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Na^+ Transport in *Acetabularia* Bypasses Conductance of Plasmalemma

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Abstract. Na^+-selective microelectrodes with the sensor ETH 227 have been used to measure the cytoplasmic Na^+ concentration, $[Na^+]_c$, in *Acetabularia*. In the steady-state, $[Na^+]_c$ is about 60 mM (external 460 mM). Steps in external Na^+ concentration, $[Na^+]_o$, cause biexponential relaxations of $[Na^+]_c$ which have formally been described by a serial three-compartment model (outside ↔ compartment 1 ↔ compartment 2). From the initial slopes (some $mMsec^{-1}$) net uptake and release of about 3 $\mu molm^{-2}sec^{-1}$ Na^+ are determined. Surprisingly, but consistent with previous tracer flux measurements (Mummert, H., Gradmann, D. 1991. *J. Membrane Biol,* **124:**255–263), these Na^+ fluxes are not accompanied by corresponding changes of the transplasmalemma voltage. $[Na^+]_c$ is neither affected by the membrane voltage, nor by electrochemical gradients of H^+ or Cl^- across the plasmalemma, nor by cytoplasmic ATP. The results suggest a powerful vesicular transport system for ions which bypasses the conductance of the plasmalemma. In addition, transient increases of $[Na^+]_c$ have been observed to take place facultatively during action potentials. The exponential distribution of the amplitudes of these transients (many small and few large peaks) points to local events in the more ore less close vicinity of the Na^+ recording electrode. These events are suggested to consist of disruption of endoplasmic vesicles due to a loss of pressure in the cytoplasm.

Key words: *Acetabularia* — Compartmentation — Net Na^+ fluxes — Na^+-selective microelectrodes — Vesicular shuttle

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Introduction

Cells keep their cytoplasmic Na^+ concentration low. Animal cells accomplish this by the Na^+/K^+ ATPase in the plasmalemma which is generally accepted to be absent in plants. The majority of plant cells live without a major Na^+ problem in an environment with low salt concentration, and operate a H^+-ATPase instead. Marine algae, however, live in high concentrations of external Na^+, $[Na^+]_o$, of about 0.5 M and at alkaline pH (8). Under these conditions, Na^+ extrusion cannot take place via an electroneutral Na^+/H^+ antiporter in the plasmalemma. The alternative strategies of vascular plants, as Na^+ sequestration into the vacuoles of disposable organs, cannot be used by unicellular organisms.

From a theoretical point of view, one might expect that marine cells try to circumvent the difficulty with internal Na^+ by minimizing Na^+ entry beforehand, and handle the remaining problem by coupling the desired uphill Na^+ export through the plasmalemma to a stronger downhill gradient by an appropriate cotransporter. However, previous attempts to confirm this view by tracer flux measurements in *Acetabularia* (Mummert & Gradmann, 1991*a*) have failed for the following reasons. The experiments resulted in rates of uptake and release of $^{22}Na^+$, which corresponded to unexpectedly large unidirectional Na^+ influxes and effluxes around 2 $\mu molm^{-2}sec^{-1}$. In addition, these fluxes turned out to be insensitive to the voltage across the plasmalemma. To explain this problem, one might argue that these unidirectional fluxes reflect vigorous Na^+/Na^+ exchange without net translocation of Na^+. This auxiliary hypothesis can be examined by recordings of $[Na^+]_c$. In particular, the relaxation kinetics of $[Na^+]_c$ upon stepping $[Na^+]_o$ from control level to zero and back, should provide the essential information about the actual capacity of net Na^+ uptake and export.

Also, the effects of electrochemical driving forces on $[Na^+]_c$ should be re-examined.

In the present study, the measurements of $[Na^+]_c$ have been accomplished by means of Na^+-selective microelectrodes. To our knowledge, this is the first report on application of this method in plants. Therefore, the technique is described here in some detail. We also describe and apply here the formalism of compartmental analysis which uses the recordings of cytoplasmic concentrations, instead of the release of radioactive tracers (Walker & Pitman, 1976).

Although we cannot offer an ultimate explanation of how *Acetabularia* solves the Na^+ problem, the results are clear enough to exclude the initial view of small inward leakage of Na^+ and its extrusion by electrochemical transport coupling at the plasmalemma. On the other hand, the results indicate a powerful vesicular system for ion uptake and release in *Acetabularia* which bypasses the conductance of the plasmalemma. Furthermore, local and transient increases in $[Na^+]_c$, seem to be elicited by disruption of Na^+-loaded vesicles upon a facultative pressure drop in the cytoplasm during action potentials.

Materials and Methods

Cells, Solutions and Setup

Cells of *Acetabularia acetabulum* have been cultured according to Lüttke and Grawe (1984). The cells used had an average volume of about 10^{-9} m^3, a surface area of about $3 \cdot 10^{-5}$ m^2, and a cytoplasmic volume of about $5 \cdot 10^{-11}$ m^3, i.e., about 5% of the cell volume (Goldfarb, Sanders & Gradmann, 1984).

