Biochimica et Biophysica Acta, 436 (1976) 128–139

BBA 77320

DEFECTIVE ION REGULATION IN A CLASS OF MEMBRANE-EXCITATION MUTANTS IN *PARAMECIUM*

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(Received October 28th, 1975)

SUMMARY

The "paranoiac" mutants of *Paramecium aurelia* show prolonged backward swimming in solutions containing Na^+, unlike wild-type paramecia, which jerk back and forth in Na^+ solutions. The paranoiac mutants in Na^+ solutions also show large losses of cellular K^+ and large influxes of Na^+. Three different paranoiac mutants all show similar defects in ion regulation but to different degrees. Wild-type *Paramecium*, in contrast, shows no Na^+-dependent loss of cellular K^+ and a much smaller Na^+ influx. In K^+-containing solutions, there is no difference between wild-type and paranoiac paramecia with respect to their cellular K^+ content.

The Na^+ influx, the K^+ loss, and the duration of backward swimming are all proportional to the extracellular Na^+ concentration. Electrophysiologically, the backward swimming of the paranoiac mutants corresponds to a prolonged depolarization of the membrane potential, while the backward jerks of wild-type *Paramecium* correspond to a series of transient depolarizations. We propose that the large Na^+ influxes and the large K^+ effluxes in paranoiacs occur during the periods of backward swimming, while the membrane is depolarized.

INTRODUCTION

Paramecium has many advantages for studying the biochemistry of membrane excitation. The electrophysiology of this excitable membrane has been extensively studied [1, 2]. Furthermore, the membrane potential changes in *Paramecium* can be readily observed by their effects on the swimming behavior of the cell [1, 3]. Thus, biochemical data can be easily correlated with electrophysiological data through simple behavioral observations. Since *Paramecium* is a unicell, there is no problem with interfering cell types; and large numbers of identical cells can be cultured for biochemical analyses.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

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Kung and co-workers have used the behavioral correlates of membrane activity to "dissect" genetically the excitable membrane of *Paramecium* [4–6]. They have isolated mutants of *Paramecium* with various types of abnormal behavior. Many of these mutants have corresponding abnormalities in membrane excitation. They thus provide a unique system for studying the mechanisms of membrane functions and the mechanisms of simple types of behavior.

The class of mutants studied here is called "paranoiac". The name describes the mutants' over-reaction to Na^+ stimulation. In Na^+ solutions, the paranoiac mutants swim backward for long distances [4, 7]. When normal (wild-type) paramecia are transferred to Na^+ solutions, in contrast, they jerk backward and forward but show little discernable backward swimming. The transient backing of wild type and the sustained backing of the mutants are both due to a reversal of the direction of the ciliary beat [4]. Intracellular recordings of membrane potential show that the wild-type membrane has repeated transient depolarizations and the mutant membrane has sustained depolarizations in Na^+ solutions [7].

We have found that the cation fluxes of three independent paranoiac mutants are grossly abnormal. The abnormalities in their ion fluxes correlate well with their behavioral abnormality.

MATERIALS AND METHODS

Supplies. Cerophyl (powdered, dehydrated rye leaves) was obtained from Cerophyl Laboratories, Inc., Kansas City, Mo. U.S.A. Tris (tris(hydroxymethyl)-aminomethane) and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) were both obtained from Sigma Chemical Co., St. Louis, Mo. U.S.A. Sodium-22 as NaCl in 0.5 M HCl was obtained from ICN Life Sciences Group, Irvine, Ca. U.S.A.

Cell stocks. All strains were *Paramecium aurelia*, species 4. The wild-type *Paramecium* were stock 51s (non-kappa bearing). The following paranoiac mutants were also used: d4-90, genotype *PaA PaA*, (designated as strain '*PaA*'); d4-149, genotype *fna*^{P} *fna*^{P}, ('*fna*^{P}'); and d4-150, genotype *PaC PaC*, ('*PaC*') (Van Houten, J., Chang, S.-Y. and Kung, C., in preparation). These mutants were derived from 51s and were isogenic except for the single mutations.

