*J. exp. Biol.* (1980), **84**, 73–87
*With 9 figures*
*Printed in Great Britain*



# $Ba^{2+}$ INFLUX MEASURES THE DURATION OF MEMBRANE EXCITATION IN *PARAMECIUM*

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(*Received 27 April* 1979)

SUMMARY

We have developed an assay for the duration of membrane excitation in *Paramecium tetraurelia* by tracing the influx of $^{133}Ba^{2+}$. This assay is performed at physiological temperatures and in physiological solutions. $Ba^{2+}$ enters the paramecia through the Ca channels, since mutants defective in Ca channels show no significant $Ba^{2+}$ influx. $Ba^{2+}$ enters only when the Ca channels are opened during excitation which can be triggered by $Na^+$ or $Ba^{2+}$ itself. The ionic species and relative concentrations determine the duration of the action potentials and hence the duration of spinning or backward swimming. The longer the average period of excitation, the larger the $Ba^{2+}$ influx. In a Ba-Ca solution the cells spend 30% of their time in the excited state. The rate of $Ba^{2+}$ entry into the paramecia in that state is 1·3 mM/min. $Ba^{2+}$ influx occurs over a 50-fold range of $Ba^{2+}$ concentration. There is very little $Ba^{2+}$ efflux. The fate of the entered $Ba^{2+}$, the consequences of the large and rapid influx, the advantages and drawbacks of the $Ba^{2+}$ influx assay, and the possible use of the assay for Ca channel function in cell-free preparations are discussed.

## INTRODUCTION

Jennings (1906) found that paramecia reverse the beating direction of their cilia when exposed to certain stimuli. Transient ciliary reversal causes the cell to back up for a distance. Such behaviour is called the 'avoiding reaction'. By intracellular electrical recordings, Kinosita and co-workers (1965) showed that the paramecium membrane is excitable, i.e. it can generate action potentials. They also showed that ciliary reversal is coupled to the action potential. Further electrophysiological studies by Naitoh & Eckert showed that this membrane is permeable to $Ca^{2+}$ and $Ba^{2+}$ during excitation (Naitoh & Eckert, 1968*a*, *b*; Eckert, 1972). $Ca^{2+}$ is the natural ion that carries the action current. This transient internal accumulation of $Ca^{2+}$, through an as yet unknown mechanism, leads to ciliary reversal (Naitoh & Kaneko, 1972; Naitoh & Eckert, 1974). $Ba^{2+}$, as an analogue of $Ca^{2+}$, presumably passes through the Ca channel and has effects similar to those of $Ca^{2+}$ on ciliary movement (Naitoh & Eckert, 1968*b*; Naitoh & Kaneko, 1972). Studying the influx of $Ca^{2+}$ or $Ba^{2+}$ is clearly the first step toward biochemical analyses of excitation in paramecium.

Naitoh & Yasumasu (1967) studied Ca binding components in *P. caudatum* in

solutions of millimolar $Ca^{2+}$. They found that other cations compete with $Ca^{2+}$ for the binding sites and concluded that the $Ca^{2+}$ displaced from these sites triggered ciliary reversal. Although their hypothesis remains to be tested, it is clear that the major component they measured is surface binding of $Ca^{2+}$. Browning & Nelson (1976) and Browning, Nelson & Hansma (1976) showed a steady accumulation of $Ca^{2+}$ in *P. tetraurelia* incubated in solutions of 10–40 $\mu$M-$Ca^{2+}$. By various criteria they showed that this $Ca^{2+}$ is internal, comes through the membrane and is related to excitation and ciliary reversal. This influx of $Ca^{2+}$ can only be measured at 0 °C and not 24 °C presumably because of an energy- and therefore temperature-dependent Ca-extrusion mechanism. Since the volume of literature on the physiology and behaviour of paramecium deals with events at room temperature and in media of millimolar cations, it would be desirable to have a biochemical assay of excitation under these physiological conditions.

The amplitude of the action potential of paramecium is usually graded with the stimulus. In Ba-Ca solutions, however, all-or-none action potentials are generated even without other stimuli (Kinosita, Murakami & Yasuda, 1965; Naitoh & Eckert, 1968*b*). The duration of the action potential is proportional to the ratio of the concentration of the two divalent cations, $[Ba^{2+}]/[Ca^{2+}]$, in the bath (Naitoh & Eckert, 1968*b*). Resistance measurements show that the membrane is highly permeable during the prolonged action potential (Satow, Hansma & Kung, 1976). Naitoh & Eckert (1968*b*) proposed that barium had its effect by entering through the Ca channel, and then blocking the short-circuiting $K^+$ efflux. Browning & Nelson (1976) showed that $Ba^{2+}$ is a competitive inhibitor of $Ca^{2+}$ influx. In this paper, we report the $Ba^{2+}$ influx in *P. tetraurelia* at room temperature, and compare the rate of influx with the duration of backward swimming.

## MATERIALS AND METHODS

### *Stocks*

Four strains of *Paramecium tetraurelia* (formerly *P. aurelia*, species 4) were used. They were the wild-type strain, stock 51s (non-kappa-bearing); a pawn mutant, stock d4-95 (genotype *pwB/pwB*); a temperature-sensitive pawn, d4-132 (*pwA/pwA*); and a paranoiac mutant, d4-90 (*PaA/PaA*). The mutants were all derived from stock 51s. The behavioural and electrophysiological phenotypes and the genetics of these strains have been described (Kung, 1971; Kung & Eckert, 1972; Chang & Kung, 1973; Satow, Chang & Kung, 1974; Satow *et al.* 1976).