The experimental setup as well as fabrication, calibration and use of ion-selective microelectrodes has already been described in a corresponding Ca^{2+} study (Amtmann, Klieber & Gradmann, 1992). For experiments, we used artificial sea-water (ASW, in mM: 10 KCl, 460 NaCl, 10 $CaCl_2$, 25 $MgCl_2$, 28 $MgSO_4$, buffered with 10 MES/Tris at pH 8.0 or 5.8). In Na^+-free (<10 μM) ASW, NaCl was replaced by cholinechloride (ChlnCl). For examination of possible effects of the energy metabolism on $[Na^+]_c$, 50 μM of the uncoupler carbonylcyanide-3-chlorphenylhydrazone (CCCP) was used by adding 0.1% (v/v) 5 mM ethanolic stock solution to ASW. Butyrate (5 mM at pH 5.8 in ASW) was used to test possible effects of cytoplasmic acidification (Sanders, Hansen & Slayman, 1981).

Na^+-selective Microelectrodes

The Na^+-selective microelectrodes for intracellular use contained the ionophore ETH 227 (Sodium Ionophore I-Cocktail A, 71176 Fluka, CH-Buchs) and were calibrated in solutions which correspond to the cytoplasm of *Acetabularia* (ACA); all calibration solutions contained 5 mM $MgCl_2$, pCa 6.2 buffered with 2.5 mM $CaCl_2$ and 5 mM ethyleneglycolbis(2-aminoethyl)tetraacetic acid (EGTA), pH 7 buffered with 10 mM HEPES/Tris; solutions with different Na^+ concentrations and constant ionic strength were prepared with the following NaCl/KCl ratios (in mM); pNa 1: 100/300, pNa 2: 10/400, pNa 3: 1/400.

For an ionic strength of about 0.5 M in the cytoplasm of *Acetabularia,* the activity coefficient for Na^+, γ_{na}, of 0.7 was calculated according to Robinson and Stokes (1968). If necessary, activities and concentrations of the free ions will be specified by the prefixes *a* and *c*, respectively, i.e., $_a[Na^+]$ or $_c[Na^+]$. In equations, activities will be given by italics, e.g., *Na*.

The relationship between the voltage, V_{Na}, of a Na^+-selective microelectrode and $[Na^+]$ in the presence of competing ions *J* is described by the equation:

$$V_{Na} = V_0 + s \cdot \log \frac{Na_b + \sum KJ_b}{Na_p + \sum KJ_p} \quad (1)$$

with a slope *s* (theoretically: 2.3 $RT/(zF)$) between 53 mV (given by Fluka) and 59 mV (theoretical value), the reference voltage V_0, ΣKJ being $\Sigma K_{J/Na} \cdot {}_a[J]^{1/zJ}$ with $K_{J/Na}$ being relative potentiometric selectivity coefficients with respect to Na^+, and the indices *b* and *p* marking bath and pipette.

Calculations of V_{Na} of ETH 227 electrodes as a function of $_a[Na^+]$ in the presence of competing ions in realistic experimental solutions yielded corresponding detection limits, *dl,* (as defined by IUPAC, 1976) using potentiometric activity coefficients according to Steiner et al. (1979): for 100 mM KCl, the calculated *dl* was at 28 μM Na^+; for ACA (with little Ca^{2+}), the theoretical *dl* was at 0.3 mM Na^+, and for ASW (in the presence of 10 mM Ca^{2+}), a *dl* of about 6 mM was calculated by Eq. (1). The corresponding curves are shown by Fig. 1*A*.

Experimental calibrations of 26 Na^+-selective microelectrodes in ACA before and after impalement resulted in *dl* of 2.6 ± 1.6 SD mM in precalibrations, and was slightly increased to 4.3 ± 3.2 SD mM in postcalibrations. According to these empirical results, the electrodes were suited to record $_a[Na^+]_c$ in *Acetabularia,* which is usually more than an order of magnitude higher than these detection limits. The slopes, *s*, and the reference voltages, V_0, of the electrodes did not differ significantly between pre- and postcalibration; *s* ± SEM: pre 56.2 ± 0.4 mV, post 56.7 ± 0.4 mV; V_0 ± SEM: pre 31.78 ± 3 mV, post 36.4 ± 0.4 mV. An example is shown by Fig. 1*B*.

For recordings of $_a[Na^+]_c$ with ETH 227, possible interference of Ca^{2+} has been examined empirically in ACA with different contents of free Ca^{2+} (p_aCa between 2.5 and 6.5 according to Amtmann et al., 1992). The V_{Na} recordings were analyzed by a version of Eq. (1) which treats Ca^{2+} explicitly

$$V_{Na} = V_0 + s \cdot \log \frac{Na_b + KCa_b + \sum KJ_b}{Na_p + KCa_p + \sum KJ_p} \quad (2)$$

The results from four precalibrations and three postcalibrations amounted to $\log K_{Ca/Na} = 2.4 \pm 0.3$ SD. Figure 1*C* shows the average results and the fitted calibration curve. These results mean that Ca^{2+} permeates the membrane of the Na^+-selective electrode about 250 times better than Na^+ does.

It is pointed out that the microelectrode recordings given here refer to the cytoplasmic compartment because in *Acetabularia* the electrode tips do not enter the vacuole, but are surrounded by cytoplasm (Gradmann & Klemke, 1974). Experiments with vacuole-depleted cell segments according to Goldfarb et al. (1984) have been carried out in the course of this study. Since these experiments did not yield additional insights, the corresponding results are not presented separately.

