TURNOVER OF MONOVALENT CATIONS IN
PARAMECIUM

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SUMMARY

Regulation of sodium and potassium by Paramecium was studied by measuring fluxes of $^{22}$Na and $^{86}$Rb respectively. Fluxes of these tracers had the following similarities. (1) Steady-state turnover showed approximately single-pool kinetics with 50% turnover in $\frac{1}{2}$ h. (2) Efflux of both cations showed a specific exchange diffusion when the medium contained the corresponding non-radioactive cation. (3) The net efflux of both cations was slow in the absence of exchange diffusion.

Fluxes showed the following differences. (1) The cells had 2 to 4 times as much $K^+ \cdot Rb$ as $Na$. Therefore, (2) the $K^+ \cdot Rb$ fluxes at steady state were 2–4 times larger than the $Na$ fluxes, since the fractional turnover was the same for both cations. (3) Membrane excitation by $Ba^{2+}$ stimulated the efflux of $Rb$ but not $Na$. The efflux of $^{86}Rb$ during membrane excitation appeared to represent the outward or delayed rectification that repolarizes the membranes after an action potential, since it was absent in a pawn mutant that is lacking in membrane excitation.

INTRODUCTION

Ionic currents are now implicated in a wide range of cellular functions, including embryonic development, morphogenesis, mitochondrial energy production and circadian rhythms (Jaffe, Robinson & Nuccitelli, 1974; Sweeney, 1974; Harold, 1977). In Paramecium, ionic currents control swimming behaviour (Eckert & Brehm, 1979). When its membrane is at the resting potential level, Paramecium swims forward. When its membrane is hyperpolarized, Paramecium swims forward faster. When its membrane is depolarized, Paramecium swims backward. Threshold depolarization triggers a Ca action potential, in which Ca enters the cell through Ca channels located only on the ciliary membranes (Dunlap, 1977; Ogura & Takahashi, 1976). The resulting rise in intraciliary Ca causes the cilia to beat backward, which causes the Paramecium to swim backward. Backward swimming lasts only as long as the membrane is depolarized and can be observed easily with a dissecting microscope. This correlation of membrane potential with swimming behaviour makes it easy to measure directly the ionic fluxes involved in membrane excitation. Changes in membrane potential in Paramecium are caused by changes in external stimuli.
such as temperature or chemicals. Therefore the ion currents in *Paramecium* carry out one step in the sensory transduction of external stimuli into altered swimming responses.

The beauty of *Paramecium* for investigating ion currents and membrane excitation is that it is one of the few organisms that can be studied by all of the following techniques: genetic dissection, intracellular electrophysiology, radioactive ion fluxes, and biochemical analyses of purified excitable membrane (Kung *et al.* 1975). It has been particularly useful to measure ion fluxes in different mutants with specific defects in membrane excitation and swimming behaviour. For example, by measuring fluxes in the ‘paranoic’ mutants it has been found that *Paramecium* has a voltage-dependent Na-current which was not seen by electrophysiology (Hansma, 1979). In the present work, the regulation of Na and K has been studied in the wild type *Paramecium* and a pawn mutant lacking in functional Ca channels.

**MATERIALS AND METHODS**

**Supplies**

Sodium-22 and rubidium-86 were obtained from New England Nuclear. RbCl was ‘Suprapur’ from EM Reagents. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) and CCCP (carbonylcyanide, m-chlorophenylhydrazone) were obtained from Sigma.

**Cell stocks**

The strains used were *Paramecium tetraurelia* (formerly *P. aurelia*, syngen 4). The wild type stock was 51s, from which all mutants were derived. The pawn mutant was d4~95, genotype pwb pwb. The K-resistant mutants d4~52i and d4~524 were used for some experiments on the steady-state turnover of $^{86}$Rb. All strains are homozygous at all loci and were kindly supplied by Ching Kung.

**Culturing and harvesting cells**

Cells were grown in Cerophyl medium inoculated with *Enterobacter aerogenes* (Sonneborn, 1950). This medium contained 5 mM-K+, 0.5 mM-Na+ and 0.2 mM-Ca2+. Cells were harvested by centrifuging for 2 min at 300 g.

