Volume-regulatory Responses of *Amphiuma* Red Cells in Anisotonic Media

The Effect of Amiloride

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ABSTRACT *Amphiuma* red cells were incubated for several hours in hypotonic or hypertonic media. They regulate their volume in both media by using ouabain-insensitive salt transport mechanisms. After initially enlarging osmotically, cells in hypotonic media return toward their original size by losing K, Cl, and H_2O. During this volume-regulatory decrease (VRD) response, K loss results from a >10-fold increase in K efflux. Cells in hypertonic media initially shrink osmotically, but then return toward their original volume by gaining Na, Cl, and H_2O. The volume-regulatory increase (VRI) response involves a large (>100-fold) increase in Na uptake that is entirely blocked by the diuretic amiloride (10^{-3} M). Na transport in the VRI response shares many of the characteristics of amiloride-sensitive transport in epithelia: (*a*) amiloride inhibition is reversible; (*b*) removal of amiloride from cells pretreated with amiloride enhances Na uptake relative to untreated controls; (*c*) amiloride appears to act as a competitive inhibitor (K_i = 1–3 μM) of Na uptake; (*d*) Na uptake is a saturable function of external Na (K_m ~29 mM); (*e*) Li can substitute for Na but K cannot. Anomalous Na/K pump behavior is observed in both the VRD and the VRI responses. In the VRD response, pump activity increases 3-fold despite a decrease in intracellular Na concentration, while in the VRI response, a 10-fold increase in pump activity is observed when only a doubling is predicted from increases in intracellular Na.

INTRODUCTION

Red blood cells from a number of species regulate their cell volume when incubated in anisotonic media (Kregenow, 1971*a*, *b*, 1978; Schmidt and McManus, 1977*a*, *b*; Cala, 1977; Parker, 1973; Parker et al., 1975; Fugelli, 1967; Fugelli and Reierson, 1978). After undergoing initial osmotic shrinkage or swelling, they return to or toward their original size. These volume-regulatory responses involve ouabain-insensitive changes in electrolyte content. By altering the number of intracellular osmotic particles, the cells change their water content

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and thus their cell volume. In this paper, we demonstrate that the giant red cells of the salamander *Amphiuma means* regulate their volume in both hypotonic and hypertonic media by using ouabain-insensitive salt transport mechanisms. The response of *Amphiuma* red cells to hypertonic media differs from analogous responses of other cells in that it involves K-independent Na movements that are completely blocked by the diuretic amiloride. Importantly, several characteristics of Na transport that are shared by amiloride-sensitive epithelia (Cuthbert and Fanelli, 1978; Benos, 1982) also characterize the Na movements of the volume-regulatory increase (VRI) response. The findings of this study have been presented in preliminary form (Siebens and Kregenow, 1978, 1980, 1982) and reviewed (Kregenow, 1981; Benos, 1982; Cala, 1983; Siebens, 1985). Other investigators using both *Amphiuma* (Cala, 1980) and frog (Palfrey and Greengard, 1981) red cells have confirmed and expanded on some of these findings. Amiloride-sensitive volume regulation has also been recently identified in dog red cells (Parker, 1983; Parker and Castranova, 1984), lymphocytes (Grinstein et al., 1983), and *Necturus* gallbladder (Ericson and Spring, 1982).

METHODS

Salamanders of both sexes of the species *Amphiuma means tridactylum* were obtained from Wauban Laboratories (Schriever, LA) or Rand McNally (Somerset, WI). Animals were maintained at room temperature (20–25°C) in aquaria that contained either dechlorinated tap water with added NaCl (19 mM) or pond water (no additives). In most experiments, blood was obtained by decapitating the animal and collecting blood from the torso. Additional blood was then collected from an incision in the heart after pithing the animal. In a few experiments, blood was obtained by cardiac puncture (Stoner and Kregenow, 1980). Heparin served as an anticoagulant. The blood was then strained through gauze to remove any clots and centrifuged at 700 *g* in a centrifuge (RC2, Sorvall, Wilmington, DE). An aliquot of plasma was removed for osmometry (Advanced Instruments, Needham Heights, MA), while the remaining plasma and the buffy coat were removed by suction. In experiments in which the blood from a single animal was insufficient, a second animal was bled. In all two-animal experiments, the difference between plasma osmolalities was <10 mosmol. About one-fourth of the experiments used two animals, with no apparent differences observed between these and single-animal experiments.

All media contained the following common constituents: 3.0 mM K, 2.0 mM Ca, 9.2 mM HCO_3, 1.1 mM Mg, 0.6 mM SO_4, 1.8 mM PO_4, 5.6 mM dextrose, 15 g/liter bovine serum albumin, and 1 mg/liter chloramphenicol. Three standard media were used: an isotonic medium (A), a hypotonic medium (B), and a hypertonic medium (C). Because the plasma osmolality was found to vary among animals from 195 to 238 mosmol, the NaCl concentration of the standard isotonic medium A was varied (range of [Na], 94–115 mM) to match within 4 mosmol the plasma osmolality of the animal (or the average plasma osmolality in two-animal experiments). The standard hypotonic medium B contained 40 mM less NaCl than medium A, and the standard hypertonic medium C contained 60 mM more NaCl than medium A. The osmolality of the standard hypotonic medium (~140 mosmol) was therefore about two-thirds that of the isotonic medium (~215), while the osmolality of the standard hypertonic medium (~325 mosmol) was about two-thirds that of the isotonic medium. Three "choline media," whose osmolalities matched those of solutions A, B, and C, were often used as wash media to remove trapped extracellular Na. In these choline media, Na was replaced by equimolar amounts of choline. Na originally associated with the powdered albumin, however, resulted in a final [Na] of ~2.5

mM in these and other media to which no NaCl was added. The other media used in this study, all of which were modifications of the hypertonic medium C, will be described in the appropriate figure legends. The modifications included the omission of K, Ca, and Mg, the replacement of Na with K or Li, partial replacement of Na with choline, and the use of choline Cl to change the medium osmolality at constant Na. In solutions other than the standard solutions, choline Cl was used to make the minor adjustments of medium osmolality necessary to match the osmolality of the appropriate standard solution. Some of the modified hypertonic media contained 5 mM HEPES (titrated to pH 7.7 with NaOH) to increase the medium buffering power. The subscript "H" denotes media containing HEPES, e.g., A_H, C_H. Solutions were made fresh within 24 h of the experiment and gassed with 1% CO_2/99% O_2. Cells were washed two or three times with 5–10 vol of medium A, and preincubated in medium A for 2–4 h before experimentation. All incubations were performed at pH 7.7 ± 0.05 in a shaking water bath at 25°C. The hematocrit was 10% during preincubation and 1–3% during the experiments. Incubation flasks were either gassed continuously or stoppered after gassing to prevent evaporation. Minor pH adjustments were made in some experiments by the addition of HCl or NaOH.

Experimental Procedures

Well-mixed, preincubated cells were introduced into flasks containing incubation media. Except where noted, if ouabain, amiloride, ^{42}K, ^{22}Na, or ^{24}Na were used in the experiment, these substances were added before the addition of cells. Samples were then taken at the times indicated in the figures.