Net Fluxes

Usually, fluxes are determined by uptake and release of radioactive tracers under steady-state conditions (for review, *see* Walker & Pitman, 1976). This approach provides *unidirectional* fluxes (e.g., Na^+/Na^+ exchange would cause unidirectional fluxes in both direc-

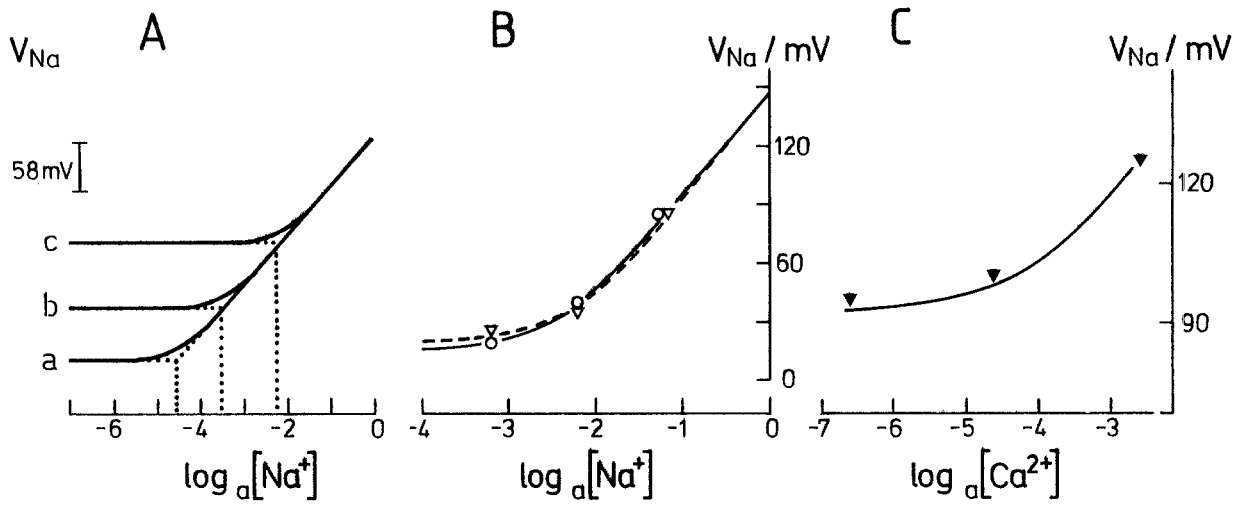

Fig. 1. Voltage response of Na^+-selective microelectrodes with sensor ETH 277. (*A*) Theoretical behavior (Eq. 1 with coefficients from Steiner et al., 1976) in the presence of competing ions; (*a*) 100 mM KCl; (*b*) simulated cytoplasm of *Acetabularia* (ACA: components given in text); (*c*) artificial sea-water (composition in text). (*B*) Example of real behavior in ACA, data measured, curves fitted with Eq. (1); (○—○) precalibration, V_0: 40 mV, *s:* 53 mV, ΣK: $3.4 \cdot 10^{-3}$; (▽---▽) postcalibration, V_0: 39 mV, *s:* 56 mV, ΣK: $5.3 \cdot 10^{-3}$. (*C*) Example of effect of competing Ca^{2+} in ACA, solutions according to Amtmann et al. (1992), data measured, line fitted by Eq. (2) with average parameters of Eq. (1) and $\log K_{Ca/Na} = 2.4$.

tions without net flux). In this study, changes in cytoplasmic Na^+ are measured, which provide *net* fluxes.

In our experiments, the cytoplasmic Na^+ concentration, C_1, changes with a biexponential response on rectangular steps in external Na^+.

$$C_1(t) = c_{11}exp(-\lambda_1 t) + c_{12}exp(-\lambda_2 t) \tag{3}$$

Such relaxation kinetics can sufficiently be described by a serial model with three elements ($C_0 \leftrightarrow C_1 \leftrightarrow C_2$), where the indices 0 and 1 denote the external medium and the cytoplasm, respectively, and 2 the stands for a system of endoplasmic vesicles and vacuoles (for details, *see* Discussion).

Under steady-state conditions, $[Na^+]$ in compartment 1 (C_1) and in compartment 2 (C_2) equilibrate with the external concentration, C_0, following the relationships

$$C_1/C_0 = P_{01}/P_{10} \tag{4a}$$

$$C_2/C_1 = P_{12}/P_{21} \tag{4b}$$

with the permeabilities P_{21}, P_{12}, P_{10}, and P_{01} describing the transition of Na^+ from one compartment (first index) to another one (second index).

For the description of the temporal changes, $dC/dt = \dot{C}$, of C_1 and C_2 upon a step of C_0 to zero, the following differential equations can be written

$$-\lambda C_1 = \dot{C}_1 = -(k_{10} + k_{12})C_1 + k_{21}C_2 \tag{5a}$$

$$-\lambda C_2 = \dot{C}_2 = k_{12}C_1 - k_{21}C_2 \tag{5b}$$

where the coefficients k_{10}, k_{12}, and k_{21} reflect permeabilities, *P*, surface areas, *A*, and volumes, *Vol*, according to the following relationships:

$$k_{21} = P_{21}A_2/Vol_2 \tag{6a}$$

$$k_{12} = P_{12}A_1/Vol_1 \tag{6b}$$

$$k_{10} = P_{10}A_1/Vol_1 \tag{6c}$$

and k_{01} is irrelevant at the moment because of $C_0k_{01} = 0$.