### *Culture methods*

Cells were cultured in cerophyl medium inoculated with *Enterobacter aerogenes* (Sonneborn, 1970) at either room temperature (23 ± 1 °C) or 35 ± 1 °C.

### *Chemicals*

$^{133}BaCl_2$ in 0·5 M-HCl (0·4–1·0 mCi/m-mole) was from New England Nuclear Corp. All other chemicals were analytical reagent grade.

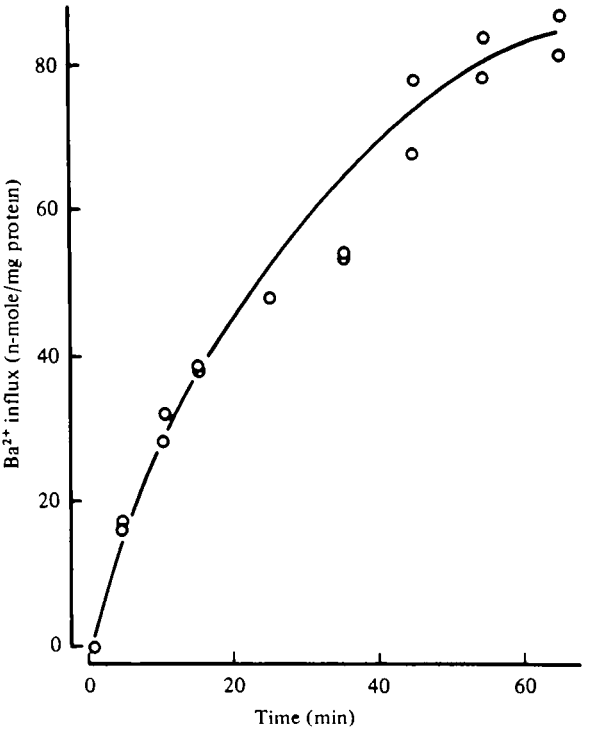


Fig. 1. Time course of barium accumulation in wild-type *Paramecium* (51s) in influx medium containing 7·3 mM [$Ba^{2+}$] and 0·85 mM [$Ca^{2+}$]. Each point is the average of duplicate samples from the same experiment. The results of two experiments are presented.

$Ba^{2+}$ in the medium, by sampling at various times using the above centrifugation technique.

*Behavioural observations*

Swimming behaviour of paramecia was monitored under a stereomicroscope as the ion flux experiments were carried out. In separate experiments, the duration and frequency of avoiding reactions in forms of jerks, spins or backward swimming in various influx media were monitored and registered with an event recorder equipped with hand-press switches. We estimate that there was a fairly uniform lag of ~0·3 s between the occurrence and the registration of an event. Durations of backward swimming over 1 s in each influx medium were measured from the records, tabulated, and compared.

## RESULTS

*Time course of $Ba^{2+}$ influx*

Fig. 1 shows the accumulation of $Ba^{2+}$ in wild-type *P. tetraurelia* bathed in Ba-Ca solution. This solution contains 0·85 mM free $Ca^{2+}$ and 7·3 mM free $Ba^{2+}$. It is known to trigger prolonged action potentials (Naitoh & Eckert, 1968*b*) and sustained backward swimming or spinning (see below). The $Ba^{2+}$ accumulation is rapid. 42 n-mole/mg protein (S.D. 9, $n$ = 12) are accumulated in the first 15 min. Within this 15 min,

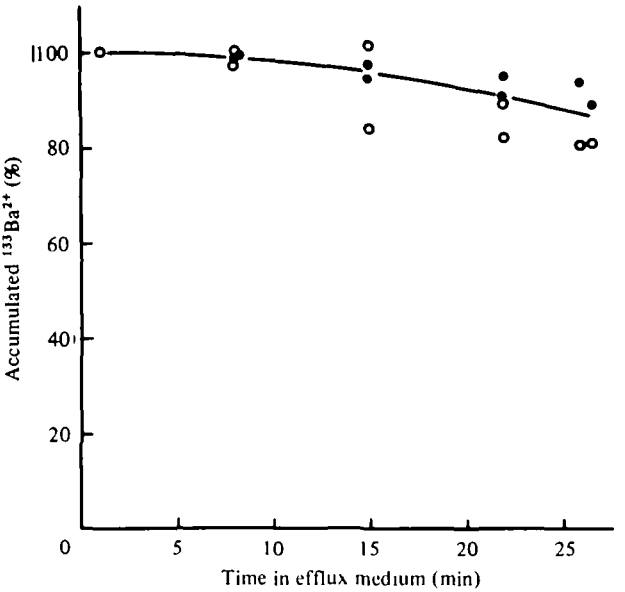


Fig. 2. Time course of barium efflux in wild-type *Paramecium* (51s). Cells were prepared and preloaded with $^{133}Ba^{2+}$ in 7·3 mM [$Ba^{2+}$]–0·85 mM [$Ca^{2+}$] influx medium for 15 min as described in Materials and Methods. Cells were resuspended in efflux medium with (○) and without (●) unlabelled 7·3 mM [$Ba^{2+}$]. Amount of radioactivity remaining in samples is represented as percentage of barium accumulation measured at time zero. Each point is the average of duplicate samples from the same experiment. The results of two experiments are presented.

the rate of accumulation does not change (Figs. 3*a* and 7*a*). The rate decreases gradually after 15 min.