In some experiments, the extracellular fluid trapped in the red cell pellet was removed by washing. In other experiments, [^{14}C]inulin was used as an extracellular marker, and the ion content of the inulin space was subtracted. In both methods, ~10 cc was poured from an incubation flask into a 50-cc plastic tube. In the [^{14}C]inulin method, 2 μCi of [^{14}C]inulin was immediately added and the well-mixed suspension was transferred to a special tube, which was then centrifuged for 5 min at 17,500 *g* in a centrifuge at 4°C. In the wash method, the 50-cc tube containing the cell suspension was immediately centrifuged at 700 *g* and an aliquot of the supernatant was removed for analysis of radioactivity and electrolyte concentration. The remaining supernatant was then aspirated, and the pellet was resuspended in ice-cold wash medium, transferred to the special tubes, and centrifuged for 5 min at 17,500 *g*. The wash medium was either ice-cold incubation medium that was drug- and tracer-free, or drug- and tracer-free ice-cold choline medium of the same tonicity as the incubation medium. In a series of paired experiments, the [^{14}C]inulin, Na-containing wash, and choline wash methods were compared by measuring ^{24}Na uptake in cells undergoing the VRI response. There was no statistically significant difference between any two of these three methods (Siebens, 1983). In addition, there was no significant difference between the [^{14}C]inulin and choline wash methods with respect to cell Na content (Siebens, 1983). The [^{14}C]inulin method was always used in determinations of cell Cl content, while either the [^{14}C]inulin method or the wash method was used in measurements of cell ^{22}Na, ^{24}Na, ^{42}K, or chemical Na content. The extracellular space correction was ~6%. No correction was made for trapped chemical K because the quantity trapped was small (~0.2 mmol/L_{cells}).

In experiments in which cell volume was measured, two samples were taken at each sample time. One sample was used to measure cell volume, while the other was used to measure ion content and tracer uptake. After removing an aliquot of the supernatant, care was taken to repeatedly aspirate all the supernatant before removing the cells (50–250 μl) from each centrifuge tube. The special Lucite centrifuge tubes used held ~13 ml and were tapered to a 3-mm-diam cylindrical core. This core opened at the base and

could be occluded with a plastic plug. Cells for gravimetric cell volume determination were removed from the top of the special tubes with a Pasteur pipette. The cell pellet was then weighed, dried at 60°C to constant weight (at least 12 h), cooled in a dessicator, and reweighed for the dry weight determination (Riddick et al., 1971). To obtain cells for chemical or tracer ion measurements, the plug was removed and the cells were washed out with deionized water through the opening at the base into a centrifuge tube. A drop of the nonionic detergent Cutscum (Fisher Scientific Co., Fairlawn, NJ) and 3 drops of 1 N NH_4OH were then added to facilitate hemolysis and to form a gel of the residual cellular debris (Riddick et al., 1971). After separating the gel by centrifugation, the hemolysate was assayed for hemoglobin, K, and Na. Aliquots of hemolysate were also analyzed for Cl, Li, ^{42}K, ^{24}Na, and [^{14}C]inulin when appropriate. In each experiment, we took a reference sample at the initial sample time by removing an aliquot of cells from isotonic media. These cells were centrifuged for 5 min at 17,500 *g* and sampled volumetrically to determine the quantity of hemoglobin per unit volume of packed cells. Because the quantity of hemoglobin in a well-mixed sample is directly proportional to the number of cells, this reference hemoglobin quantity is an indicator of the number of cells in isotonic media originally occupying one liter. All calculations concerning ion movements were related to this reference quantity of hemoglobin, giving ion (or tracer) content per original number of cells (onc) in one liter (mmol/L_{onc}).

To measure hemoglobin, an aliquot of hemolysate was treated with Drabkin's cyanide reagent and the hemoglobin was measured as cyanomethemoglobin at 540 nm on a spectrophotometer (Beckman Instruments, Inc., Irvine, CA). K and Na were measured on an internal Li standard flame photometer (model 143, Instrumentation Laboratories, Lexington, PA). Cl was measured in duplicate on a Cotlove chloridometer after deproteinating aliquots of hemolysate by adding 0.1 times the hemolysate volume of two solutions: 0.7 N $ZnSO_4$/0.25 N H_2SO_4 and 0.75 N NaOH. To measure Li, an aliquot of hemolysate was deproteinated with 1.6% trichloracetic acid (TCA) and diluted, and the emission at 6,714 nm was measured on an atomic absorption spectrophotometer (Techtron, Cupertino, CA). Standards were prepared by adding known amounts of Li to Li-free hemolysates. The final TCA concentration was 0.8%. Li measurements were accurate to within 5%. ^{22}Na and ^{24}Na were counted in a Beckman Instruments Gamma 9000. To measure [^{14}C]inulin, aliquots of lysate and supernatant were deproteinated using $ZnSO_4$/NaOH or 1.6% TCA and counted in a Beckman Instruments Beta 9200 counter using Aquasol (New England Nuclear, Boston, MA) as the scintillation fluid.

Materials

^{24}Na, ^{22}Na, and ^{42}K were obtained as Cl salts from New England Nuclear along with [^{14}C]inulin. Ouabain was obtained from Aldrich Chemical Co. (Milwaukee, WI). Amiloride was a gift of Merck, Sharpe & Dohme (West Point, PA), and chloramphenicol (Chloromycetin) was donated by Parke-Davis (Santurce, Puerto Rico). Choline Cl was obtained from Eastman Kodak Co. (Rochester, NY) and recrystallized from ethanol. All salts were analytic grade.

Calculations

In all calculations, the subscript "r" denotes the isotonic reference sample taken at the initial sample time, while "u" represents any other sample. The equation for calculating the cell volume of sample u relative to the reference cell volume is:

$$\text{percent original volume} = \frac{(V_u)}{(V_r)} \cdot (100) = \frac{(W_{Dr}/W_{Wr})}{(W_{Du}/W_{Wu})} \cdot \frac{(P_r)}{(P_u)} \cdot (100), \quad (1)$$

where V is cell volume, P is the density of packed cells relative to water, W_W is the "wet" weight of an aliquot of packed cells, and W_D is the weight of cell solids after drying the aliquot of packed cells to constant weight.

The fractional cell water content on a weight basis, w, is given by $1 - (W_D/W_W)$. The relative density, P, of the sample u was calculated using the experimentally determined relationship between w and P such that:

$$P_u = 1.094 + 0.3(0.682 - w_u). \tag{2}$$

1.094 ± 0.001 (SEM) is the mean reference density of packed cells relative to water ($P_{water} = 1$), which was derived from triplicate measurements of the relative density of isotonic reference cells in three experiments. 0.682 ± 0.003 is the mean fractional water content on a weight basis obtained from isotonic reference samples from 21 experiments.

Hemoglobin was used to normalize the quantity of a ion or tracer [^{14}C]inulin in an aliquot of lysate such that:

$$\text{ion or tracer content}/L_{onc} = Hb_r \frac{(S)}{Hb_u}, \tag{3}$$

where Hb_r is the hemoglobin reading of an aliquot of the lysate from the volumetric reference sample, Hb_u is the hemoglobin reading from sample u, and S is the measured ion concentration (in millimoles per liter) or tracer concentration (in counts per minute per liter). Since Hb_r is proportional to the number of cells originally occupying one liter (L_{onc}), the units of ion contents normalized to Hb_r are millimoles per L_{onc}, while the units of tracer content are counts per minute per L_{onc}.