Culturing and harvesting cells. Cells were grown in flasks of autoclaved Cerophyl medium [8, 9] bacterized with *Enterobacter aerogenes*. Cultures were filtered through gauze and centrifuged for 2 min at $330 \times g$ in an oil-testing centrifuge (IEC Model HN-S). The cell pellets were removed and washed by recentrifugation in one of the salt solutions described below.

Cellular potassium analyses. This procedure was developed for studying the effect of different salt solutions upon the cellular K^+ content of *Paramecium* [9]. Cells were washed and suspended in salt solution A containing 1 mM KCl. (Solution A = 1 mM Ca^{2+}/1 mM citrate/1 mM Tris, pH approx. 7.2.) After 15–30 min in this solution, the cells were recentrifuged and the cell pellet was resuspended to a density of $1–5 \cdot 10^5$ cells/ml. 1-ml aliquots of this cell suspension were pipetted into narrow-tipped centrifuge bottles containing 100 ml of different incubation solutions (Solution A with various concentrations of NaCl or KCl).

At the end of the incubation period, the samples were centrifuged for 2 min at $2000 \times g$ in an oil-testing centrifuge. The supernatants were decanted, and the insides

of the inverted bottles were rinsed with glass-distilled water to remove as much of the contaminating salt solution from the cell samples as possible.

The cell pellets were then suspended in distilled water. Aliquots of each suspension were taken for protein determinations [10]. Inorganic ions were extracted from the remaining cells by the method of Dunham and Child [11, 12]: 2 drops of glacial acetic acid were added, and the tubes were heated for 5–10 min in a water bath at 85–90 °C. This procedure extracted 99–100 % of the total cellular potassium [9, 11].

Before flame photometric analysis, the samples were centrifuged 10–15 min at 25 000 $\times g$ to pellet the cell debris. The concentrations of K^+ in the supernatants were measured with an Eppendorf flame photometer. Standard solutions of known K^+ concentrations were measured with each set of samples. The results were expressed as μmol K^+ per g of cellular protein.

The Na^+ content of the samples was also measured by flame photometry. These data were unreliable, however, since the flame photometer is much less sensitive for Na^+ than K^+ and the contamination with extracellular Na^+ was high [9].

^{22}Na uptake. This procedure was developed for measuring Na^+ uptake by cells incubated in solutions of different Na^+ concentrations.

Cells were washed and suspended in salt solution B (1 mM Ca^{2+}/1 mM citrate/1 mM Tris/1 mM HEPES, pH approx. 7.2). After 10–20 min, the cells were

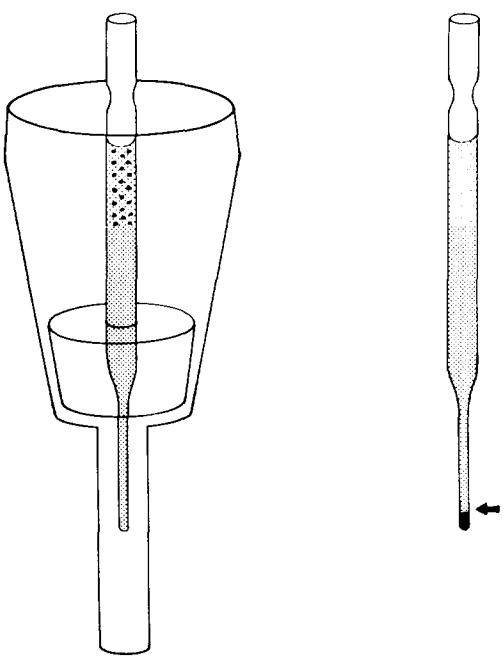

Fig. 1. Disposable centrifuge tubes for studying tracer uptake by cells in suspension. Centrifuge tubes are 6-inch disposable Pasteur pipets with the tip sealed in a flame; tubes are a simple version of those developed by Baadenhuijsen et al. [15]. Left: cell suspension layered over wash solution; centrifuge tube is held in rubber-stopper adapter in centrifuge cup for pear-shaped oil tube. Right: after centrifuging, the cell pellet can be cut off by inverting the centrifuge tube and nicking the tube at the arrow with a diamond-tipped glass-marking pen.

recentrifuged; and the cell pellet was removed. Aliquots of the cell pellet were transferred to vials containing the incubation solutions (Solution B with ^{22}Na and non-radioactive NaCl added at various concentrations).