The morphology and behaviour of the paramecia were closely monitored during the influx experiments. The paramecia swell slightly when first transferred into the Ba-Ca solution. Within 10–15 min, their contractile vacuoles slow down. By 30 min, blisters begin to form on the surface of some cells. The proportion of damaged cells increases with time. By 45 min, some cells begin to lyse. In this Ba-Ca solution, paramecia swim backward or spin tens of seconds at a time. Between bouts of backing or spinning, they swim forward. The degree of deterioration corresponds to the amount of backing or spinning in these and the following experiments. It appears that some correlates of prolonged membrane excitation, most likely the accumulation of $Ba^{2+}$ and/or $Ca^{2+}$, poison the paramecia. The decline in the accumulation rate after 15 min may be a secondary effect of this Ba- (or Ca-) poisoning. Thus, all 'influx rates' described below refer to the rate of $Ba^{2+}$ accumulation during the first 15 min.

### *$Ba^{2+}$ efflux*

Paramecia were preloaded with $^{133}Ba^{2+}$ in the above Ba-Ca solution for 15 min and then washed and resuspended in media with or without the added $Ba^{2+}$. Cells were sampled at different times for their radioactivity. As shown in Fig. 2, the cells release little of the preloaded $Ba^{2+}$ whether or not there is $Ba^{2+}$ in the medium. In either case, less than one-tenth of the accumulated $Ba^{2+}$ has left the cells in 15 min. Since little

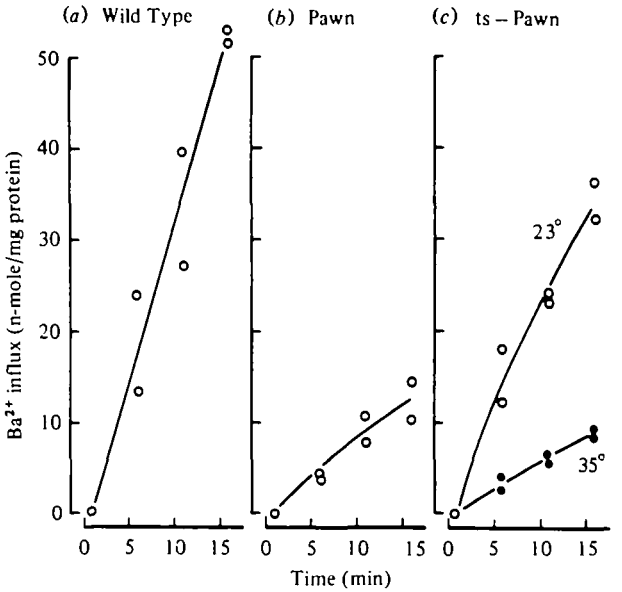


Fig. 3. Barium influx assays of (*a*) wild type, 518 at 23 °C; (*b*) pawn, d4-95 at 23 °C; and (*c*) temperature-sensitive pawn, d4-132 grown at 23 °C (○) and at 35 °C (●) in influx medium containing 7·3 mM [$Ba^{2+}$] and 0·85 mM [$Ca^{2+}$]. Each point is the average of duplicate samples from the same experiment. The results of two experiments are presented.

efflux occurs, the net $Ba^{2+}$ accumulation rate measured in the influx experiments is a close estimate of the true influx rate.

*Pawn mutants*

Pawns are mutants that have lost the function of their voltage-sensitive Ca channels (Kung & Eckert, 1972; Schein, Bennett & Katz, 1976; Oertel, Schein & Kung, 1977). Consequently, they do not generate action potentials and do not jerk or back up. Fig. 3*b* shows that they also do not show significant $Ba^{2+}$ influx as compared to the wild-type cells in Fig. 3*a*. The temperature-sensitive pawn mutant shows $Ba^{2+}$ influx when grown at 23 °C, but fails to accumulate $Ba^{2+}$ when grown at 35 °C (Fig. 3*c*). The temperature-sensitive pawns show subnormal membrane excitability when grown at 23 °C (Satow & Kung, 1976), but lose their excitability completely when grown at 35 °C (Satow *et al.* 1974; Satow & Kung, 1974). These experiments show that significant $Ba^{2+}$ influx can occur only when there are functional Ca channels.

*Effects of [$Ba^{2+}$] and [$Ca^{2+}$] on the $Ba^{2+}$ influx rate*

The kinetics of $Ba^{2+}$ influx were examined by studying the influx rate in both wild-type and pawn mutant (d4-95) as a function of $Ba^{2+}$ concentration in the medium. Fig. 4 shows that $Ba^{2+}$ influx is concentration dependent, but is clearly not a first-order, saturable process. Little $Ba^{2+}$ is accumulated in the pawn over the range of $Ba^{2+}$ concentration studied. $Ba^{2+}$ is accumulated very slowly when wild-type paramecia are bathed in media of 1·7 or 3·4 mM [$Ba^{2+}$]. Above 4 mM, the influx rate increases abrubtly. Beyond 12 mM, the paramecia are damaged, as judged by morphological criteria, within the first 15 min in which the rate is measured.