Eq. 4 was used to calculate iM_K, the influx of K between zero time and time t:

$$iM = \frac{R_c}{\bar{x}_m}. \tag{4}$$

R_c is the change in counts per minute per L_{onc} between zero time and time t, and x_m is the mean specific activity of the medium during this interval (Kregenow, 1971*a*). Although this equation assumes a steady state two-compartment system, flux values corrected for changes in cell K content using the non-steady state method of Tosteson et al. (1955) yielded values that differed by at most 4% from those calculated with Eq. 4. Eq. 4 was also used to calculate tracer Na uptake. The nonlinearity of Na uptake as a function of time (Fig. 7) and the rapid changes in the specific activity of cell Na precluded a reliable estimation of the tracer backflux required for the calculation of Na influx. In experiments designed to analyze the mechanism of action of amiloride, the flux period was shortened as much as possible and care was taken to analyze Na uptake when Na uptake was a linear function of time (see the legend to Fig. 15).

Direct microelectrode measurements in *Amphiuma* red cells have shown that the Cl concentration ratio can be used to predict the membrane potential, V_m, under steady state conditions (Lassen, 1977; Stoner and Kregenow, 1980; Cala, 1980). These studies show that there is an ~33-mV change in V_m per 10-fold change in the Cl concentration ratio. This relationship is given by:

$$V_m = 33 \log[rCl], \tag{5}$$

where V_m is the potential difference in millivolts across the cell membrane (inside-negative), and rCl is the ratio of intracellular to extracellular Cl concentration, $[Cl^-]_i/[Cl^-]_o$ (Stoner and Kregenow, 1980). Note that this equation is a modification of the Nernst equation in which the coefficient 33 has replaced the usual 59.2 [i.e., $2.30(RT/F)$ at 25°C]. Possible explanations for the non-Nernstian slope include influences of ions other than Cl on the

membrane potential, exclusion of Cl from the nucleus, and differences in activity coefficients for Cl between the cell and the medium (Stoner and Kregenow, 1980). Microelectrode studies of V_m in *Amphiuma* red cells have also been performed on cells recovering their volume under anisotonic conditions similar to those of the present study (Cala, 1980). Cala (1980) found that there were no detectable changes in V_m during volume recovery, which suggests that the volume-regulatory mechanisms are electroneutral. It must be emphasized that if volume-regulatory Na and K movements were through conductive pathways, rCl would not be an accurate indicator of the membrane potential (Lassen, 1977; Wieth and Brahm, 1980). The relative conductances of Cl, Na, and K in *Amphiuma* red cells are 2:1:1 (Lassen, 1977). Thus, if G_{Na} increased >100-fold, as would be required to account for the Na movements observed in the VRI response, V_m would approach E_{Na}, and Cl would not equilibrate rapidly enough through the Cl conductance to reflect V_m. On the other hand, if the volume-regulatory mechanisms of *Amphiuma* red cells are electroneutral, as suggested by the data of Cala (1980), the high transport capacity of the anion exchanger would, in parallel with other Cl pathways, re-equilibrate Cl within seconds (see Fig. 3 of Kregenow et al., 1985). Thus, given the prior information that the volume-regulatory mechanisms are electroneutral (Cala, 1980), rCl can be used to predict changes in V_m semiquantitatively. The major advantages of using rCl to estimate V_m are technical simplicity and sensitivity in detecting small changes. Our V_m values estimated using Eq. 5 are in good agreement with the microelectrode measurements of Cala (1980), but suggest that minor gradual changes in V_m occur during volume recovery that were not detected in the microelectrode study (Cala, 1980).

The intracellular Cl concentration, $[Cl]_i$, in millimoles per $L_{cell\ water}$ or millimolar, was calculated from the cell Cl content and cell volume of sample u using the following rearrangement of Formula 1 of Stoner and Kregenow (1980):

$$[Cl]_i = \frac{(\text{Cl content in mmol/L}_{onc})_u}{w'_u} \cdot \frac{V_r}{V_u}, \tag{6}$$

where w'_u, the fractional water content on a volume basis, is equal to $w_u \cdot P_u$, the fractional water content on a weight basis times the relative density. By substituting Na values for Cl, this equation was also used to calculate the intracellular Na concentrations.

K effluxes were calculated according to the equation:

$$oM_K = iM_K = \Delta K_c, \tag{7}$$

where oM_K is the unidirectional K efflux, iM_K is the unidirectional K influx, and ΔK_c is the change in cell K content between zero time and time t.

Kinetic analysis of tracer Na uptake as a function of extracellular Na was performed by using the method of least squares to fit the data to linear transformations of the Michaelis-Menten equation assuming rapid equilibrium kinetics (Segel, 1975; Colton, 1974). These values were verified using nonlinear least-squares fitting of the data to the Michaelis-Menten equation (Knott, 1979). Inasmuch as our data (Siebens and Kregenow, 1980; Kregenow et al., 1985) and those of Cala (1980) strongly suggest that Na enters in exchange for H^+ (or is cotransported with OH^-), special care was taken to keep pH constant. For example, in addition to the routine use of low (1–3%) hematocrits, the medium buffer power in experiments such as that shown in Fig. 15 was increased by the addition of 5 mM HEPES. As discussed by Segel (1975), a bireactant system, such as a Na/H exchanger, can be analyzed using Michaelis-Menten analysis, provided one of the reactants (in this case H^+) is kept constant and the data fit the Michaelis-Menten equation.

All experiments presented are representative of at least three experiments of similar design. The two-tailed paired t test was used for all statistical analysis, with $P < 0.05$ considered statistically significant. In figures and tables in which results from groups of

experiments are analyzed, results are expressed as means ± SEM. When cells incubated under various conditions are compared, the term "isotonic control cells" refers to cells incubated in drug-free isotonic medium A (or A_H). The following notation is used to express changes in measured parameters caused by a given experimental variable: $\Delta parameter_{variable}$. For example, $\Delta Na + K_{ouab} = 4.8 \pm 0.7$ mmol/L_{onc} ($P < 0.01$) indicates that ouabain caused a change in the Na + K content of the cells that was statistically significant at the $P < 0.01$ level using a paired t test.