The four observables, λ_1, λ_2, c_{11} and c_{12}, correlate with the system parameters by the following independent relationships

$$\lambda_1 + \lambda_2 = k_{10} + k_{12} + k_{21} \tag{7a}$$

$$\lambda_1\lambda_2 = k_{21}k_{12} \tag{7b}$$

$$c_{11} = C_{1,t=0}\frac{k_{21}\lambda_2 - \lambda_1\lambda_2}{k_{21}(\lambda_2 - \lambda_1)} \tag{7c}$$

$$c_{12} = C_{1,t=0} - c_{11} \tag{7d}$$

which have been used to identify the three permeabilities P_{10}, P_{12}, and P_{21} by fitting k_{10}, k_{12}, and k_{21} to the four observables, and conversion by Eqs. (6). Eventually, P_{01} was obtained by Eq. (4a). For convenience, the rate coefficients, λ, are frequently replaced by $1/\tau$ (τ: time constants).

For numerical calculations, the volume of compartment 2 (Vol_2) was taken to be 10^{-9} m^3 like the cell volume, and its surface area, A_2, was assumed to be $3 \cdot 10^{-5}$ m^2 like the cell surface, although the real surface area of the folded and invaginated endomembranes is assumed to be larger by a large but unknown factor. The volume of compartment 1 was taken to be $5 \cdot 10^{-11}$ m^3 as the cytoplasmic volume.

Results

Basic Observations

Figure 2 shows a protocol over the first minutes of an experiment to measure the membrane voltage, V_m, si-

multaneously with $[Na^+]_c$ in *Acetabularia,* including precalibration and impalement of the electrodes. The upper tracing, V_{Na}, is the voltage difference between the Na^+-selective microelectrode (which records the sum of the voltages over the Na^+-selective membrane *plus* over the plasma membrane) and a conventional V_m-recording electrode (lower tracing). Immediately after impalement of the electrodes (marked by arrow), some transient changes appear in V_{Na}, which are artifacts due to the slower temporal response of the Na^+-selective electrode compared with the faster reference electrode which records V_m only. These artifacts in V_{Na} have the opposite sign to the corresponding events in V_m. V_{Na} reaches a stable level corresponding to a steady-state concentration of 106 mM Na^+ in the cytoplasm. Similar recordings from 43 cells resulted in a large variance. Thus, these results are better summarized in logarithmic terms: $p_aNa = 1.22 \pm 0.4$ SD, corresponding to a geometric mean activity of about 60 · /3 mM. The numeric results actually ranged from 4 to 295 mM $_a[Na^+]_c$ in ASW.

Replacing Na^+ by $Chln^+$ in the external medium (28 experiments) resulted in a decline of $[Na^+]_c$ which could be described by two exponential components (Eq. 3): a fast one with a mean time constant τ_1 of 21 ± 4 SD sec and a mean amplitude c_{11} of 22 ± 9 SD% of the starting concentration, followed by a slow component of the remaining amplitude (c_{12} about 78%) with a time constant τ_2 of about half an hour which has not been analyzed in detail, due to limited observation time with respect to possible injury to the cell.

Analysis of these data with the formalism described in Materials and Methods resulted in the approximate permeabilities (in $nmsec^{-1}$): $P_{01} = 4$, $P_{10} = 20$, $P_{12} = 60$, and $P_{21} = 70$. For the calculation of these permeabilities, the voltage terms for the Na^+ gradients are not considered, because the major Na^+ fluxes are voltage-independent as demonstrated below, and the voltages across the endomembranes are not known.

When Na^+ was readded after several minutes of exposure to Na^+-free ASW, $[Na^+]_c$ increased again from $[Na^+]_c(t_1)$ towards its control level in ASW. This recovery could also be described by two exponentials. For a kinetic analysis of these recoveries, it would be necessary to know $[Na^+]$ in compartment 2 at the beginning of readdition of external Na^+. In principle, this information emerges from the analysis of the washout experiment. However, due to the very long observation times required for unambiguous determination of the exponential parameters, the starting conditions for the readdition experiments have not been known well enough to render significant a systematic analysis of the latter experiments.

Figure 3 shows an example of such an experiment where $[Na^+]_c$ was recorded during withdrawal and readditon of external $[Na^+]$. In this experiment, the amplitude of the first exponential component was extremely