At the end of the incubation period, duplicate or triplicate aliquots of the cell suspensions containing 3–10 · 10^4 cells were layered over a washing solution in centrifuge tubes made from disposable Pasteur pipets (Fig. 1). The wash solution was Solution B with 1 % sucrose added to increase the density; in some experiments, the wash solution also contained non-radioactive NaCl at the same concentration as the incubation solutions (see figure captions). The tubes were centrifuged, and the tip of the tube containing the packed cell pellet was broken off. The radioactivity of the cell pellet was measured directly in a well-type gamma counter (Nuclear Chicago). The radioactivity of the total cell suspension was also measured to determine the specific activity of the extracellular Na^+. Protein determinations were made on aliquots of the cell suspension. The results were expressed as μmol Na^+ taken up per g of cellular protein.

Measurements of swimming behavior. Cells in culture fluid were suspended in several volumes of Solution B for at least 15 min before beginning behavioral observations. To observe behavior, we transferred individual cells from Solution B to a depression slide containing one of the Na^+ solutions. We observed the cells under a dissecting microscope and timed with a stopwatch the duration of backward swimming of the cells immediately after transfer to the Na^+ solution [5].

RESULTS

Swimming behavior

The characteristic feature of the paranoiac mutants is their abnormally prolonged backward swimming in Na^+-containing solutions [4, 5, 12]. The behavior of wild type and three paranoiac mutants was examined in a series of solutions with Na^+ concentrations ranging from 0 to 20 mM (Fig. 2). We used the same set of solutions for the ion-regulation experiments below, in order to correlate the two membrane-related processes, the excitation-driven behavior and the ion fluxes.

Wild-type *Paramecium* shows brief backward jerks in these solutions, which usually last less than 1 s; and the cells seldom swim backward for more than 3 s. Three paranoiac mutants, in contrast, show prolonged backward swimming in solutions of high Na^+ concentration. The duration of this backward swimming increases as the Na^+ concentration of the solution increases (Fig. 2). The paranoiacs *PaA* and *PaC* swim backward longer in 20 mM Na^+ than *fna*^{P}. After this period of backward swimming, the cells show alternating periods of backward and forward swimming. The three paranoiac mutants, *PaA*, *PaC*, and *fna*^{P} have mutations in three different genes. The mutations of *PaA* and *PaC* are closely linked (3 map units apart) and are unlinked to *fna*^{P} (Van Houten, J., Chang, S.-Y. and Kung, C., in preparation).

Most, but not all of the paranoiac cells swim backward immediately after transfer to the solutions containing 8 or 20 mM Na^+. The percentage of cells that immediately swims backward is dependent upon the external Na^+ concentration. 70–85 % of the paranoiac cells swim backward immediately after transfer to 8 mM Na^+, while 85–95 % swim backward after transfer to 20 mM Na^+. The variability in response to Na^+ is probably due in part to the variable stimulation of the cells'

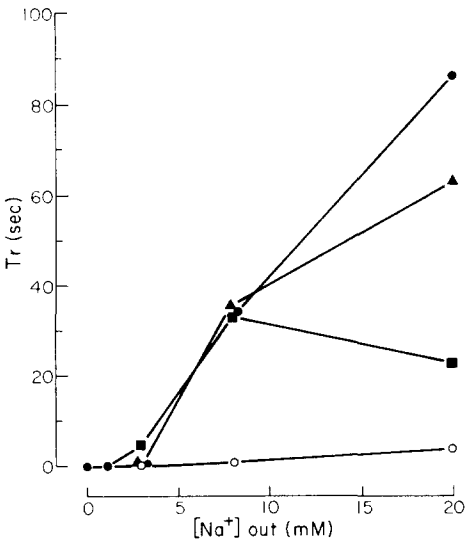

Fig. 2. Backward swimming of *Paramecium* in Na^+ solutions. Data show the duration of backward swimming in seconds (T_r) immediately after cells were transferred into solutions of the specified Na^+ concentrations. ○, wild-type *Paramecium*. Closed symbols, paranoiac mutants *PaA* (●), *PaC* (▲), and *fna*^{P} (■). Each point shows mean for 20 cells. Since all populations contained some cells that did not swim backward initially, the distributions of the individual data were not Gaussian. Therefore, the standard deviations were not calculated. For the paranoiacs that did swim backward immediately in 20 mM Na^+ (85–95 % of cells), the standard deviations were 20–50 % of the means.