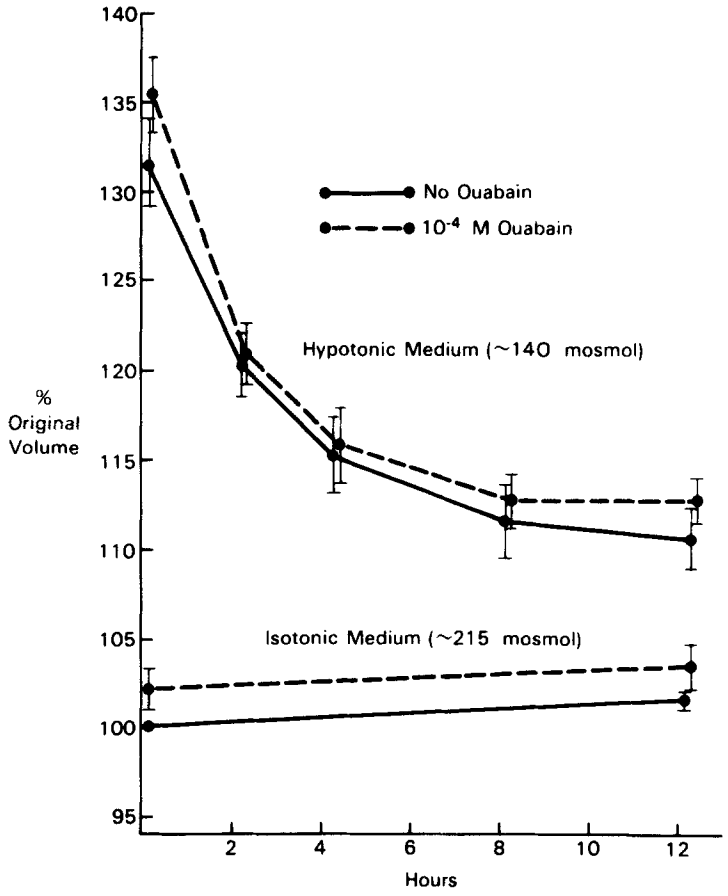

FIGURE 1. Time course of cell volume changes during incubation in hypotonic media: the effect of ouabain. Cells were incubated in medium A or medium B in the presence or absence of 10^{-4} M ouabain. The initial samples were taken 8–11.5 min after the addition of cells to the incubation media. $N = 4$. In this and all other figures, brackets represent the standard error of the mean.

RESULTS

Response to Hypotonicity: Volume-regulatory Decrease (VRD)

When *Amphiuma* red cells were placed in the standard hypotonic medium B (~140 mosmol), they initially enlarged osmotically by ~33%. Fig. 1 shows that with continued incubation in the hypotonic medium, the cells gradually returned

toward their original volume. Cells in medium B reached a new stable volume in ~8 h, which was 5–15% larger than that of isotonic control cells. Fig. 1 also demonstrates that the magnitude of the volume recovery phase was not affected by the cardiac glycoside ouabain.

Volume recovery is brought about mainly through the loss of cellular K, as shown in Fig. 2*A*. The magnitude of the cellular K loss in hypotonic media depends on whether the Na/K pump is blocked by ouabain, with ouabain-treated cells losing more K than untreated cells. In both the presence and absence of ouabain, however, the K loss reduces the Na + K content to virtually the same extent.

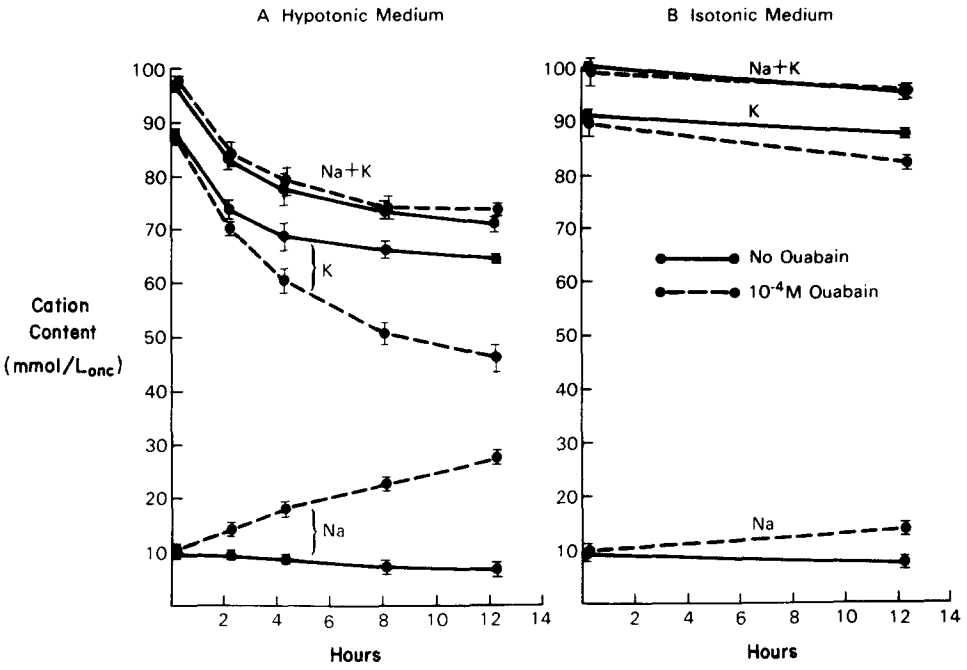

FIGURE 2. Changes in cation content during incubation in hypotonic media: the effect of ouabain. This figure shows the cellular K, Na, and Na + K contents for the four experiments whose volumes are depicted in Fig. 1.

If ouabain is present, cells undergoing the VRD response (Fig. 2*A*) gain about three times as much Na as ouabain-treated cells in isotonic media (Fig. 2*B*). This suggests that Na uptake increases during volume regulation. Inasmuch as the Na content of untreated cells undergoing the VRD response does not increase, the data of Fig. 2*A* also suggest that increased Na/K pump activity results in the extrusion of this additional Na in the untreated cells. The Na content of untreated cells in hypotonic media actually decreased ~3.4 mmol/L$_{onc}$ ($P < 0.05$) over 12 h (Fig. 2*A*), but a similar small decrease, 1.7 mmol/L$_{onc}$ ($P < 0.05$), was also observed in isotonic control cells during this period (Fig. 2*B*).

Fig. 3 depicts the K effluxes observed during the initial 2 h of the VRD response. The efflux of K was 10–20-fold larger in cells undergoing the VRD

response than in isotonic control cells. This resulted in the observed K loss (Fig. 2).

In the experiments shown in Fig. 3, the ouabain-sensitive K influx (an indication of Na/K pump activity) was increased approximately threefold in cells undergoing the VRD response compared with that of isotonic control cells (0.92 ± 0.14 compared with 0.34 ± 0.09 mmol/L_{onc}/h, $N = 4$). These K influx data and the Na and K data of Fig. 2 support the conclusion that there is a threefold increase in pump activity during the VRD response. This stimulation of pump

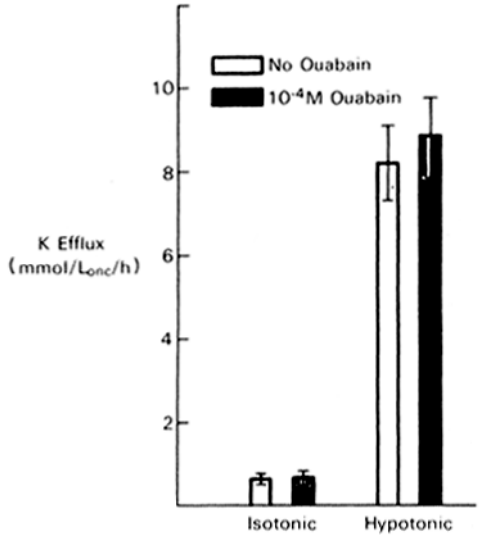

FIGURE 3. The effects of hypotonicity and ouabain on K efflux. K effluxes were calculated from ^{42}K influxes and net K movements in the four experiments shown in Figs. 1 and 2 (see Methods). Values for cells in hypotonic media were calculated from samples taken at zero time and 2.1 h,[1] while zero time and 12.1-h samples were used for cells in isotonic media.

activity is noteworthy in that the intracellular Na concentration was decreased from ~13 mM in isotonic control cells to ~9 mM upon initial osmotic enlargement in solution B. $[Na^+]_i$ then remained below control levels throughout the VRD response. In human red cells, stimulation of Na/K pump activity is usually associated with an increase in $[Na]_i$ (Post and Jolly, 1957; Garay and Garrahan, 1973).