**Steady-state turnover of $^{22}$Na and $^{86}$Rb**

Cells were concentrated 15-fold and incubated in culture medium containing either $^{22}$Na + NaCl or $^{86}$Rb + RbCl + KCl (see figure captions). After overnight incubation, the cells were centrifuged gently and resuspended in non-radioactive culture medium containing the same concentrations of salts. $^{86}$Rb or $^{22}$Na efflux was measured by centrifuging duplicate 1 ml aliquots of the cell suspension through a wash solution (culture medium with 1% sucrose and the same concentrations of salts as the efflux medium) in centrifuge tubes made by flaming shut the tips of six-inch Pasteur pipettes (Hansma & Kung, 1976). After centrifugation, the tips of the centrifuge tubes containing the cell pellets were cut off. The radioactivity of tips containing $^{22}$Na was measured directly in a gamma counter. Tips containing
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Rb were crushed in vials containing scintillation fluid (Anderson & McClure, 1973) before measuring their radioactivity in a scintillation counter.

The data were plotted as log \((D_t - D_x)/(D_0 - D_w)\) v. time, where \(D_t\) and \(D_0\) are the specific activities of \(^{22}\)Na or \(^{88}\)Rb at time \(t\) and time zero, respectively, and \(D_x\) is the specific activity of \(^{22}\)Na or \(^{88}\)Rb in cells plus efflux medium, i.e. the minimum level to which the cellular specific activity will drop.

Efflux of \(^{22}\)Na and \(^{88}\)Rb into buffer

Cells were concentrated 15-fold and incubated in culture medium containing either \(^{22}\)Na or \(^{88}\)Rb + 1 mM-RbCl. After overnight incubation, the cells were centrifuged gently and suspended in buffer (10 mM-HEPES, 6 mM-Tris, 0.1 mM-CaCl₂, 0.02 mM-EDTA, pH 7). A pair of 1 ml aliquots were centrifuged to obtain the 'time zero' data. Then the indicated amounts of other salts were added (see figure captions), and pairs of aliquots were centrifuged at subsequent times. Radioactivity was measured in cell pellets and/or supernatants.

Statistical analyses

Data are presented as means ± standard deviations. Student's \(t\) test (Campbell, 1974) was used to determine the statistical significance of differences between means.

RESULTS

Total cellular Na varies with extracellular Na

In medium containing 0.5 mM-Na, the total cellular Na was 0.62 ± 0.24 nmol/10³ cells. Assuming a uniform distribution of Na throughout the cell, this amounts to a cytoplasmic concentration of 3–6 mM. In medium containing 20 mM-Na, the total cellular Na doubled to 1.27 ± 0.44 nmol/10³ cells, amounting to 6–12 mM. In media containing 5 and 10 mM-Na, the Na content was similar: 1 nmol/10³ cells in 5 mM-Na and 0.9 nmol/10³ cells in 10 mM-Na.

When cells equilibrated with 20 mM-Na were incubated on ice for 10–30 min, their total cellular Na almost doubled. When the cells were returned to room temperature, their Na content fell to near the 'pre-ice' level in 20–30 min. This suggests that cells in 20 mM-Na may have cytoplasmic concentrations of approximately 10 mM-Na. Incubation on ice might be inhibiting an energy-dependent mechanism for the extrusion of Na, since ice and the metabolic inhibitors NaN₃ and CCCP all had similar effects on the Na and Rb fluxes of cells in buffer. The two metabolic inhibitors decrease the ATP in Paramecium to low levels (Browning & Nelson, 1976; Browning, 1976; and H. G. Hansma, unpublished observations).

When cells equilibrated with 0.5 mM-Na were incubated on ice, they lost only 20–30% of their Na after 1 h.

Steady-state turnover of Na

The steady-state turnover of \(^{22}\)Na showed approximately first-order kinetics, suggesting that there is a single cellular pool of Na. The time for 50% efflux \((t_d)\) was determined graphically. The deviations from first-order kinetics were not inter-
Fig. 1. Steady-state turnover of $^{22}$Na in wild type Paramecium. Extracellular Na$^+$ = 0.5 mM (○) or 10 mM (●). Data points are means of paired determinations.