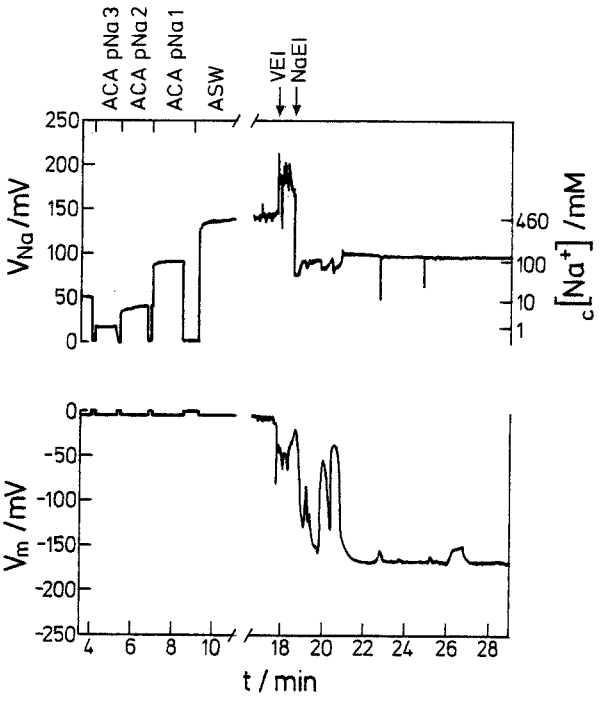

Fig. 2. Example of experimental protocol for measurement of $[Na^+]_c$. Lower tracing, V_m: conventional voltage recording microelectrode.; upper tracing, V_{Na}: difference between voltage from Na^+-selective microelectrode and V_m. 4th–10th min: precalibration in ACA pNa 3–1 and ASW; 18th–24th min: recordings with both electrodes in ASW, impalement of the two electrodes (*VEl* and *NaEl,* marked by arrows), transient instabilities and eventual steady-state recordings of V_m and $c[Na^+]_c$ via calibrated V_{Na}.

large. If the observed changes in $[Na^+]_c$ are assumed to reflect uniform fluxes through the plasmalemma, the average initial slope $\dot{C}_{t=0}$ (about 2 $mMsec^{-1}$) and the volume/surface ratio of the cytoplasm (about $1.5 \cdot 10^{-6}$ m) yield an initial flux $\Phi_{t=0}$ of about 3 $\mu molm^{-2}sec^{-1}$ by the relationship

$$\Phi = \dot{C}_1 \cdot Vol_1/A_1 \qquad (8)$$

The nature of these large Na^+ fluxes will be discussed below.

Factors which May Act on $[Na^+]_c$

To identify possible mechanisms for keeping $[Na^+]_c$ well below its thermodynamic equilibrium with $[Na^+]$ in the normal external environment, we examined some factors which could possibly affect $[Na^+]_c$.

pmf

It is widely accepted that plant cells maintain a *pmf* across the plasmalemma to drive H^+-coupled transport,

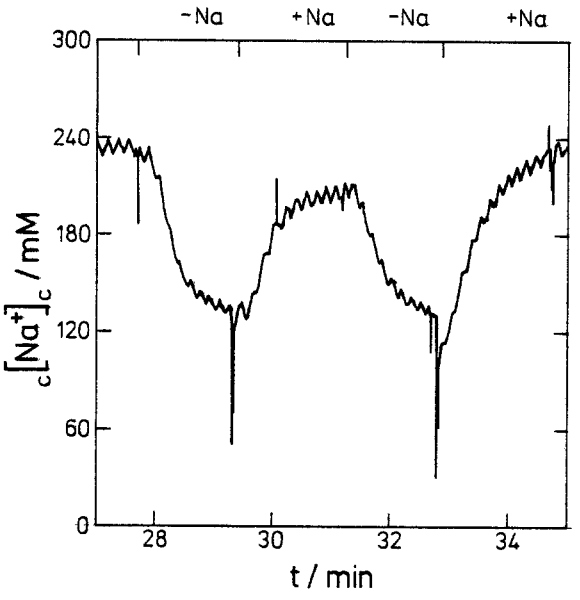

Fig. 3. Examples of relaxations in $c[Na^+]_c$ upon temporal withdrawal and readdition of external Na^+. Fitted parameters of two exponentials (Eq. 3) of washout relaxation during section I: c_{11} 100 mM, τ_1 20 sec, c_{12} 130 mM, τ_2 2,000 sec; $[Na^+]$ scale corresponds to calibration solutions. The superimposed, *ca.* 0.1 Hz oscillations are an apparative artifact.

e.g., of anions, sugar, and amino acids. Thus, Na^+ export against its electrochemical gradient might take place through a Na^+/H^+ antiporter, which is known to exist in plants (Blumwald & Poole 1985; Katz, Kaback & Avron, 1986; Clint & MacRobbie 1987; Garbarino & Du Pont 1988; Hassidim et al., 1990). In the case of *Acetabularia,* however, the chemical gradient for H^+ is outward directed by about one pH unit. Under these conditions, an electroneutral Na^+/H^+ antiporter cannot export Na^+. However, an electrophoretic Na^+/nH^+ antiporter with a stoichiometry factor $n > 1$, could fulfill the task to extrude Na^+ by coupling to an *electro*chemical H^+ inward gradient. To test this possibility, we recorded $[Na^+]_c$ at various V_m and external pH. The results of a typical experiment are shown in Fig. 4 with a spontaneous transition of V_m from the diffusion-dominated resting potential, RP′ of about −70 mV to the pump-dominated resting potential *RP* of about −180 mV (Gradmann, 1970), and with experimentally imposed changes in external pH between pH 5.8 and 8.0. Apparently, neither external pH, nor V_m have an appreciable effect on $[Na^+]_c$. The slight increase in $[Na^+]_c$ by about 10 nMsec^{-1} at V_m upon the polarization from −70 to −180 mV in Fig. 4 is not significant, especially with respect to the results in Fig. 3, which demonstrate the actual capability (about 2 mMsec^{-1}) of the dominating Na^+ transport system.