[1] The small increase in the mean 2.1-h efflux value for ouabain-treated cells in hypotonic medium compared with untreated cells was not statistically significant. Significant differences in tracer efflux values were obtained, however, at later times. Ouabain-treated cells presumably have a larger K efflux to explain the 20-mmol difference in the K content of ouabain-treated and untreated cells that have undergone the VRD response for 12 h (see Fig. 2*A*). A larger efflux is expected because complete blockage of the ouabain-sensitive component of K influx (0.58 mmol/L_{onc}/h) accounts for less than half of this 20-mmol difference. The fact that both ouabain-treated and untreated cells complete the response with the same Na + K loss, then, is presumably a consequence, in part, of a compensatory increase in the K efflux of ouabain-treated cells.

^{24}Na was used to confirm that the net Na gain observed in ouabain-treated cells undergoing the VRD response results from increased inward Na movements. Fig. 4 demonstrates that the Na uptake of cells in hypotonic media was elevated two- to sevenfold relative to cells in isotonic media whether or not ouabain was present. The difference between the two right-hand columns of Fig. 4 indicates that ouabain caused a small, statistically significant difference in the ^{22}Na uptake ($P < 0.05$, $N = 4$) of cells in hypotonic media. Rather than resulting from an actual difference in inward Na movements, this calculated flux difference

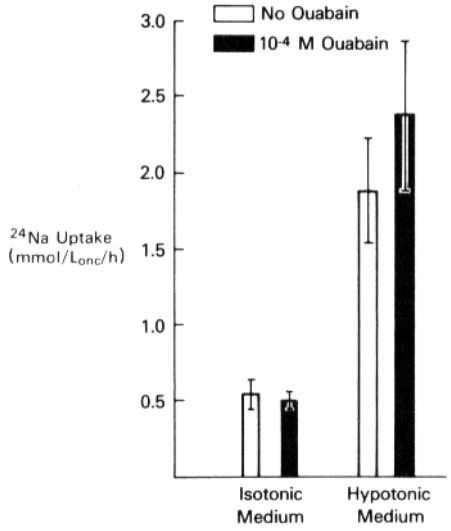

FIGURE 4. The effects of hypotonicity and ouabain on Na influx. Cells were incubated for 1 h in medium A or medium B in the presence or absence of 10^{-4} M ouabain. Although four experiments were performed, the data from one were not included in this figure since the control influx was 2.3 mmol/L_{onc}/h, about fivefold greater than normal. Cells in the hypotonic media exhibited the usual increase in Na uptake of ~1.5 mmol/L_{onc}/h, and the data were included in performing a paired *t* test.

was probably an artifact and resulted from the stimulation of the pump previously shown in Fig. 2. In this interpretation, no actual difference in Na influx exists; part of the ^{22}Na that enters cells not treated with ouabain is subsequently extruded by a more active pump. This effect is not detectable under isotonic conditions when the pump rate is not elevated.

Because amiloride blocks Na uptake in the response of these cells to hypertonic media (see Fig. 10), its effect on Na uptake during the VRD response in hypotonic media was tested. 10^{-3} M amiloride did not appear to inhibit the Na movements of the VRD response (^{22}Na $\text{uptake}_{amil}/^{22}$Na $\text{uptake}_{no\ amil} = 1.19 \pm 0.21$, $N = 3$). Net K loss was also unaffected by amiloride in these experiments (K loss_{amil}/K $\text{loss}_{no\ amil} = 0.95 \pm 0.06$).

Table I (*B*) summarizes the changes in cell Cl content associated with changes in cell Na + K content during the VRD response. Cl concentration ratios (rCl)

and membrane potentials estimated from Cl ratios (see Methods) are also included. Table I (*A*) indicates values for control cells in isotonic media. A gradual decline in cell Cl content was observed during the VRD response, with the magnitude of the Cl loss being about one-third that of the cation loss. Assuming that the Cl and bicarbonate concentration ratios are the same (Funder and Wieth, 1966), a loss of ~1.3 mmol/L_{onc} of bicarbonate is also expected. Changes in the charge of cellular buffers such as hemoglobin presumably account for the difference between the anion loss and the cation loss (see Discussion). At 12 h (i.e., after completion of the VRD response), there was a small but statistically significant increase in the Na + K contents of ouabain-treated compared with untreated cells ($\Delta Na + K_{ouab} = 3.2 \pm 1.0$ mmol/L_{onc}, $P < 0.05$). The possible

TABLE I

Effect of Incubation in Hypotonic Media on Na + K Content, Cl Content, rCl, and Estimated Membrane Potential

	10^{-4} M ouabain	(*A*) Isotonic (~215 mosmol)		(*B*) Hypotonic (~140 mosmol)		
		Time 0	12 h	Time 0	2 h	12 h
Na + K (mmol/L_{onc})	−	100.2±1.1	95.0±0.9	97.6±1.2	82.5±1.0	70.0±0.7
	+	98.9±2.0	94.2±0.9	98.0±0.9	83.7±1.7	73.2±0.9
Cl (mmol/L_{onc})	−	18.2±0.9	18.3±1.6	18.0±1.0	13.3±1.0	10.2±0.8
	+	17.8±1.3	17.2±1.0	15.7±0.7	12.8±1.2	10.9±0.6
rCl	−	0.266±0.013	0.260±0.023	0.308±0.018	0.255±0.020	0.217±0.017
	+	0.253±0.022	0.244±0.018	0.258±0.011	0.241±0.021	0.229±0.011
Estimated V_m (mV)	−	−19.0±0.7	−19.3±1.2	−16.9±0.8	−19.6±1.1	−21.9±1.1
	+	−19.7±1.2	−20.2±1.0	−19.4±0.6	−20.4±1.2	−21.1±0.7

Cells were incubated in medium A or medium B in the presence or absence of 10^{-4} M ouabain. Zero time was 5–11.5 min after the addition of cells. $N = 6$. The membrane potential was estimated from the Cl concentration ratio as described in the Methods. The extracellular [Cl] was 93 mM in the isotonic media and 53 mM in the hypotonic media.

implications of this and a similar small increase observed in cells that have completed the VRI response will be mentioned in the Discussion.

Table I also shows that there is no initial change in rCl in the VRD response. However, rCl is expected to decrease slightly during volume recovery because the relative contribution of the impermeant anions (such as hemoglobin) to the total anion content of the cell increases as the cell cation content decreases (Van Slyke et al., 1923; Jacobs and Stewart, 1947). Analysis of the pooled data from ouabain-treated and untreated cells indicates a small but statistically significant ($P < 0.01$, $N = 6$) decrease in rCl of 0.060 and an increase in the estimated V_m of 3.4 mV at 12 h in cells in hypotonic media compared with cells at zero time in hypotonic media. The difference in rCl (0.029) and V_m (1.8 mV) between cells in hypotonic and isotonic media at 12 h was also statistically significant ($P < 0.05$, $N = 6$).