Preceding Na pools since they were not very pronounced and could be artefactual. Two sources of experimental error were: (1) the cells were gradually starving during the course of the experiment, so that the total Na content was gradually decreasing, and (2) the extracellular solution contained $^{22}$Na with 1 to 3% of the initial specific activity of Na, so that there was a small influx of $^{22}$Na counterbalancing some of the efflux. Cation turnovers in erythrocytes show similar deviations from first-order kinetics which also have not been fully explained (Passow, 1964).

The steady-state turnover of Na was not significantly different for cells in high Na and low Na media. The $t_1$ was 53 ± 15 min for cells in 0.5 mM-Na (Fig. 1), 41 ± 7 for cells in 5 mM-Na, 30 for cells in 10 mM-Na (Fig. 1) and 40 ± 11 for cells in 20 mM-Na.

The kinetics of Na turnover were not affected by a 10-fold increase in the extracellular Ca concentration: $t_1$ = 44 min for cells in medium containing 2 mM-Ca, 5 mM-Na.
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**K+ inhibits Rb+ turnover**

$^{86}$Rb was used as an analogue for K, since its 19-day half-life is much longer than the 12-h half-life of $^{42}$K. There are three lines of evidence that Rb is a good analogue for K in Paramecium. First, Browning (1976) has shown that the kinetics of uptake of $^{86}$Rb and $^{42}$K are similar. Second, Naitoh & Eckert (1968) have shown that K and Rb have the same effect on membrane potential, resting resistance, and current-voltage curves in Paramecium. Third, Shusterman, Thiede & Kung (1978) have found that K and Rb have similar effects on the survival of wild type and K+-resistant mutants of Paramecium. However, the results given below show that exchanges of K and Rb are not identical in Paramecium.

The kinetics of steady-state turnover of Rb were measured at different Rb concentrations and at different ratios of Rb to K (Fig. 2). When the Rb to K ratio in the medium was high (Rb/K = 4), $t_1$ was $49 \pm 6$ min, which is the same as that for Na. This medium contained $20 \text{ mM-Rb} + 5 \text{ mM-K}$ ($25 \text{ mM-Rb+K}$). When the Rb to K ratio was low (0·2) but the total Rb + K was kept at 25 mM (4·2 mM-Rb + 20·8 mM-K) the $t_1$ was 73 ± 14 min, which is significantly higher. The $t_1$ was the same (77 ± 11 min) for cells in a medium of only 6 mM-Rb + K, when the Rb/K ratio remained at 0·2. These results are interpreted as follows: (1) Rb + K turnover is independent of the extracellular Rb + K concentration, if the ratio of Rb to K is kept constant. (2) K inhibits Rb turnover; it appears that K is exchanged in preference to Rb.

The kinetics of Rb turnover were unaffected by a 10-fold increase in extracellular Ca: $t_1 = 85$ min for cells in medium containing 2 mM-Ca, 6 mM-Rb + K with Rb/K = 0·2.

The total cellular K + Rb concentration was the same over a four-fold change in the extracellular K + Rb concentration. Cells in medium containing 6 mM-Rb + K had $2·2 \pm 0·6 \text{ nmol Rb+K/10}^3 \text{ cells}$, and cells in 25 mM-Rb + K had $2·4 \pm 0·4 \text{ nmol Rb+K/10}^3 \text{ cells}$. These are equivalent to cytoplasmic concentrations of 12–24 mM, assuming uniform distribution of Rb + K. These results for cells equilibrated overnight in different concentrations of Rb + K are similar to earlier results on the total cellular K measured by flame photometry of cells incubated briefly in solutions of different K concentrations (Hansma & Kung, 1976). The total cellular Rb + K was relatively independent of the Rb/K ratio; there was no significant difference in Rb + K for cells in the two media of 25 mM-Rb+K, one with Rb/K = 4 and the other with Rb/K = 0·2.