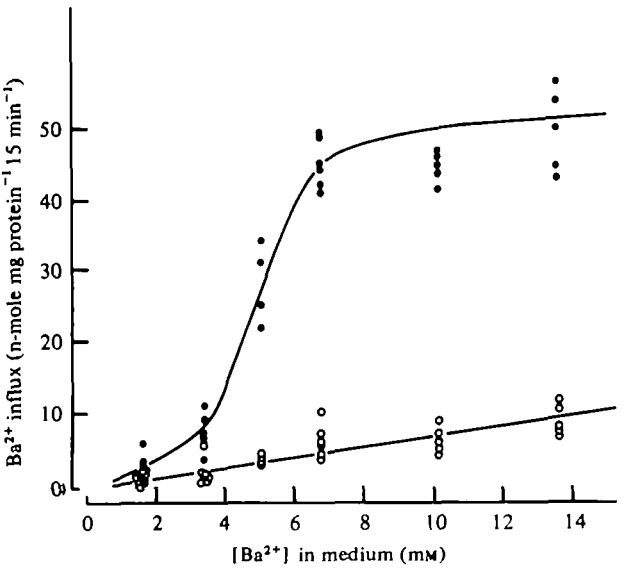


Fig. 4. Rate of barium influx in wild type 51s (●) and pawn mutant d4-95 (○) *Paramecium* in influx media containing 0·85 mM free calcium and various free barium concentrations. The results of four to six experiments are shown. Each point represents the average of duplicate samples in each experiment.

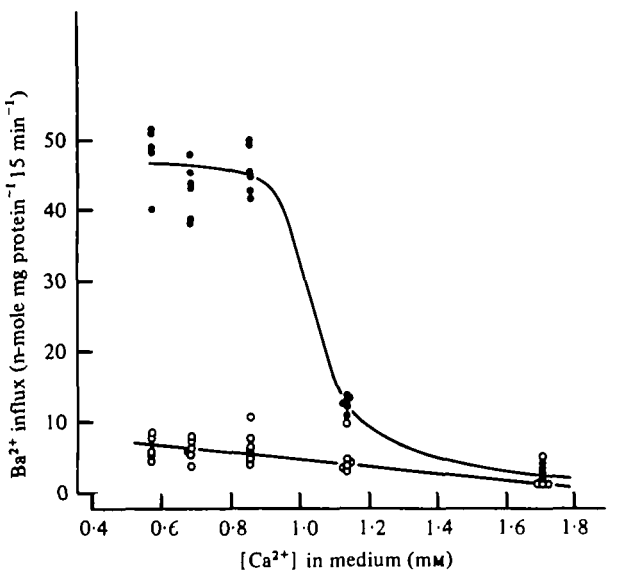


Fig. 5. Rate of barium influx in wild type 51s (●) and pawn mutant d4-95 (○) in influx medium containing 6·8 mM free barium and various free calcium concentrations. The results of four to six experiments are shown. Each point represents the average of duplicate samples in each experiment.

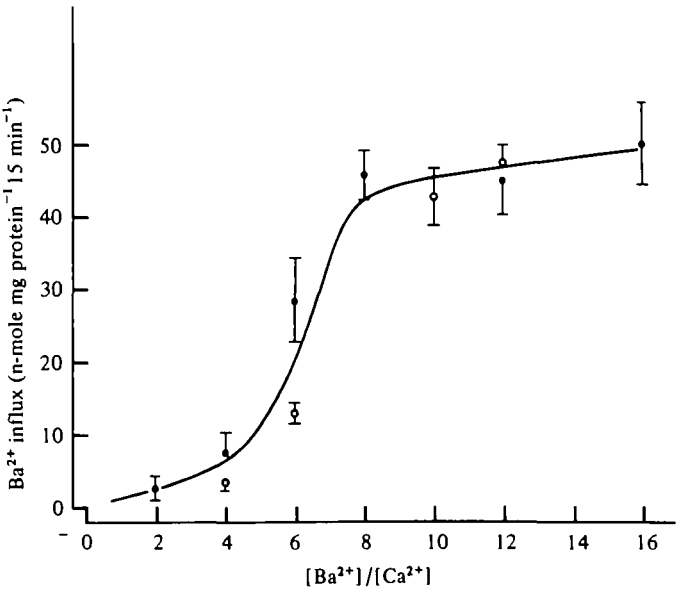


Fig. 6. Effect of the Donnan ratio, $[Ba^{2+}]^i/[Ca^{2+}]^i$, on the rate of $Ba^{2+}$ influx in wild type (518). Open symbols and bars are means ± S.D. ($n$ = 4–6) from experiments with influx media containing 0·85 mM $[Ca^{2+}]$ and various $[Ba^{2+}]$; closed symbols represents experiments with media containing 6·8 mM $[Ba^{2+}]$ and various $[Ca^{2+}]$. Note that the rate of $Ba^{2+}$ influx is largely a function of the ratio and not the absolute concentration of ions.

When $[Ba^{2+}]$ is kept at 6·8 mM and $[Ca^{2+}]$ varied, it is clear that the $Ba^{2+}$ influx rate is also dependent upon $[Ca^{2+}]$. While the influx rate of the pawn does not change significantly, that of wild-type cells drops precipitously as the $[Ca^{2+}]$ increases from 0·85 to 1·13 mM (Fig. 5). Toxic effects of media with less than 0·5 mM $[Ca^{2+}]$ preclude meaningful measurements in them.