Response to Hypertonicity: VRI

When *Amphiuma* red cells were placed in the hypertonic medium C (~325 mosmol), they initially shrank osmotically ~20%. Fig. 5 shows that with continued incubation in the hypertonic medium, the cells returned toward their original volume. As in the VRD response, the changes in cell size during this VRI

response were not affected by inhibition of the Na/K pump with ouabain. The VRI response was considerably more rapid than the VRD response, a new stable volume being reached in 40–90 min compared with 8 h for the VRD response. As in the VRD response, volume recovery was incomplete. The final (6 h) volumes of both ouabain-treated and untreated cells in hypertonic media remained slightly smaller than those of cells in isotonic media under the same drug conditions. Pooling the data from ouabain-treated and untreated cells, the mean difference between cells in isotonic vs. hypertonic media was 3.8 ± 0.9%, N = 10, $P < 0.01$. In addition, the ouabain-treated cells that completed the response in hypertonic media were slightly larger at 6 h than untreated cells ($\Delta volume_{ouab}$ = 3.1 ± 0.9%, $P < 0.05$, $N = 5$).

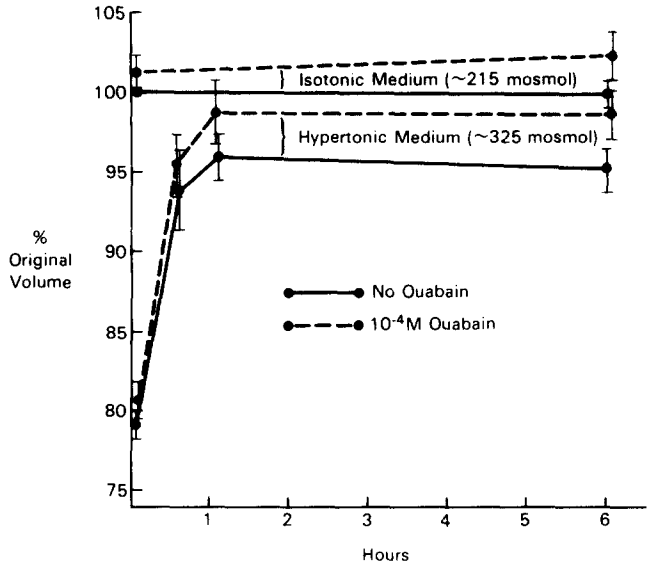

FIGURE 5. Time course of cell volume changes during incubation in hypertonic media: the effect of ouabain. Cells were incubated in medium A or medium C in the presence or absence of 10^{-4} M ouabain. The first sample was taken 2–7 min after the addition of cells. $N = 5$.

Fig. 6 shows the cation changes associated with the VRI response for the same experiments depicted in Fig. 5. Fig. 6*A* demonstrates that the cells in medium C enlarged by rapidly gaining ~35 mmol/L_{onc} Na. This Na gain increased the cell Na content to about five times its normal value and increased the Na + K content by ~40%. The Na gain was regulated in that the cells did not continue to enlarge beyond their isotonic control volume, even though both the chemical gradient for Na ($[Na]_o/[Na]_i$ = ~160 mM/~60 mM) and the membrane potential (~20 mV, inside-negative; Cala, 1980; Table II) favored continued Na entry. Experiments in which volume recovery was prevented by replacing medium Na with choline (data not shown) further support the concept that changes in cell Na content bring about the observed volume changes.

Fig. 6 shows that the effect of ouabain in the VRI response was similar to its effect in the VRD response; it blocked the exchange of Na for K without substantially affecting Na + K content. The gain of Na and loss of K produced by ouabain was nearly eightfold greater in cells undergoing the VRI response than in cells in isotonic media (compare 6-h samples, Fig. 6, *A* and *B*). These data are consistent with an eightfold stimulation of the Na/K pump during the VRI response. Another characteristic shared with the VRD response is that the Na + K content of ouabain-treated cells in hypertonic media was slightly greater than that of untreated cells several hours after the apparent stabilization of cell volume (at 6 h, $\Delta Na + K_{ouab} = 4.8 \pm 0.7$ mmol/L_{onc}, $P < 0.01$).

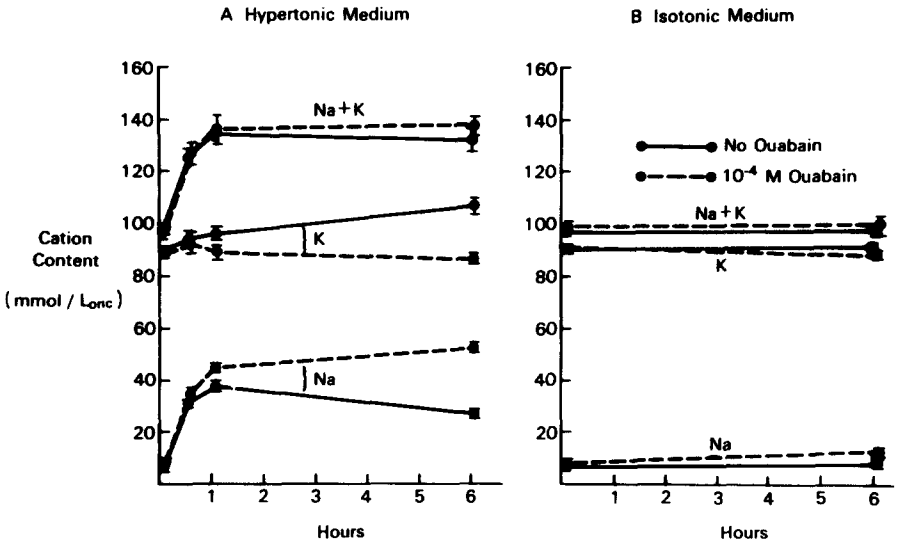

FIGURE 6. Changes in cation content during incubation in hypertonic media: the effect of ouabain. This figure shows the cellular K, Na, and Na + K contents for the five experiments whose volumes are depicted in Fig. 5.

The effect of medium osmolality on the time course of the cell Na increase is shown in Fig. 7. In these experiments, the medium osmolality was increased with choline Cl, while $[Na]_o$ was kept constant at 94 mM. Because of the variability among experiments in the time course and rate of Na gain, the results from a single experiment are shown. Three observations are noteworthy. First, the rate of Na uptake increased as a function of increasing medium osmolality, with the rates at the higher osmolalities being well over 100-fold greater than isotonic control values. In simultaneously performed ^{22}Na measurements, the maximal relative rates of Na uptake (hypertonic/isotonic) by cells in the 292-, 326-, and 383-mosmol media were 92, 173, and 265, respectively. Second, the amount of Na gained also increased with medium osmolality, while the final volume (indicated at the right of the figure) was nearly the same. The final Na content of cells in the 383-mosmol medium was about twice that of cells in the 292-mosmol

medium. This provides further evidence that the cells use Na to restore their volume. Third, the rate of Na uptake by cells in all three hypertonic media was not maximal immediately: there was a "delay period" between zero time and the period of maximal Na gain. In the experiment shown, the delay period was ~10 min for cells in the 383- and 326-mosmol media and ~15 min for cells in the 292-mosmol medium. There was considerable variation among experiments in both the duration of the delay period and the maximal rate of Na uptake observed. In nine experiments performed in ~325-mosmol media, the duration of the delay period ranged from 5 to >25 min, while the maximal rate of ^{22}Na uptake ranged from 5 to 13 mmol/L_{onc}/5 min, 100–450-fold greater than the uptake of paired isotonic control cells. In addition, the apparent decrease in the