mechanical receptors during transfer to the solutions, since mechanical stimulation of *Paramecium* is known to cause ciliary reversal under some conditions [1].

Potassium regulation by Paramecia in sodium solutions

The paranoiac defect is characteristically expressed only in Na^+ solutions [5]. We have found that this behavioral defect is paralleled by a defect in the regulation of cellular K^+ levels by paranoiacs in Na^+ solutions.

Wild-type *Paramecium* maintains a high cellular K^+ content even in solutions of high Na^+ concentration (Fig. 3a). There is a small, but statistically significant, decrease in the K^+ content of cells incubated in 1 mM Na^+ solution. The cause of this decrease is unknown. Since the cells were adapted in a solution of 1 mM K^+, however, this is the only Na^+ solution in which the cells experience no change in ionic strength.

At both extremes of the Na^+ concentrations tested, i.e., 0 mM (approx. 0.01 mM [9]) and 20 mM, the cellular K^+ content is approx. 120 μmol/gm protein. Given the protein content (0.015 μg/cell) and cell volume ($1.5 \cdot 10^{-7}$ ml), the K^+ content is equivalent to a K^+ concentration of 12 mM, assuming that the cell is a single equimolar compartment.

In contrast to wild type, the three strains of paranoiac mutants (*fna*^{P}, *PaA*, and *PaC*) all show large losses of cellular K^+ when incubated in solutions of high Na^+ concentration (Fig. 3b, c, d). The K^+ loss in all three paranoiac mutants is proportion-

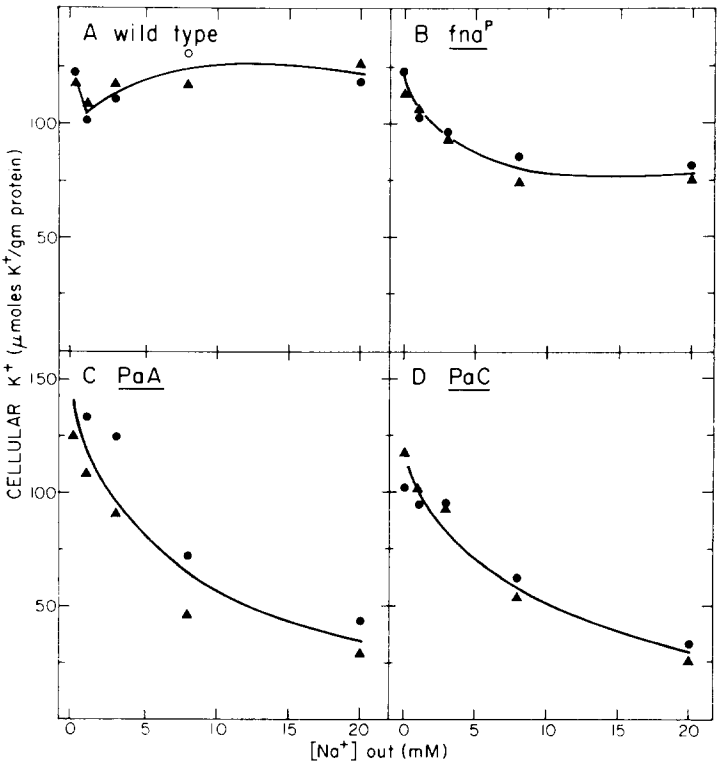

Fig. 3. Cellular K^+ content of *Paramecium* after incubation for 30–50 min in solutions of different Na^+ concentrations. Each point is the mean of duplicate incubations of cells. The two symbols (● and ▲) represent data from two experiments run on different days using different batches of cells. A, wild-type *Paramecium*. B, C, and D are the paranoiac mutants *fna*^{P}, *PaA*, and *PaC*, respectively.

al to the external Na^+ concentration. Both *PaA* and *PaC* lost approx. three quarters of their cellular K^+ after 30 min in 20 mM Na^+ solution.