Figs. 4 and 5 show that increasing $[Ca^{2+}]$ has opposite effects to those obtained by increasing $[Ba^{2+}]$. However, the opposite effects cannot be explained by a simple substrate competition for common carriers alone. Behavioural experiments in this paper and physiological studies in the literature (see Discussion) show that the ratio $[Ba^{2+}]/[Ca^{2+}]$ determines the duration of excitation during which the Ca channels presumably open. The curve obtained by replotting the data in Figs. 4 and 5 in terms of the ratio of $[Ba^{2+}]/[Ca^{2+}]$ in the medium is consistent with this view (Fig. 6). To test this view directly, influx rates were measured in media with a tenfold concentration change of $[Ba^{2+}]$ and $[Ca^{2+}]$. Fig. 7 shows the rate of $Ba^{2+}$ influx in two sets of solutions, one being one-tenth the concentration of the other. The two solutions have a $[Ba^{2+}]/[Ca^{2+}]$ ratio of approximately 8/1 in Fig. 7*a*, 6/1 in Fig. 7*b*, and 4/1 in Fig. 7*c*. (The actual $[Ba^{2+}]/[Ca^{2+}]$ ratios for full-strength media are 8·5, 6·2, and 4·0; those for the one-tenth strength media are 8·4, 6·3 and 4·2). Nevertheless, the tenfold dilution has no effect on the rate of $Ba^{2+}$ influx, and the rate of $Ba^{2+}$ influx is determined mainly by the $[Ba^{2+}]/[Ca^{2+}]$ ratio.

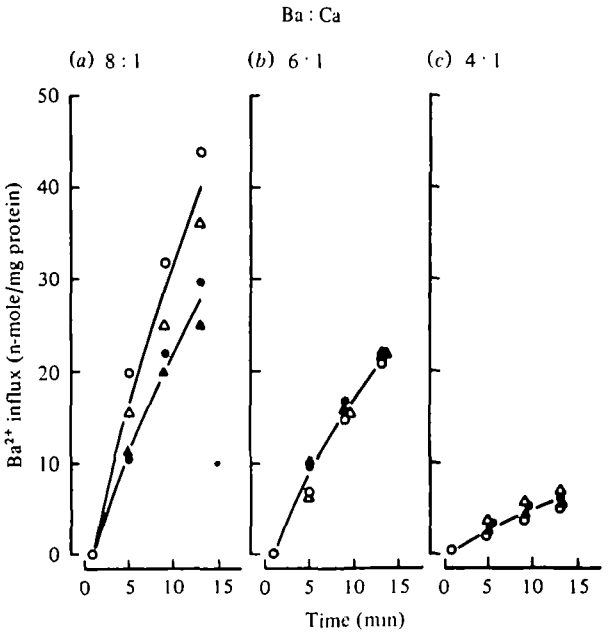


Fig. 7. Effects of the $[Ba^{2+}]/[Ca^{2+}]$ ratio on the $Ba^{2+}$ influx. The ratio is approximately 8 in (*a*), 6 in (*b*), and 4 in (*c*). Note that the rate of $Ba^{2+}$ influx decreases as the ratio decreases. Open symbols (○ and △) are from experiments with full-strength media; closed symbols (● and ▲) are from media of 1/10 the ionic strength. Note that the influx rate is independent of the absolute concentrations of ions. (The actual $[Ca^{2+}]$ is 0·85 mM in the full-strength and approximately 0·09 mM in the diluted media. The actual $[Ba^{2+}]$ is 7·3 mM and 0·76 mM in (*a*), 5·3 mM and 0·57 mM in (*b*), and 3·4 mM and 0·38 mM in (*c*).) The results of two sets of experiments are shown. Each point is the average of duplicate samples from the same experiment.

*Behaviour and membrane excitation of paramecia in the Ba-Ca solutions*

We monitored the duration and frequency of forward and backward locomotion of paramecia with an event recorder for 20 min after the cells were transferred into various Ba-Ca solutions. Samples of the record are shown in Fig. 8. When the $[Ca^{2+}]$ is kept at 0·85 mM (Fig. 8*a*) the cells respond with increasing vigour as $[Ba^{2+}]$ increases. At 1·7 mM $[Ba^{2+}]$, paramecia perform quick, almost rhythmic jerks of high frequency (~1 Hz; data not shown). This type of behaviour corresponds to action potentials lasting only a few tens of milliseconds and is often dubbed the 'barium dance'. At 3·4 mM $[Ba^{2+}]$, the jerks are less frequent and the backing step of the dance is slightly longer but still too short to be measured by eye. At 5·1 mM, backing or spinning up to 60 s is occasionally seen besides short jerks. At 6·8 mM and 10·2 mM $[Ba^{2+}]$, 20–50% of the cells are in backing or spinning bouts at any time. These results are summarized in Table 1. Note that below 5 mM $[Ba^{2+}]$, paramecia do not swim backward for longer than 1 s. This correlates with the lack of significant $Ba^{2+}$ influx at similar concentrations (Fig. 4).