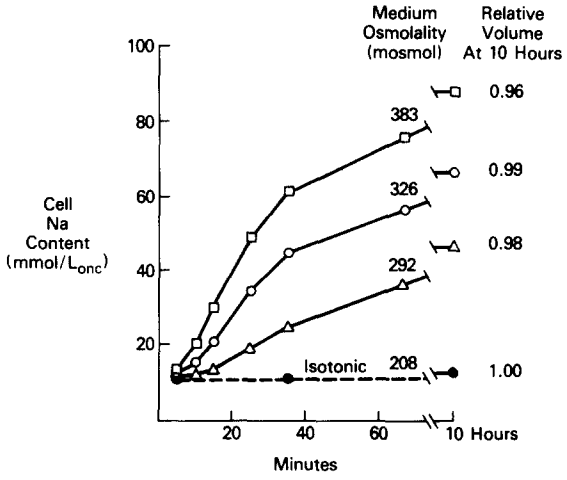

FIGURE 7. The effect of medium osmolality on the time course of changes in Na content of cells in isotonic or hypertonic media. Cells from a single animal were divided into four portions and incubated in flasks containing isotonic (208 mosmol) or hypertonic (292, 326, or 383 mosmol) media. Cells were added at zero time and samples were taken simultaneously from the four flasks at the indicated times. The Na concentration in all media was 94 mM, with choline Cl used to vary the medium osmolality. The remaining constituents were those of medium A_H.

delay period as the medium osmolality was increased was much more pronounced in some experiments than in others. In one experiment, there was no detectable effect of osmolality on the duration of the delay period. These differences among experiments remain to be explained.

Table II (*B*) summarizes the changes in cell Cl content associated with changes in Na + K content for cells undergoing the VRI response. Values of rCl and V_m (estimated from rCl) are also included. The data for control cells in isotonic media are shown in Table II (*A*). As the cells in 325-mosmol media enlarge, they gain about half to three-quarters as much Cl as they gain Na + K. If the bicarbonate ratio is the same as the Cl ratio, a further anion gain of ~2 mmol/L_{onc} bicarbonate is expected. The zero-time column in Table II (*B*) indicates that

there was no initial change in rCl or estimated V_m upon osmotic shrinkage. However, the data from subsequent sample times indicate that rCl moves toward unity and the estimated membrane potential becomes slightly less negative as the cells gain Na, K, and Cl. A shift in rCl toward unity is predicted from the osmotic and ionic equilibria considerations (Van Slyke et al., 1923; Jacobs and Stewart, 1947) mentioned with respect to the VRD response. Thus, the relative contribution of the cellular impermeant anions decreases as the total Na + K + Cl content increases, and $[Cl]_i$ moves toward $[Cl]_o$. The mean difference between the value of rCl at zero time and that at 6 h for cells in hypertonic media was 0.139, which is consistent with a change in V_m of 6.6 mV ($P < 0.01$). The mean difference in rCl (0.120) and V_m (5.5 mV) at 6 h between cells in isotonic and hypertonic media was also statistically significant ($P < 0.01$).

TABLE II

Effect of Incubation in Hypertonic Media on Na + K Content, Cl Content, rCl, and Estimated Membrane Potential

	10^{-4} M ouabain	(*A*) Isotonic (~215 mosmol)		(*B*) Hypertonic (~325 mosmol)			
		Time 0	6.0 h	Time 0	0.5 h	1.0 h	6.0 h
Na + K	−	98.6±2.2	97.4±1.7	97.4±0.9	125.2±4.2	135.3±2.8	135.0±4.0
(mmol/L_{onc})	+	99.7±2.6	99.0±3.9	99.7±2.7	123.4±5.7	136.2±5.8	138.1±4.0
Cl (mmol/L_{onc})	−	17.7±0.9	17.9±0.9	18.7±1.2*	39.6±3.0	39.0±2.0	38.0±0.9
	+	18.2±1.2	18.8±1.3	19.0±0.8	35.9±3.5	42.5±3.9	43.6±1.2
rCl	−	0.255±0.018	0.255±0.022	0.246±0.025	0.382±0.039	0.364±0.024	0.359±0.015
	+	0.251±0.022	0.257±0.022	0.228±0.016	0.337±0.024	0.382±0.022	0.393±0.014
Estimated V_m	−	−19.6±1.0	−19.6±1.2	−20.1±1.4*	−13.8±1.4	−14.5±0.9	−14.7±0.6
(mV)	+	−19.8±1.2	−19.5±1.2	−21.2±1.0	−15.6±1.0	−13.8±0.8	−13.4±0.5

Cells were incubated in medium A or medium C in the presence or absence of 10^{-4} M ouabain. Zero time was 2–6.5 min after the addition of cells. $N = 4$. The extracellular [Cl] content was 95 mM in the isotonic media and 151 mM in the hypertonic media.

* $N = 3$.

In Fig. 6, changes in the Na and K contents of cells treated with ouabain suggested about an eightfold increase in Na/K pump activity during the VRI response. Fig. 8 provides additional evidence that the Na/K pump is stimulated during the VRI response. The total ^{42}K influx of cells incubated in ouabain-free hypertonic medium C was dramatically increased relative to that of isotonic controls. Blockage of the Na/K pump with ouabain completely inhibited this increase. In the experiments of Fig. 8, the ouabain-sensitive (pump) component of ^{42}K influx for cells in hypertonic media was ~10-fold greater than that for cells in isotonic media.

As in the VRD response, the Na/K pump responded to changes in $[Na]_i$ in an atypical fashion during the VRI response, which suggests that factors in addition to $[Na]_i$ influence pump activity. Studies of Na/K pump activity in human red cells (Garay and Garrahan, 1973) and muscle fibers (Mullins and Frumento, 1963; Nelson and Blaustein, 1980) indicate that Na/K pump activity is a saturable function of $[Na]_i$ and is half-maximal when $[Na]_i$ = 12–20 mM. Extrapolating from the data of Garay and Garrahan (1973) and assuming uniform distribution

of Na throughout the cell water, the expected increase in pump activity for the observed increase in $[Na]_i$ during the *Amphiuma* red cell VRI response (from ~12 to ~60 mM) is about 2-fold, in contrast to the observed 10-fold increase.

In some volume-regulating red cells, the ability of the cells to regulate their volume is dependent upon the presence of cations other than Na in the medium. A medium [K] of >2.5 mM is required for cell enlargement in the duck VRI response (Kregenow, 1971*b*), while the dog red cell VRD response involves Ca-dependent Na extrusion that is half-maximal at a medium [Ca] of ~2 mM (Parker, 1973; Parker et al., 1975). In addition, an interaction between Ca and Na transport has been demonstrated in some tissues (Blaustein, 1974; Taylor