When cells equilibrated with 6 mM-Rb + K were cooled on ice, they lost 40–60% of their $^{86}$Rb in 1 h, and the cellular Rb increased to near the ‘pre-ice’ level when the cells were returned to room temperature. This suggests that the cells have an energy-dependent mechanism for concentrating Rb + K above the extracellular level. Similarly, cells in buffer lost Rb when incubated on ice, in 1 mM-NaNO$_3$, or in 2 mM-CCCP.

When cells equilibrated with 25 mM-Rb + K were chilled on ice for one hour, they showed less than 10% gain or loss of $^{86}$Rb. This suggests that the cytoplasmic concentration of Rb + K may be close to 25 mM, which is approximately the concentration calculated from total Rb + K assuming a uniform distribution in ‘thin’,
Fig. 2. Steady-state turnover of $^{86}$Rb in wild type Paramecium. Extracellular medium contains: 5 mM-K$^+$ and 1 mM-Rb$^+$ (O, Rb/K = 0.2) or 20 mM-K$^+$ and 42 mM-Rb$^+$ (x, Rb/K = 0.2), or 5 mM-K$^+$ and 20 mM-Rb$^+$ (●, Rb/K = 4).

starved cells with volumes of $10^{-7}$ ml. The volume of Paramecium changes with growth condition and has been reported as 1 to $2 \times 10^{-7}$ ml/cell (Hansma, 1979; Hansma & Kung, 1976; Ling & Kung, 1980).

**Effect of cations on Rb and Na efflux**

Cells equilibrated with $^{22}$Na (Fig. 3) or $^{86}$Rb (Figs. 4 and 5) were transferred to buffer with or without inorganic cations to determine the dependence of cation efflux on extracellular cations. In buffer with no added cations, the net efflux of Rb was very slow: less than 5% of the total cellular Rb was lost in 1 h. There was also little net efflux of Rb into buffer containing 1 mM-Na$^+$ or Ca$^{2+}$. These buffers contained about 30-50 μM-$^{86}$Rb + K.

The net efflux of Na into buffer containing no added cations was somewhat faster: about 25% of the total cellular Na was lost in 1 h. The buffer contained 5-10 μM-$^{22}$Na. The efflux of $^{22}$Na was not affected by the addition of 1 mM-Ca, K, or Rb (Fig. 3).

The efflux of $^{86}$Rb was stimulated by adding 1 mM-Rb or K to the medium, and the efflux of $^{22}$Na was stimulated by adding 1 mM-Na to the medium. This represents a one-for-one exchange of Na for $^{22}$Na and Rb for $^{86}$Rb, since there was no loss of $^{22}$Na or $^{86}$Rb from cells incubated in buffer containing 1 mM-Na or Rb at the same
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Fig. 3. Efflux of $^{22}$Na from wild type Paramecium in buffer (A) or buffer supplemented with chloride salts of the cations listed in B, C and D. Data points are the means of paired determinations. The mean Na content of cells in buffer (control) at zero time (100%) is 0.5 nmol Na/10^6 cells.

Specific activity as in the cells. The kinetics of both Rb influx and efflux show half-maximal rates with 0.1 mM or less extracellular Rb. These kinetics are approximate, since the earliest ‘initial rates’ that can be measured are not linear. The kinetics of Na efflux show half-maximal saturation at less than 0.5 mM extracellular Na.

Membrane excitation stimulates Rb efflux

Ba^{2+} causes prolonged membrane excitation in wild type Paramecium, and the cells show correspondingly long periods of backward swimming: in 0.8 mM-Ba they swim backward 30–60% of the time for at least the first 5 min; the individual episodes of backward swimming vary in duration from less than 1 s to more than 1 min (Hansma, 1981; Ling & Kung, 1980).