When $[Ba^{2+}]$ is held at 6·8 mM and $[Ca^{2+}]$ varied (Fig. 8*b*), behavioural response weakens as the $[Ca^{2+}]$ increases. At 1·7 or 3·4 mM $[Ca^{2+}]$, there are barium dances but no backing or spinning. This lack of backing or spinning corresponds to the lack

Table 1. *Percentage time that wild-type* P. tetraurelia *spent on backing or spinning bouts of various lengths* for the first* 20 *min in different Ba-Ca solutions*

| Bout length‡ (s) | [$Ba^{2+}$] in Ba-Ca solutions† | | | |
|---|---|---|---|---|
| | 1·7 mM | 3·4 mM | 5·1 mM | 6·8 mM |
| 1–20 | 0 | 0 | 6·2 | 8·6 |
| 20–40 | 0 | 0 | 4·0 | 11·5 |
| 40–60 | 0 | 0 | 2·2 | 5·2 |
| 60–80 | 0 | 0 | 3·8 | 3·2 |
| >80 | 0 | 0 | 0 | 0 |

* The behaviour of paramecia after the transfer from the preinflux solution to the Ba-Ca solution was monitored for 20 min. Each entry in this table is derived from the results from at least 19 paramecia. Forward swimming between bouts accounts for the major portion of the time.

† See Materials and Methods for complete compositions.

‡ Jerks lasting less than a second cannot be measured accurately.

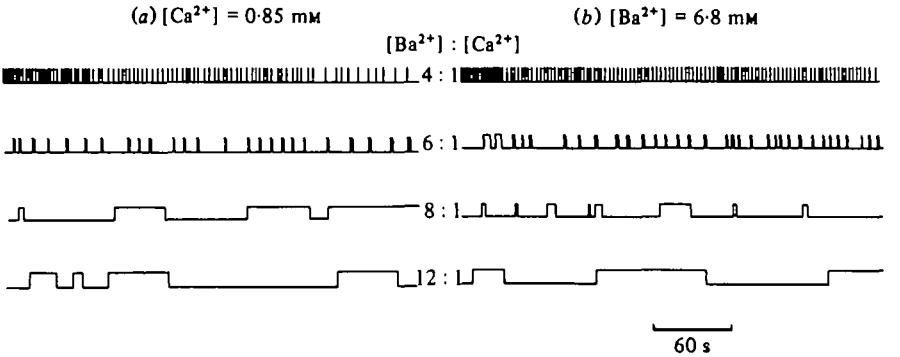


Fig. 8. Frequency and duration of forward and backward swimming of wild-type paramecia in various Ba-Ca media. The behaviour of paramecia were monitored with a microscope and their directions of movement were registered with a manually operated event recorder. The lower level of the traces indicates the paramecium is swimming forward; the upper level backward. Short spikes are jerks and long plateaus are sustained backward swimming. Note that when the [$Ba^{2+}$]/[$Ca^{2+}$] ratio is above 5, long bouts of backward swimming are observed.

of significant $Ba^{2+}$ influx at these [$Ca^{2+}$] (Fig. 5). At 8 mM-$Ca^{2+}$ even the dance is eliminated (data not shown).

*$Na^+$-stimulated $Ba^{2+}$ influx*

In the previous experiments of $Ba^{2+}$ influx, the excited state of the membrane is induced and maintained by $Ba^{2+}$ itself. Paramecia can also be excited by high [$Na^+$] relative to [$Ca^{2+}$] (Naitoh, 1968; Naitoh & Eckert, 1968*a*; Satow & Kung, 1974; Satow *et al.* 1976) especially in solutions having a small amount of $Ba^{2+}$. Mutants (the 'paranoiacs') exist which have prolonged excitation in $Na^+$ solution (Kung, 1971; Satow & Kung, 1974; Hansma & Kung, 1976; Satow *et al.* 1976). Fig. 9*a* shows the background $Ba^{2+}$ influx of the wild type and a paranoiac mutant in a medium containing 1·6 mM [$Ba^{2+}$] and 0·7 mM [$Ca^{2+}$]. Paramecia of both strains swim forward with occasional avoiding reactions with no sustained backward swimming in this medium. When 16 mM-$Na^+$ is added to the Ba-Ca medium, there is an increase in

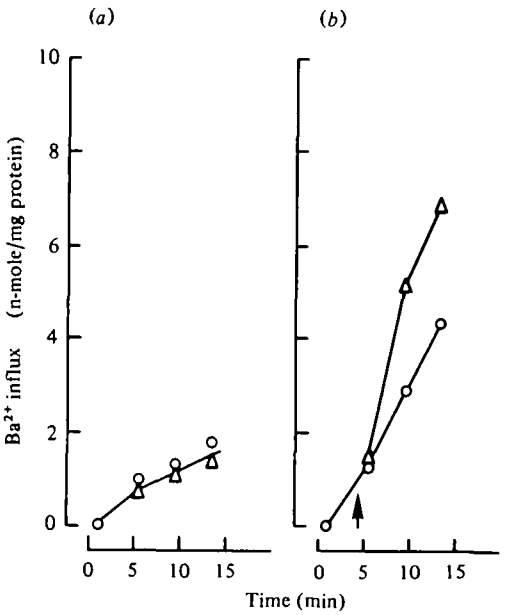


Fig. 9. Na-stimulated $Ba^{2+}$ influx in wild-type and paranoiac mutant of *Paramecium*. Barium influx was assayed in medium containing 1·6 mM [$Ba^{2+}$] and 0·7 mM [$Ca^{2+}$]: (*a*) No $Na^+$ was added, and (*b*) final concentration of 16 mM-NaCl was added to the influx assay mixture at minute 4 as denoted by the arrow. Circles are the wild-type (51s) cells and triangles are the paranoiac mutant (d4-90). One representative experiment is presented here with each point being the average of duplicate samples. Four independent experiments gave similar results.