Theoretically, these negative results do not strictly exclude the operation of an effective Na^+/nH^+ antiporter, because the effects of V_m and pH_o in the investigated ranges might be saturated already (Sanders et al., 1984). In this case, at least pH_c should have an effect on $[Na^+]_c$. However, 5 mM butyrate in the external medium, which causes cytoplasmic acidification by about half a pH unit in other plants (Sanders et al., 1981), did not cause any visible change in $[Na^+]_c$, neither in the presence of external Na^+ nor in Na^+-free ASW. Under these circumstances, a Na^+/nH^+ antiporter can now be excluded to be the main mechanism for Na^+ extrusion in *Acetabularia.*

By the same token, changes in $[Na^+]_c$ have no immediate effect on V_m. An example is shown by Fig. 5 with considerable changes in $[Na^+]_c$ which are not accompanied by corresponding V_m changes. These results suggest that the major changes in $[Na^+]_c$ do not represent a corresponding movement of charges through the plasmalemma.

ATP

To identify the energy source for maintaining the Na^+ gradient in *Acetabularia,* we tested a possible effect of ATP on $[Na^+]_c$ by means of the uncoupler CCCP. In *Acetabularia,* 50 μM CCCP in the external medium causes a decrease in cytoplasmic ATP by about 60% and a concomitant depolarization from −170 mV by about 100 mV within a few seconds (Gradmann, 1975). In our experiments, this treatment also caused a rapid depolarization to about −70 mV within *ca.* 10 sec but no effect >10 nMsec^{-1} on $[Na^+]_c$ could be observed within about 300 sec of exposure to CCCP. Compared to the striking changes in $[Na^+]_c$ of around 2 mMsec^{-1} in other experiments (e.g., Figs. 3 and 5), these results suggest that in *Acetabularia,* ATP is not the immediate energy source for the major export mechanism of Na^+ from the cytoplasm to the external medium. This point will be discussed below again.

Cl^-

Since the prime ATPase in the plasmalemma of *Acetabularia* does not export H^+ (e.g., Fig. 4: changes in pH_o caused no change in V_m) but imports Cl^- (for review, *see* Gradmann & Wolf 1994), it might be the ATP-generated chloride motive force which drives Na^+ export by a Cl^-/Na^+ symporter. Previous results render this possibility unlikely: if it were an electroneutral mechanism, the nonexisting chemical Cl^- gradient (both $[Cl^-]_c$ and $[Cl^-]_o$ are about 0.5 M; Saddler, 1970), could not establish a Na^+ gradient; and the major Na^+ transport system does seem, in fact, to be electroneutral, according to the results in Figs. 4 and 5. Nevertheless, the possible effect of $[Cl^-]_o$ on $[Na^+]_c$ was tested, by replacing 85% of external Cl^- by I^- (460 mM NaI instead

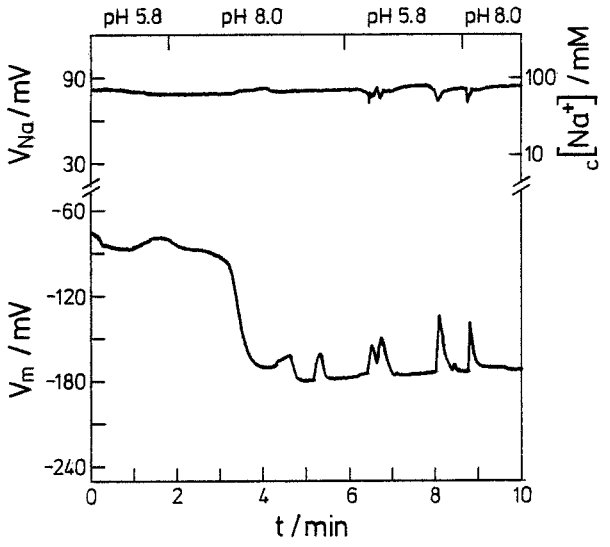

Fig. 4. Recording of V_m and V_{Na} during changes in external pH and during a spontaneous transition of V_m from RP′ (diffusion-dominated around −80 mV) and RP (pump dominated around −180 mV); main result: $[Na^+]_c$ is rather independent of V_m and of pH_o.

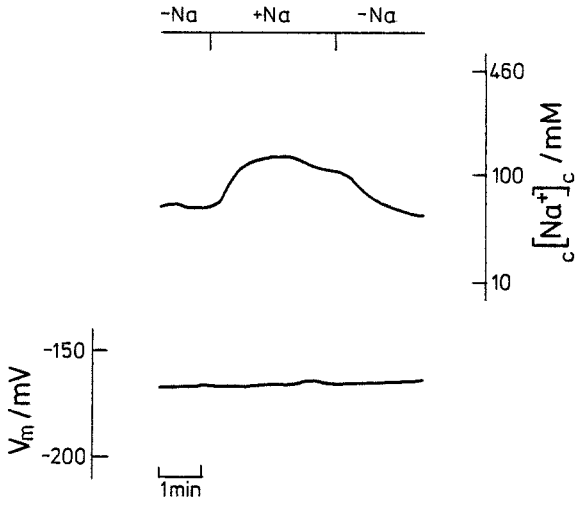

Fig. 5. Transient increase in $[Na^+]_c$ during stable resting voltage; right $[Na^+]$ scale corresponds to calibration solutions.

of NaCl). This treatment depolarized the membrane by about 35 mV—confirming previous results (Hansen et al., 1982)—however, $[Na^+]_c$ did not change under these conditions. In conclusion, the main Na^+ transport through the plasmalemma of *Acetabularia* is not directly coupled to Cl^- transport.