When wild type equilibrated with $^{85}$Rb were transferred to buffer with 0.8 mM-Ba, they showed a rapid net loss of 10–15% of the total cellular Rb in the first 5 min, followed by a slower loss at longer times (Fig. 4D). Higher concentrations of Ba were lethal. In 1 mM-Ba-buffer, wild type lost 20% of its Rb in 5 min; by 15 min, many of the cells were dead, and the cellular Rb had dropped to a low level.
Fig. 4. Efflux of $^{86}$Rb from wild type *Paramecium* in buffer (A) or buffer supplemented with chloride salts of the cations listed in B, C and D. The mean Rb + K content of cells in buffer is 2.4 nmol/10^6 cells. Lower line in D is dashed because many cells died between 5 and 15 min.

The pawn B mutant has little or no membrane excitation. In 1 mM-Ba-buffer these pawn B cells swam forward slowly or pivoted, and they survived more than an hour. Pawn B also showed little $^{86}$Rb efflux even in 1 mM-Ba (Fig. 5D). The Rb efflux from pawn B was normal in buffer containing cations that did not cause extensive membrane excitation in wild type (Fig. 5A–C).

Membrane excitation stimulates Na uptake in *Paramecium* when the electrochemical gradient favours Na influx (Hansma, 1979). If the electrochemical gradient favours Na efflux, will Na also flow out when the membrane is depolarized? Ba causes prolonged membrane excitation but little Na efflux in wild type (Fig. 3D). The Na efflux in Ba-buffer is less than 10% as large as the Rb efflux in Ba-Buffer. Na efflux during membrane excitation is also less than 10% as large as the Na uptake during membrane excitation reported earlier (Hansma, 1979). Therefore, it appears that membrane excitation causes little, if any, Na efflux.
DISCUSSION

Comparison of Na and K(Rb) regulation

Na and K(Rb) regulation in Paramecium have the following similarities: (1) Steady-state turnover shows approximately single-pool kinetics with $t_1/2$ near 3 h. (2) Efflux of both cations shows a specific exchange-diffusion when the medium contains the corresponding non-radioactive cation. (3) The uptake of both cations shows saturation kinetics with apparent $K_m = 0.15$ mM for Na (Hansma, 1979) and 0.03 mM for K or Rb, with perhaps a second system of lower affinity (Browning, 1976; Nelson & Kung, 1978). These systems work against the electrochemical gradients for the ions, and there is evidence that both are energy-dependent. The apparent affinities for exchange-diffusion are similar to those for the corresponding uptake systems. (4) The net efflux of both Na and K(Rb) is slow in medium with Ca as the only cation.

There are also differences between Na and K regulation in Paramecium: (1) The cells have 2-4 times as much K+Rb as Na. Therefore, (2) the K+Rb fluxes at
steady state are 2-4 times larger than the Na fluxes since $t_j$ is the same for both ions. Also, (3) membrane excitation stimulates $^{86}$Rb efflux but not $^{22}$Na efflux. These differences are consistent with the electrophysiologist's picture that *Paramecium* has a high permeability to K and a low permeability to Na and that membrane excitation activates a K-efflux.

The steady-state turnover of K + Rb or Na in low-Na medium can be explained completely by a transmembrane exchange diffusion: the rates of $^{22}$Na or $^{86}$Rb efflux are approximately the same during both steady-state turnover (Figs. 1, 2) and exchange-diffusion (Figs. 3C and 4B), although Rb efflux is faster than Na efflux in both types of experiment. The turnover of Na and K(Rb) must involve a transmembrane flux, since 90% or more of the total cellular cation turns over during the course of the experiment. It appears that surface-bound $^{22}$Na and $^{86}$Rb turn over quickly and are removed by centrifuging the cells through wash solution. Preliminary results suggest a different picture for Ca (H. G. Hansma, unpublished observations). Steady-state turnover of Ca shows 2 pools: one pool has a $t_j$ of several minutes and may represent surface binding, while the other pool has a $t_j$ of several hours and may represent transmembrane fluxes.

The steady-state turnover of Na in high Na medium is more complicated. Cells in 5 to 20 mM-Na have twice as much Na as cells in 0.5 mM-Na, and their steady-state fluxes are twice as large. These high-Na cells may have a temperature-dependent system for extrusion of Na in addition to exchange-diffusion, since their Na content doubles at 0 °C and returns to normal after the cells are warmed to room temperature. Cells in high Na also have a voltage-dependent Na uptake (Hansma, 1979) which may account for their higher Na content.