$Ba^{2+}$ influx (Fig. 9*b*). Furthermore, the increase is significantly larger in the paranoiac mutant than in wild type. This observation is consistent with the finding of Browning *et al.* (1976) where paranoiac mutants accumulate more $Ca^{2+}$ than wild type when excited with $Na^+$. Wild-type paramecia show rapid jerks as well as repeated backward swimming for tens of seconds in this Na-Ba medium. The paranoiac mutants swim backward for even longer periods in tighter helices than wild type. This is similar to the previously observed 'paranoiac' behaviour in a Na-containing solution (Kung, 1971; Browning *et al.* 1976; Satow *et al.* 1976).

These results again show that significant $Ba^{2+}$ influx only occurs when there is prolonged excitation. With $Ba^{2+}$ behaving as a $Ca^{2+}$ analogue, the $Ba^{2+}$ influx assay measures the duration of the excited state, in which the Ca channels open, regardless of how the excited state is triggered and sustained.

## DISCUSSION

### *Surface binding or influx?*

We believe that the $Ba^{2+}$ influx reported in this paper represents internal accumulation and not $Ba^{2+}$ binding on the cell surface. The reasons are the following: (1) The $Ba^{2+}$ accumulation continues after 30 min (Fig. 1), whereas most surface binding, including the cation binding in *Paramecium* studied by Naitoh & Yasumasu (1967), is saturated within 1 min. (2) Once accumulated, most of the $Ba^{2+}$ cannot be

released even after prolonged incubation in Ba-free medium (Fig. 2). It is not likely that $Ba^{2+}$ becomes adsorbed on the surface since the physiological and behavioural effects of $Ba^{2+}$ are reversible and can be counteracted by $Ca^{2+}$. (3) If $Ba^{2+}$ accumulates on the cell surface, one would expect a $^{133}Ba^{2+}$-$^{137}Ba^{2+}$ exchange when $^{133}Ba^{2+}$-loaded cells are transferred into the efflux medium with unlabelled $Ba^{2+}$. This expected exchange is not observed (Fig. 2). (4) The amount of $Ba^{2+}$ accumulated is large (below). It is difficult to imagine that the paramecium surface has such a large capacity for $Ba^{2+}$ binding.

*The amount of $Ba^{2+}$ accumulated*

In the influx experiments with 7·3 mM [$Ba^{2+}$] and 0·85 mM [$Ca^{2+}$] (Fig. 1), the influx of $Ba^{2+}$ causes an increase in internal $Ba^{2+}$ by 0·4 mM/min, considering a cell volume of $10^{-7}$ ml. If the accumulation is within the lumen of the cilia, the concentration will rise 100 times as fast. This rapid and large influx of $Ba^{2+}$ has three implications. (1) The membrane is highly permeable to $Ba^{2+}$ during excitation. (2) Much of the $Ba^{2+}$ accumulated is not free $Ba^{2+}$, otherwise the internal concentration of $Ba^{2+}$ will exceed the external concentration in 20 min and influx will cease. The $Ba^{2+}$ is most likely precipitated since most Ba salts dissociate poorly. (3) There must be fluxes of at least one other ion to balance the influx of $Ba^{2+}$. Taking the membrane capacitance of 1 $\mu$F/cm and a total surface of $4 \times 10^{-4}$ cm$^2$ (Eckert & Naitoh, 1980; Eckert, 1972), the paramecium membrane will be depolarized by 20 *volts* per minute. In reality, the membrane depolarizes and repolarizes by 10–20 *millivolts* at the up- and down-strokes of the action potentials. A counterion flux, such as a $K^+$ efflux, must occur. In the cases of the 'paranoiac' mutants which show prolonged excitation, loss of cellular $K^+$ has been shown (Naitoh & Eckert, 1968*b*; Hansma & Kung, 1976).

*When does the $Ba^{2+}$ enter?*

When we increase the [$Ba^{2+}$]/[$Ca^{2+}$] ratio by increasing the [$Ba^{2+}$] or decreasing the [$Ca^{2+}$], prolonged backing or spinning occurs only in solutions of ratios bigger than 4:1 (Fig. 8*a*, *b* and Table 1). Only in these solutions do we observe significant $Ba^{2+}$ influx (Figs. 4, 5 and 7). Prolonged backing or spinning corresponds to prolonged action potentials. At the plateaus of these action potentials the membrane is highly permeable as shown by the very low electrical resistance (Naitoh & Eckert, 1968*b*; Satow *et al.* 1976). Therefore, we conclude that the $Ba^{2+}$ enters the cells when the membrane is excited, i.e. during the action potentials. The membrane excitation can be stimulated by ions other than $Ba^{2+}$. Fig. 9 shows that as long as the membrane is excited, in this case due to the presence of $Na^+$, $Ba^{2+}$ will enter the cell regardless of how the excitation is triggered.