There was no cell death during incubation or centrifugation of the cells in any of the solutions. All cells were alive at the end of the centrifugation, as determined by counts of swimming cells before and after the experiment [9]. Thus, the loss of K^+ by paranoiacs cannot be attributed to a K^+ release by lysed cells. Cell death was observed only after incubation in solutions of 50 mM Na^+ or more.

The cellular K^+ as shown in Fig. 3 was measured after 30–50 min incubations in the test solutions. During this period of incubation, most of the K^+ loss occurred, as shown in Fig. 4. The cells in these experiments were transferred from 1 mM K^+ to 8 mM Na^+ solution, in which they were incubated for different lengths of time. Wild-type *Paramecium* showed a rapid K^+ loss, which was essentially complete after a 10 min incubation. This K^+ loss is probably not due to the external Na^+, since wild type has a similar K^+ content in 0 and 8 mM Na^+ (Fig. 3). The K^+ loss is probably due instead to the large decrease in extracellular K^+ at zero time (1 mM to 0.01 mM K^+). The paranoiac *fna*^{P} showed a larger K^+ loss during the first 10 min of incubation and continued to lose K^+ even after 30 min incubation. In addition, the K^+ loss from *fna*^{P} was caused in part by the extracellular Na^+.

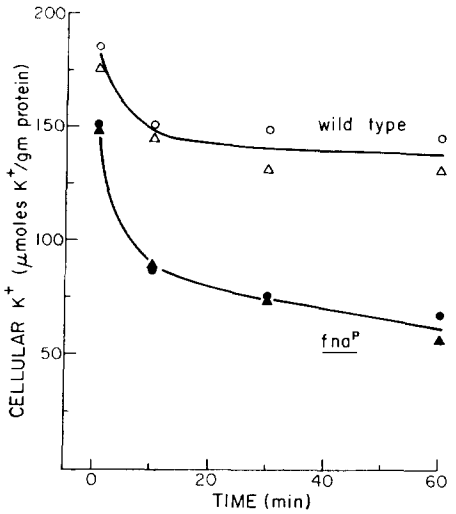

Fig. 4. Time course of K^+ efflux from *Paramecium* incubated in 8 mM Na^+ solution. Cells were transferred at time = 0 from 1 mM K^+ solution to 8 mM Na^+ solution. Open symbols = wild-type *Paramecium*. Closed symbols = paranoiac mutant *fna*^{P}. See Fig. 3 legend for additional description of symbols.

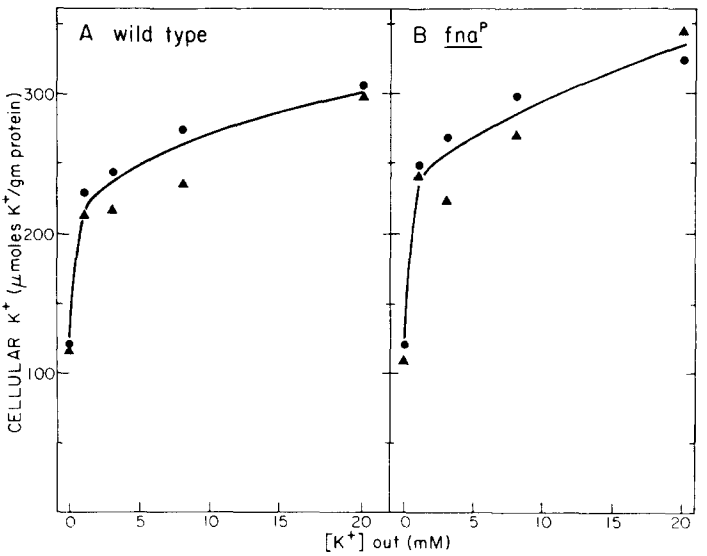

Fig. 5. Cellular K^+ content of *Paramecium* after incubation for 30–45 min in solutions of different K^+ concentrations. Cells were adapted in 1 mM K^+-solution before transfer to the incubation solutions. Actual K^+ concentration was 0.01 mM in the incubation solution of "0 mM" K^+. A, wild-type Paramecium. B, paranoiac mutant *fna*^{P}. See Fig. 3 legend for description of symbols.