Endogenous Factors

Frequently but not always, transient changes in $[Na^+]_c$ take place during spontaneous or induced action potentials. An example with three consecutive, spontaneous action potentials is shown in Fig. 6. The observed changes in V_{Na} cannot be artifacts due to the slow temporal response of the V_{Na} electrode, compared with the faster V_m electrode, because the observed events in the two electrodes had the same sign, in contrast to the mentioned artifacts in Fig. 2 with antiparallel characteristics.

From 32 observations of such transient increases in $[Na^+]_c$, the amplitude distribution was about exponential (small peaks frequent, large peaks seldom) with a mean peak amplitude of about 30% above the control concentration; the distribution of the maximum slopes was also about exponential with mean amounts of the maximum slope of some $mM sec^{-1}$ for the increase as well as for the following decrease to the control level.

Correlations between the shape of the $[Na^+]_c$ transients, the steady-state $[Na^+]_c$ levels, the resting voltage or the amplitude of the action potential could not be detected. There was neither a consistent temporal relationship between the peaks of the action potentials and the peaks of the accompanying $[Na^+]_c$ transients. The peaks of the transients in V_m and in V_{Na} appeared about simultaneously with possible differences either sign of a few seconds.

Discussion

There is no doubt that *Acetabularia* maintains low $[Na^+]_c$ against high $[Na^+]_o$ and negative membrane voltage. The average steady-state $_a[Na^+]_c$ of about 60 mM measured here and the variability of the individual recordings are consistent with previous data which have been obtained by tracer flux measurements (Saddler, 1970; Mummert & Gradmann, 1991*a*). The unidirectional steady-state in- and effluxes of Na^+, as measured by tracer fluxes (2.3 $\mu mol m^{-2} sec^{-1}$ at 22°C as well as at 5°C, according to Mummert and Gradmann, 1991*a*), do not differ significantly from the net fluxes of about 3 $\mu mol m^{-2} sec^{-1}$ determined here by the relaxations of $[Na^+]_c$ (Fig. 3). Therefore, the unidirectional fluxes from tracer flux experiments are considered to represent potential net fluxes rather than Na^+/Na^+ exchange. This means that, under steady-state conditions, there is a large net influx of about 3 $\mu mol m^{-2} sec^{-1}$, and a powerful extrusion system manages to drive the corresponding uphill export of Na^+.

There are no data available from other marine plants for comparison. The only Na^+ fluxes determined in saline conditions derive from experiments with brackish water algae. If *Chara buckelli* is transferred from pond water to 70 mM Na^+, there is an uptake of about 0.2 $\mu mol^{-2} sec^{-1}$ Na^+ (Hoffmann, Tuffariello & Bisson, 1989). With respect to the external Na^+ concentration, which was about six times lower compared to marine

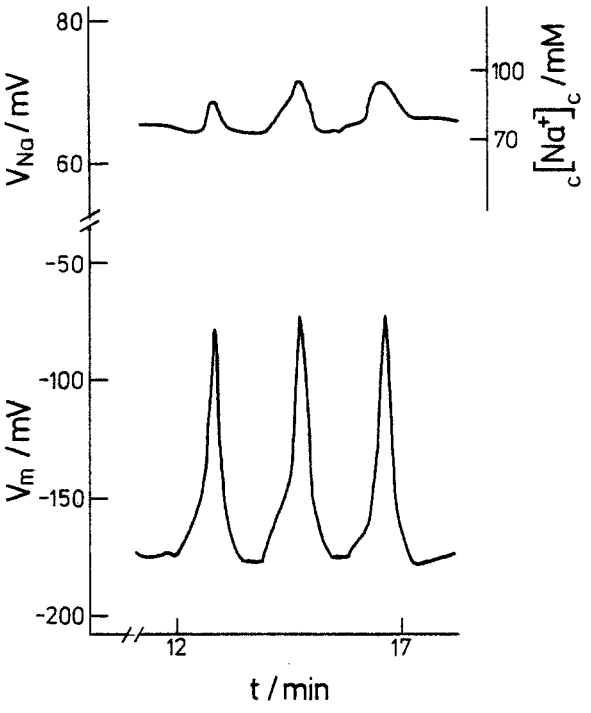

Fig. 6. Series of three spontaneous action potentials (lower tracing, virtually identical amplitudes) with associated transient increases in $[Na^+]_c$ (upper tracing, variable amplitudes).

conditions, these fluxes are within the same range as those described here. Corresponding Na^+ fluxes upon an osmotic step are also reported from *Lamprothamnium* (Kirst & Bisson, 1982; Okazaki, Shimmen & Tazawa, 1984; Reid, Jefferies & Pitman, 1984). Interestingly, this uptake of Na^+ is not paralleled by a significant Na^+ permeability, as determined by application of the Goldman equation to changes in V_m upon variations in $[Na^+]_o$ (Bisson & Kirst, 1980). This discrepancy has not been pointed out explicitly in previous studies. The same discrepancy between large Na^+ fluxes and small Na^+ conductance of the plasmalemma is evident in our measurements.