*The path of $Ba^{2+}$ influx*

Ions cross the cell membrane through specific channels. The literature on crustacean muscle (Hagiwara, Fukuda & Eaton, 1974) and *Paramecium* (Naitoh & Eckert, 1968*b*) indicates that there are Ca channels on the membranes of these cells through which $Ca^{2+}$ and its analogues, including $Ba^{2+}$, enter. This view is supported by the experiments in Figs. 3, 4 and 5 which show that paramecium mutants with membranes defective in their Ca channels have no significant $Ba^{2+}$ influx. The Ca channels

are voltage sensitive. When the membrane is depolarized, the Ca channels open, through which Ca (or Ba) ions are driven by the electrochemical gradient into the cell (Browning & Nelson, 1976; Kung & Eckert, 1972; Naitoh, Eckert, & Friedman 1972; Eckert, Naitoh & Machemer, 1976; Oertel *et al.* 1976; Satow & Kung, 1979). The experiments shown in Figs. 3–5, taken together, indicate that unless the wild-type membrane is excited (depolarized) and the Ca channels are open, there is no significant $Ba^{2+}$ influx. Therefore, we conclude that $Ba^{2+}$ enters the paramecium through its Ca channels when they are open during excitation.

Comparison of the electrophysiology of ciliated, deciliated and reciliated paramecia show that the Ca channels are largely, if not exclusively, located on the ciliary membrane and not the plasma membrane (Ogura & Takahashi, 1976; Dunlap, 1977). The exact location of these channels, the ports of the $Ca^{2+}$ or $Ba^{2+}$ entry, on the cilia has not been determined. Whether the $Ba^{2+}$ entered precipitates in the ciliary lumen or diffuses into the cell body before it is precipitated or sequestered is not known. Finally, we also do not know if the small $Ba^{2+}$ efflux (Fig. 2) is due to the energy-consuming Ca pump (Browning & Nelson, 1976).

*Donnan ratio and the duration of excitation*

The duration of backward swimming has long been used as a simple measure of the duration of excitation, and the relation between this duration of excitation and the ionic species and strength in the media has been sought. This duration is independent of the absolute concentration of $K^+$, but is dependent on the ratio of $[K^+]$ to $[Ca^{2+}]$ (Kamada & Kinosita, 1940), more precisely the Donnan ratio $[K^+]/[Ca^{2+}]^{\frac{1}{2}}$ (Naitoh & Yasumasu, 1967; Jahn, 1962; Naitoh, 1968). In $Ba^{2+}$-containing solutions, the Donnan ratio $[Ba^{2+}]^{\frac{1}{2}}/[Ca^{2+}]^{\frac{1}{2}}$ also determines the duration of excitation (Naitoh & Eckert, 1968*b*). A competition between $Ca^{2+}$ and other cations is implicated (Naitoh & Yasumasu, 1967), although the mechanism by which this competition leads to the control of ion channels is not clear (Eckert, 1972). In a voltage clamp study, Oertel *et al.* (1977) concluded that the $Ca^{2+}$ inward current subsides in time when the cell is bathed in a physiological K-Ca solution. This is known as inactivation of the Ca channel and may mean that the Ca channel closes with time (5 ms in this case) even when the membrane remains depolarized by the clamp. However, there are clearly more extreme conditions under which the excited states last for tens of seconds (Naitoh & Eckert, 1968*b*; Satow *et al.* 1976; Satow, 1978; Eckert & Brehm, 1979). Brehm & Eckert (1978) showed that $Ba^{2+}$ interferes with the inactivation and prolongs the inward current. Here, we changed the ion ratio to generate longer or shorter excited states so as to afford a comparison between the rate of $Ba^{2+}$ influx and the duration of excitation.

*Evaluation of the $Ba^{2+}$ influx asssay*

Physiological measurements of the membrane voltage and currents in various conditions allow us to deduce the species of ions which permeate through the membrane. In some instances, the quantity of the ion fluxes can also be calculated. However, tracing the fluxes of the ions with radioisotope yields the most direct measurement of the ion movements and the conclusions from such measurements depend on few assumptions. An assay of the movement of the ions which carry the action current

during excitation makes it possible to study the voltage-sensitive gating mechanism without employing electrophysiological procedures. In the *Paramecium* field, Browning & Nelson (1976) first developed an assay tracing the action current with $^{45}Ca$. The $Ba^{2+}$ influx assay described in this paper is inspired by their work and has two added advantages. (1) This assay is performed at room temperature, at which most of the previous behavioural and physiological experiments have been performed. (2) The assay is performed in solutions in the physiological range of ions, millimolar in concentration. Thus, this assay can measure the duration of membrane excitation previously measured only by behavioural or electrophysiological means.

The $Ba^{2+}$ influx assay has two drawbacks. (1) Like most biochemical assays, it cannot be used to study rapid events in millisecond scale. (2) It cannot resolve the small $Ba^{2+}$ influx during short action potentials, even when they are induced in frequency of around 1 Hz as during the barium dance. The poor resolution is due to the background $Ba^{2+}$ accumulation, as seen in the pawn experiment (Fig. 3*b*, *c*). $Ba^{2+}$ uptake unrelated to excitation may be due to surface binding or entry through food vacuole formation.

Cell-free preparations such as isolated cilia or membrane vesicles are used in cell biological and biochemical investigations. We would like to know if such preparations retain functional Ca channels of proper orientation after various fractionation procedures. Conventional physiological tests for excitability cannot be performed upon cilia or vesicles since they are smaller than the tip of a micro-electrode. With the $Ba^{2+}$ influx assay described here, we can now test these cell-free preparations for their competence in membrane excitation under physiological conditions.

We are grateful to Y. Satow and D. L. Nelson for valuable discussions and C. K. Omoto, C. S. Schobert, C. L. Shusterman, A. Adoutte, M. A. Forte, T. G. Linn and Y. Saimi for their critical review of the manuscript. This work was supported by grant GM22714 from the National Institutes of Health.

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