Potassium regulation by Paramecia in potassium solutions

Cells in 1 mM K^+ were transferred to solutions containing 0.01 to 20 mM K^+ and were incubated for 30–50 min. Similar results were obtained for wild-type *Paramecium* and the paranoiac mutant, *fna*^{P} (Fig. 5). In both wild type and this paranoiac, the cellular K^+ almost doubled as the extracellular K^+ concentration increased from 0.01 mM to 1 mM. Browning and Nelson [13] have identified an energy-dependent K^+ uptake system in *P. aurelia* with a K_m of approx. 0.03 mM. This may account for the large increase in cellular K^+ that we have observed.

We have also measured the K^+ content of cells incubated in 4 mM K^+. For all three paranoiac mutants, the mean K^+ content was within 10 % of that of wild-type *Paramecium*, which is within the variability found among different samples of wild-type cells. Thus there appears to be no difference between wild-type and paranoiac *Paramecium* in their ability to regulate cellular K^+ levels in K^+ solutions.

Sodium fluxes

The paranoiac mutants lose cellular K^+ when suspended in Na^+ solutions, as shown in Figs. 3 and 4. This K^+ efflux must be balanced by another ion flux if the cell is to maintain electroneutrality. Since the paranoiac defect is seen in Na^+ solutions, a Na^+ influx might be expected.

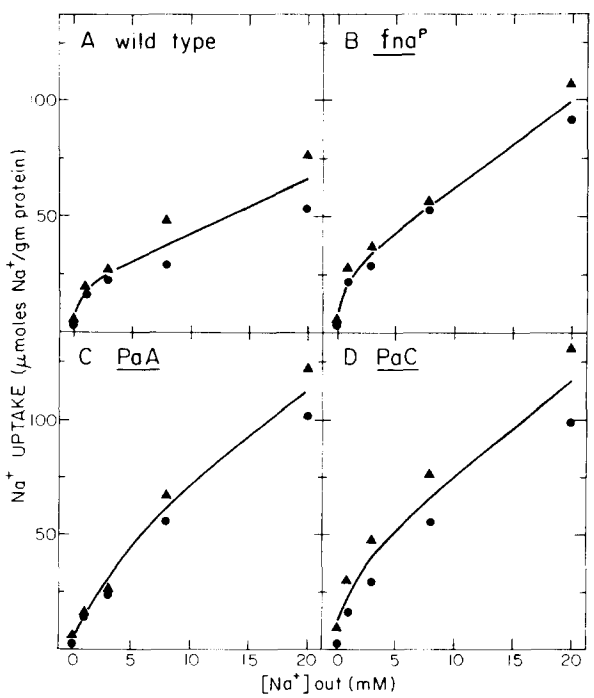

Fig. 6. Na^+ Uptake by *Paramecium* during 30–45 min incubations in solutions of different Na^+ concentrations containing ^{22}Na. The two symbols (circles and triangles) represent data obtained from two experiments run on different days using different batches of cells. Wash solutions contained non-radioactive Na^+ at the same concentrations as in the incubation solutions. A, wild-type *Paramecium*. B, C, and D are the paranoiac mutants *fna*^{P}, *PaA*, and *PaC*, respectively.

Fig. 6 shows the Na^+ uptake by wild-type and paranoiac cells after 30–45 min incubations in solutions containing different concentrations of ^{22}Na. The paranoiac mutants *PaA*, *PaC*, and *fna*^{P} all show an uptake of Na^+ similar to that of wild-type *Paramecium* in solutions of 1 mM Na^+ or less. After incubation in solutions of 8 mM Na^+ or more, however, the three paranoiacs show much larger Na^+ influxes than wild-type *Paramecium*. These results are consistent with the behavioral data of Fig. 2 and the cellular K^+ data of Fig. 3, since *PaA* and *PaC* show larger Na^+ influxes than *fna*^{P}.

