cells, their effects on swimming behavior suggest that they probably do enter. From electrophysiological studies, it was known that Ba^{2+} and Sr^{2+} can carry current both in *Paramecium* and in a similar Ca^{2+}-controlled system, barnacle muscle [20, 21]. In addition, Naitoh and Kaneko have shown that Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in eliciting the ciliary reversal response [18]. Since Ba^{2+} and Sr^{2+} (and Mg^{2+} to a smaller extent) stimulate ciliary reversal, yet completely and reversibly block Ca^{2+} influx, it appears that Ba^{2+}, Sr^{2+} and Ca^{2+} are competing for the same transport mechanism. Our attempts to measure $^{133}Ba^{2+}$ influx failed because at the Ba^{2+} concentration needed to give good inhibition of Ca^{2+} unflux, the amount of $^{133}Ba^{2+}$ binding was so large as to obscure any entry that may have occurred. Co^{2+}, Mn^{2+}, and Ni^{2+} also inhibited Ca^{2+} influx in *Paramecium*, as they do inhibit the Ca^{2+} gate of barnacle muscle [22]. Naitoh et al. [3] reported that Co^{2+} and Mn^{2+} have no effect on the electrophysiology of *Paramecium*, but this work was done in the presence of a higher concentration of Ca^{2+}.

Is Ca^{2+} influx measured at 0 °C physiological? Several lines of evidence support our belief that the Ca^{2+} influx we observe at 0 °C occurs by the same mechanism responsible for the inward Ca^{2+} current associated with membrane depolarization. (1) There is good correlation between the effects of monovalent cations upon ciliary reversal and their effects on Ca^{2+} influx. Both the effects of varying K^+ concentration on the rate of Ca^{2+} influx (Fig. 5) and the relative ability of the group IA elements to stimulate Ca^{2+} influx (Table I) paralleled the corresponding effects on the duration of ciliary reversal. (2) The concentrations of Ba^{2+} and Sr^{2+} which apparently compete with Ca^{2+} for the influx mechanism at 0 °C are the same concentrations which trigger ciliary reversal and affect the electrophysiology at 25 °C [20]. (3) The rate of Ca^{2+} influx at 0 °C is increased by galvanic stimulation of whole cells which is known to induce ciliary reversals at 25 °C [23]. (4) Behavioral mutants of the Pawn type, whose electrophysiological properties suggest a defect in the Ca^{2+} influx mechanism, do not show stimulated Ca^{2+} influx at 0 °C [24]. (5) Although swimming and ciliary reversal are sluggish at 0 °C cells incubated at 0 °C for 30 min, and then warmed, begin to swim again (albeit slower than unchilled cells) and undergo ciliary reversal normally in response to stimuli (Browning, J. L. and Nelson, D. L. unpublished). *P. aurelia* can be adapted to growth and normal swimming behavior at 0 °C, and there is therefore no reason to doubt that the excitable membrane can function at 0 °C [25]. (6) Cells loaded with $^{45}Ca^{2+}$ at 0 °C quickly extruded the label when returned to room temperature, indicating that their permeability barrier remained intact.

We conclude from these arguments that the Ca^{2+} influx which we measure at 0 °C occurs by the same mechanism which is responsible for ciliary reversal at 25 °C, and that the properties of stimulated Ca^{2+} influx which we observe at 0 °C (saturability, stimulability, specificity, and independence of metabolic energy) are properties of the Ca^{2+} conductance mechanisms at 25 °C.

Ca^{2+} binding vs. Ca^{2+} fluxes. Yamaguchi [26] and Naitoh and Yasumasu [27]

studied the distribution of $^{45}Ca^{2+}$ in *Paramecium* with and without stimulation, and both reported that the addition of Na^+ or K^+ reduced the amount of cell-bound Ca^{2+}. Their studies were done using high external Ca^{2+} concentrations (approx. 1 mM). Naitoh interpreted these results in terms of decreased surface binding of Ca^{2+} in the presence of Na^+ or K^+. This interpretation is entirely consistent with our own observations; at high external Ca^{2+} levels, most of the Ca^{2+} associated with cells is surface bound, and not released by cell lysis. In contrast, our studies of $^{45}Ca^{2+}$ fluxes were done at low Ca^{2+} concentrations (10–25 μM), where surface-bound Ca^{2+} represents only a minor fraction of total cell Ca^{2+}. Under these conditions, we were able to measure changes in internal Ca^{2+}, and thus to measure rates of ion flux. The results of Yamaguchi [26] and Naitoh and Yasumasu [27], and our present results, indicate that the addition of Na^+ or K^+ to resting cells has two effects; surface-bound Ca^{2+} is displaced, and external Ca^{2+} enters the cells.

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REFERENCES

1 Eckert, R. (1972) Science 176, 473–481
2 Kinosita, H. and Murakami, A. (1967) Physiol. Rev. 47, 53–82
3 Naitoh, Y., Eckert, R. and Friedman, K. (1972) J. Exp. Biol. 56, 667–681
4 Naitoh, Y. and Kaneko, H. (1973) J. Exp. Biol. 58, 657–675
5 Kung, C. (1971) J. Comp. Physiol. 71, 142–164
6 Kung, C. and Eckert, R. (1972) Proc. Natl. Acad. Sci. U.S. 69, 93–97
7 Dipolo, R. (1973) J. Gen. Physiol. 62, 575–589
8 Blaustein, M., Russell, J. and De Weer, P. (1974) J. Supramol. Struct. 2, 448–581
9 Chen, S. (1974) J. Physiol. Lond. 238, 313–328
10 Blaustein, M. and Russell, J. (1974) J. Gen. Physiol. 63, 144–167
11 Sonneborn, T. (1970) Methods in Cell Physiology, p. 250, Academic Press, New York
12 Ohnishi, T. and Ebashi, J. (1963) J. Biochem. Tokyo 54, 506–519
13 Hansma, H. (1975) Ph.D. Thesis, University of California, Santa Barbara
14 Patterson, M. and Greene, R. (1965) Anal. Chem. 37, 854–857
15 Naitoh, Y. (1968) J. Gen. Physiol. 51, 85–103
16 Shipley, J. and Goodeve, C. (1927) Trans. Am. Electrochem. Soc. 52, 375–389
17 Vivier, E. (1974) Paramecium, A Current Survey, p. 42, Elsevier Publishing Co., Amsterdam
18 Naitoh, Y. (1969) J. Gen. Physiol. 53, 517–529
19 Hodgkin, A. and Keynes, R. (1955) J. Physiol. Lond. 128, 28–60
20 Naitoh, Y. and Eckert, R. (1968) J. Comp. Physiol. 68, 458–472
21 Hagiwara, S., Fukuda, J. and Eaton, D. (1974) J. Gen. Physiol. 63, 564–578
22 Hagiwara, S. and Takahashi, K. (1967) J. Gen. Physiol. 50, 583–601
23 Jahn, T. (1961) J. Protozool. 8, 369–380
24 Browning, J. L., Nelson, D. L. and Hansma, H. (1976) Nature 259, 491
25 Poljansky, G. and Posnanskaya, T. (1964) Acta Protozool. 2, 271–278
26 Yamaguchi, T. (1960) J. Fac. Sci. Univ. Tokyo 8, 593–601
27 Naitoh, Y. and Yasumasu, I. (1967) J. Gen. Physiol. 50, 1303–1310