The power *Pow* of the Na^+ extrusion system in *Acetabularia* can be calculated by the product $Pow = Namf \cdot \Phi \cdot F$ of about 60 mWm^{-2}, with an electrochemical driving force for Na^+, *Namf*, of about 0.2 V, a net efflux Φ of about 3 $\mu molm^{-2}sec^{-1}$ and the Faraday constant F. This power corresponds to about 30% of the cell's ATP turnover (about 200 mWm^{-2} according to Gradmann, 1975), ignoring transport of the counter ions. For comparison, the electrogenic pump in the plasmalemma has been estimated to consume about 10% of the cells ATP turnover (Gradmann, 1975). Surprisingly, our results indicate that Na^+ extrusion, as judged by the temporal changes in $[Na^+]_c$, appears to be independent of the expected energy sources: neither V_m nor external or internal pH, nor $[ATP]_c$ seem to have a direct effect on the predominant mechanisms which cause up and downshifts in $[Na^+]_c$ by about 2 $mMsec^{-1}$ (Figs. 3, 5 and 6).

As a possible energy source for the powerful Na^+ extrusion, we can only think of the *pmf* across endomembranes. This possibility is consistent with several findings: a v-type H^+-ATPase (Ikeda et al., 1992) maintains a large pH gradient across the tonoplast (vacuolar pH is 1–2 in *Acetabularia,* according to Gradmann, 1975). According to this view, vesicles with vacuolar properties may accumulate Na^+ using the *pmf* across their membranes, and release it by exocytosis. In fact, such a vesicular system has already been suggested to cause facultative bursts of vigorous fluxes of Na^+ and Cl^- during action potentials in *Acetabularia* (Mummert & Gradmann 1976, 1991*b;* Gradmann & Mummert, 1980). Also, these fluxes have been found to be electroneutral with respect to the electrical properties of the plasmalemma (Mummert & Gradmann 1976; Wendler, Zimmermann & Bentrup, 1983; Mummert & Gradmann, 1991*b*). The actual mechanism of loading and unloading of these vesicles cannot be identified on the basis of the present information available, especially since even the fundamental questions of endo- and exocytosis in plants are under current and controversial debate (Gradmann & Robinson, 1989).

It should be pointed out that the serial three-compartment-model, though appropriate for a formal description of the $[Na^+]_c$ relaxations, does not account for several features of the Na^+-transport system. Therefore, the model needs to be extended, e.g., in the following way. Because of the insensitivity of the observed Na^+ transport to electrochemical gradients across the plasmalemma, an indirect pathway is suggested to be used by Na^+. In addition to the cytoplasm (locus of the electrodes) and the vacuole, there is a (probably inhomogeneous and dynamic) vesicular compartment which vigorously communicates Na^+ with the cytoplasm, and particularly with the external medium, whereas the Na^+ conductance of the plasmalemma is small. The additional pathway between vesicles and outside transforms the serial compartmentation model into a cyclic one including two additional rate constants. Unfortunately, determination of now six model parameters from four observable parameters is not possible. Equivalent conclusions have also been reached by analysis of tracer flux measurements in *Acetabularia* (Mummert & Gradmann, 1991*a*).

The transient increases in $[Na^+]_c$ which are seen frequently but not always associated with action potentials, vary considerably in time course and amplitude (Fig. 6). The large variation of the changes in $[Na^+]_c$ observed here may point to local, vesicular events in the cytoplasm which are recorded by the microelectrode, rather than global and homogeneous processes of the whole cytoplasm. The weak correlation with the action

potentials points to an indirect relationship. Our present interpretation is that the transients of $[Na^+]_c$ reflect an increase due to local disruptions of Na^+ stores (e.g., NaCl-loaded vesicles) in the more or less close vicinity of the Na^+-recording electrode, followed by an abatement of this local increase by simple diffusion. These disruptions are facilitated but not forced by some processes which are related to action potentials. The disruption of vesicles, for example, could be caused by a decrease of the hydrostatic pressure in the embedding cytoplasm, which is known to take place facultatively in the course of action potentials (Wendler et al., 1983). This pressure drop, in turn, has been suggested to be caused by a corresponding exocytotic discharge of NaCl-loaded vesicles (Mummert & Gradmann, 1991*b*).

Such considerations apply, of course, only for the transient increases in V_{Na}. In contrast, the temporal changes of $[Na^+]_c$ upon withdrawal and readdition of external Na^+ (Fig. 3) are relaxations towards a new steady-state, which cannot be understood as individual, local events but as global ones for the entire cell.

There is an alternative interpretation of the apparent transients in $[Na^+]_c$: Since the used ion-selective electrodes with the sensor ETH 227 have a high selectivity for Ca^{2+} (Fig. 1*C*), the observed transients might actually reflect temporal increases in $[Ca^{2+}]_c$. If the changes in V_{Na} transients (average peak about 8 mV above control level) would be interpreted as a change in $[Ca^{2+}]_c$, Eq. (2) with $\log K_{Ca/Na} = 2.4$ (Fig. 1*C*) and known control levels of cytoplasmic Ca^{2+} (p_aCa = 6.85; Amtmann et al., 1992) and Na^+ (here 70 mM), would yield a mean peak in $[Ca^{2+}]_c$ of about 50 μM. This $[Ca^{2+}]_c$ appears to be unrealistically high. It may, however, take place in the form of transient and local events.

Conclusion

The results identify a powerful transport system for ions which bypasses the electrical properties of the plasmalemma.

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