The time course of Na^+ uptake by wild type and *fna*^{P} is shown in Fig. 7. The initial rate of Na^+ uptake is much more rapid in the paranoiac *fna*^{P} than in wild type. Preliminary studies show that *PaC* also has a higher initial rate and a higher final amount of Na^+ uptake than wild type.

When grown in a medium containing 10 mM Na^+, the paranoiac mutants have a total of Na^+ content almost twice that of wild type. Isotopic equilibrium measurements at 20 h give a value of 60 μmol Na^+/gm protein for wild type and 95–110 μmol Na^+/gm protein for the paranoiacs *fna*^{P} and *PaC*. Flame photometric analysis of large samples of cells gives 65 μmol Na^+/gm protein for wild type and 130 μmol Na^+/gm protein for the paranoiac *PaC*. These data suggest that the increased uptake of ^{22}Na by paranoiacs (Figs. 6 and 7) reflects a net Na^+ uptake and not only an increased rate of Na^+ exchange.

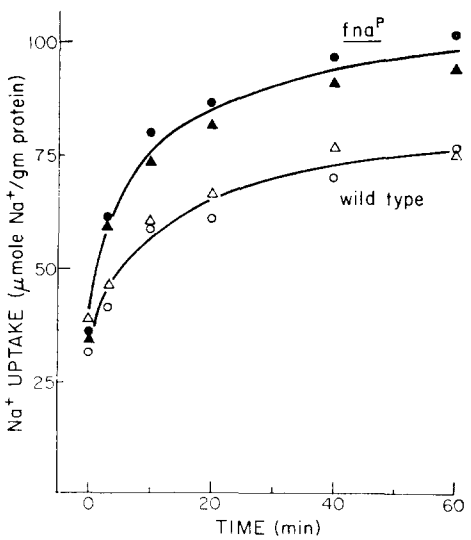

Fig. 7. Time course of Na^+ influx in *Paramecium* incubated in 8 mM Na^+-solution. Cells were transferred at time = 0 from an adaptation solution containing no added Na^+ or K^+ to an incubation solution containing 8 mM Na^+ and ^{22}Na. Aliquots of the cell suspension were analyzed for cellular ^{22}Na after the indicated incubation times, starting at $t = 20$ s. Open symbols = wild-type *Paramecium*. Closed symbols = paranoiac mutant *fna*^{P}. See Fig. 6 legend for additional description of symbols. Wash solutions did not contain Na^+. The difference in Na^+ uptake at 40 min between Figs. 6 and 7 is due to changes in the experimental procedures between the two sets of experiments (cell densities, wash solutions, etc.).

DISCUSSION

We have found a biochemical correlate for the membrane defects of a class of membrane mutants in *Paramecium* known as "paranoiacs." In solutions of 8 mM Na^+ or higher, where the electrophysiological and behavioral abnormalities are best observed, these mutants show large losses of K^+, large gains in Na^+, and prolonged backward swimming. In each case, the paranoiacs *PaA* and *PaC* differ from wild type *Paramecium* somewhat more than the paranoiac mutant *fna*^{P}. The K^+ loss from the paranoiac mutants is similar in magnitude to the Na^+ uptake, although exact comparisons cannot be made because different methods were used to measure the two ions.

Membrane excitation

The electrophysiological correlate for the backward swimming of the paranoiacs is shown in Fig. 8 (lower trace). The periods of backward swimming correlate exactly with the membrane depolarizations [7]. The paranoiac mutants in Na^+-solutions swim backward for 15 s or more at a time; thus, their membrane potentials show prolonged depolarizations. Wild type *Paramecium*, in contrast, jerks backward only briefly; and its membrane potential record shows depolarizations each lasting only about 0.5 s (Fig. 8, upper trace).

The depolarizations of both wild type and paranoiacs are due in part to an inward Ca^{2+} current [1, 4, 14, 15]. This Ca^{2+} influx raises the Ca^{2+} concentration in the cilia, which results in a reversal of the direction of the ciliary beat and, hence, backward swimming [1].