The direct measurements of ion fluxes have provided evidence for the following components of Na and K(Rb) regulation in *Paramecium*: (1) energy-dependent systems for the uptake of Na and K, with affinities near $10^{-4}$ M; (2) exchange-diffusion of Na and K, perhaps mediated by the uptake systems; (3) voltage-dependent K efflux; (4) voltage-dependent Na influx; and (5) energy-dependent Na extrusion. *Paramecium* also has a voltage-dependent K-influx or inward-going rectification that has been seen only by electrophysiological techniques (Eckert & Brehm, 1979). More research is needed to prove the existence of some of these components and to study their interrelationships.

**K-rectification can be measured with $^{86}$Rb**

The paramecium membrane, like most excitable membranes, is repolarized after an action potential by a K efflux (Eckert, 1972; Eckert & Brehm, 1979). The results show that this voltage-dependent K-efflux can be measured as $^{86}$Rb efflux stimulated by backward swimming in *Paramecium*. The evidence for this is: (1) extracellular Ba causes prolonged backward swimming and large effuxes of $^{86}$Rb in wild type; (2) the pawn B mutant, which lacks action potentials, shows neither backward swimming nor Rb efflux in Ba; (3) wild type shows neither backward swimming nor Rb efflux in buffer or buffer with 1 mM-Ca or Na; (4) it has been shown by flame photometry that the paranoiac mutants of *Paramecium* also have large losses of total cellular K during periods of prolonged backward swimming (Hansma &
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Kung, 1976; Satow, Hansma & Kung, 1975); (5) Browning (1976) has shown that the rate of K or Rb efflux is slightly faster into buffer containing Ba + K than buffer containing only K; he attributed this small difference to membrane excitation in Ba.

The membrane can be repolarized by a current that is too small to measure biochemically. When the membrane remains depolarized for many seconds or minutes, however, the voltage-dependent Rb + K efflux results in a large loss of total cellular Rb + K. This ability to measure K-rectification directly with 86Rb is a useful complement to electrophysiological techniques, which can measure very fast membrane changes but cannot separate the different currents in Paramecium in a clear-cut manner. It will be especially interesting to measure the voltage-dependent efflux of 86Rb in the TEA-insensitive mutant of Paramecium, which has been characterized by electrophysiological techniques as a K-channel mutant (Satow & Kung, 1976).

Ion regulation in Paramecium and other organisms

Na and K regulation have been studied in many other unicellular systems, including erythrocytes, bacteria and Tetrahymena. Erythrocytes have a NaK ATPase that transports K in and Na out to maintain a high intracellular concentration of K (Baker, 1972). Paramecium does not appear to have this NaK ATPase, since K does not stimulate Na efflux (Fig. 3B). It is reasonable that Paramecium does not have a pump that transports K in and Na out since it lives in ponds of low variable salt concentrations quite different from the Na-rich blood plasma. Tetrahymena also appears to have no NaK ATPase, since the specific inhibitor ouabain has no effect on Na or K transport (Dunham & Kropp, 1973); the effect of ouabain on Na and K fluxes in Paramecium has not been investigated.

Tetrahymena is a ciliate closely related to Paramecium, and it has many similarities to Paramecium in its Na and K regulation (Dunham & Kropp, 1973). The cellular concentrations of Na and K are similar in Tetrahymena and Paramecium. There is also evidence for the active uptake of Na and K in Tetrahymena and the active extrusion of Na. In Tetrahymena, the contractile vacuole seems to extrude Na when the cells are in a high-Na medium, since the osmolality of the medium affects the cellular Na content (Dunham & Kropp, 1973). The effect of changing osmolarity on Na content in Paramecium has not been studied.

In summary, Paramecium shows K uptake and exchange as in most animals and also has a Na uptake mechanism. It does not appear to have the NaK ATPase found in higher animals. Finally, the outward-going K-rectification can be measured with 86Rb. This is a useful complement to electrophysiology for understanding this aspect of membrane excitation in normal and mutant Paramecium.

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