Ion fluxes vs. membrane excitation

The K^+ losses and the Na^+ influxes of the paranoiac mutants might be occurring primarily during any one of the three phases of membrane activity: (1) while the membrane is at the resting potential level; (2) while the membrane is at the excited, depolarized state; or (3) while the membrane is depolarizing and repolarizing. We

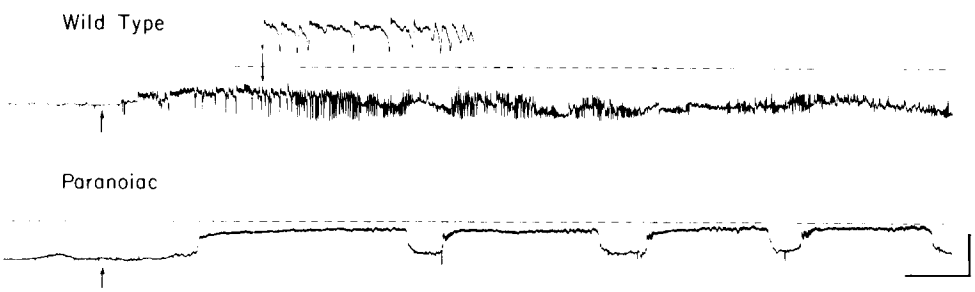

Fig. 8. Intracellular recording of membrane potentials of wild-type *Paramecium* (upper trace) and the paranoiac mutant *PaA* (lower trace) in Na^+ solutions. Na^+ solutions (4 mM NaCl, 1 mM Ca^{2+}, 1 mM citrate, 1.2 mM Tris · HCl pH 7.2) was let into the bath in place of an adaptation medium (a K^+ solution) at points marked by the arrows beneath the traces. A small part of the wild-type response is also shown in faster oscilloscope sweep-speed (insert). Broken lines mark the reference zero potential level. Calibration: vertical bar, 40 mV, horizontal bar, 25 s (5 s for the insert). (Data of Satow, Y. and Kung, C.).

believe that the large cation fluxes of the paranoiac mutants occur primarily across the depolarized membrane, for the following reasons.

First, the Na^+ uptake and the K^+ loss are directly related to the duration of backward swimming, when we vary the extracellular Na^+ concentration or when different strains of paramecia are compared (Figs. 2, 3 and 6). Although complicated hypotheses could be developed to explain this relationship, the simplest hypothesis is that the cation fluxes are occurring across the depolarized membrane and are for that reason correlated with the duration of the excited, depolarized state or the duration of backward swimming.

Second, current pulses delivered to the membrane of *PaA* show that the depolarized, excited membrane has a much higher conductance than the resting membrane [12]. Wild type membrane also has a high conductance when artificially depolarized with a long d.c. pulse. There is no obvious difference in the conductance at rest between wild-type and paranoiac membranes (Satow, Y. and Kung, C., unpublished results). If the resting conductance and the excited conductance in wild type and paranoiac are the same, then the time-averaged conductances or permeabilities of the paranoiac membrane will be greater than those of wild type, since the paranoiac membranes spend more time in the excited, high conductance state. Thus, the large cation fluxes we have measured in the paranoiacs are apparently due to the higher average conductances of the mutant membranes.

Our data, therefore, suggest that there are large Na^+ influxes and large K^+ effluxes occurring while the paranoiac mutants are swimming backward; and we have gained some understanding of how the paranoiacs swim backward. We are now beginning to find out why the paranoiac mutants swim backward; i.e., what gene is mutated in each paranoiac. For *PaA*, we have hypothesized a defect in membrane repolarization [12]. For *fna*^{P}, we have recently found an altered Na^+-"carrier" that could cause the prolonged depolarizations of this mutant (Hansma, unpublished results). Thus, this system is proving to be useful for studying the molecular mechanism of membrane excitation.

ACKNOWLEDGEMENTS

We thank Dr. Y. Satow for the use of her data in Fig. 8 and for her critical reading of the manuscript. The work is supported by NSF grant GMS75-10433 and PHS grant GM22714-01 to C.K.

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