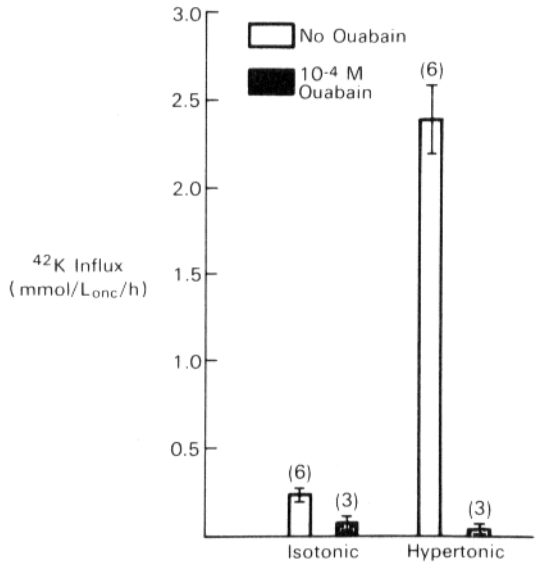

FIGURE 8. The effect of hypertonicity and ouabain on K influx. Cells were incubated in medium A or medium C in the presence or absence of 10^{-4} M ouabain. Zero time was 5–12 min after the addition of cells. The numbers above the bars indicate the number of experiments. The incubation period in the different experiments ranged from 1 to 12 h, with no apparent effect of sample time on the rate of K influx in a given medium.

and Windhager, 1979). Fig. 9 shows that omitting K or omitting K, Ca, and Mg from the medium does not affect ^{22}Na uptake during the initial 26 min of the *Amphiuma* red cell VRI response. Cell Na + K content at this and other sample times during the VRI response is similarly unaffected by the omission of K or K, Ca, and Mg from the medium.

Analysis of Amiloride-sensitive Na Transport

Fig. 10 shows the results of experiments that tested the effect of amiloride, a diuretic that blocks Na entry into many cell types, among them many epithelial cells (Cuthbert and Fanelli, 1978; Benos, 1982). Amiloride (10^{-3} M) abolishes the massive increase in Na uptake seen in the VRI response. In each of the five

experiments of Fig. 10, amiloride inhibited >97%[2] of the volume-stimulated Na uptake. By blocking Na uptake, amiloride prevents the cells from returning toward their original volume (data not shown). In experiments performed over periods ranging from 45 to 140 min, continuous exposure to amiloride prevented almost all of the increase in Na content. The Na + K content of amiloride-treated (10^{-3} M) cells in medium C (106.9 ± 3.1 mmol/L_{onc}, N = 9) was only slightly, but significantly, greater ($P < 0.01$) than that of control cells in amiloride-free isotonic media (102.7 ± 2.5). In contrast, cells in amiloride-free medium C exhibited the usual increase in Na + K content, rising to 142.0 ± 3.9 mmol/L_{onc} (N = 9). In addition, amiloride blocks further volume-regulatory Na entry if added later in the response (see Fig. 12).

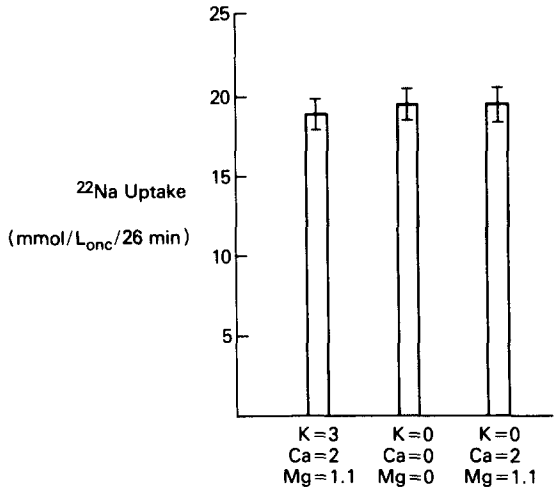

FIGURE 9. ^{22}Na uptake in the presence and absence of extracellular K, Ca, and Mg. Cells were incubated for 26 min in medium C, or medium C in which either KCl (3 mM) or K, Ca (2 mM), and Mg (1.1 mM) were isosmotically replaced with NaCl. N = 4.

In contrast to the effect of amiloride on cells in hypertonic media, the data shown in Fig. 10 suggest that amiloride does not inhibit the Na uptake of cells in isotonic media. To test this point further, additional Na uptake experiments were performed in which cells were incubated in isotonic medium A in the presence or absence of 10^{-3} M amiloride for periods ranging from 1 to 7.5 h. The ratio of the tracer Na uptake of amiloride-treated cells to that of untreated cells was 1.21 ± 0.12 (N = 9), which is not statistically different from 1.0. Thus, amiloride caused no detectable inhibition in isotonic control cells. The effect of amiloride on the Na uptake of human red cells incubated in isotonic media remains unclear; Aceves and Cereijido (1973) reported 70% inhibition, while Wessels (1969) found no inhibition.

Figs. 11 and 12 present the results of experiments designed to test whether amiloride inhibition of volume-regulatory Na uptake is reversible and whether

[2] Percent inhibition = $1 - [(\text{hyper}_{\text{amil}} - \text{iso}_{\text{amil}})/(\text{hyper}_{\text{no amil}} - \text{iso}_{\text{no amil}})]$.

Na uptake is enhanced after removal of amiloride, as has been reported in epithelia (Ehrlich and Crabbé, 1968; Turnheim et al., 1978). In the experiments of Fig. 11, cells were incubated in medium C_H in the presence or absence of 10^{-3} M amiloride, washed with amiloride-free medium C_H, and then transferred to a

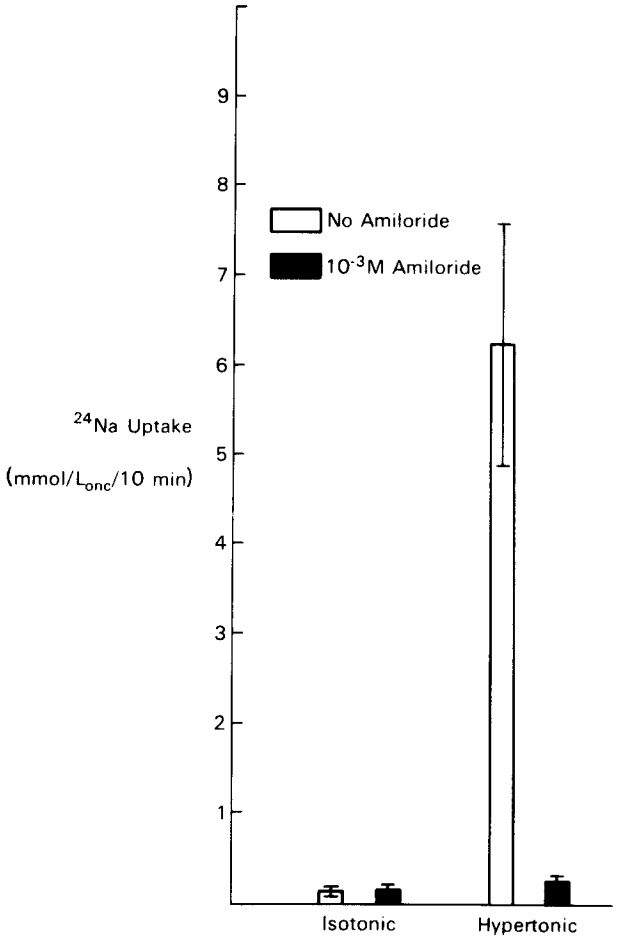

FIGURE 10. Uptake of ^{24}Na during incubation in hypertonic media: the effect of amiloride. Cells were incubated in medium A or medium C containing ^{24}Na in the presence or absence of 10^{-3} M amiloride. Two samples were taken during the first 5–13 min after adding cells to the incubation media. The second sample was taken 10 min after the first sample for cells in hypertonic media, and 60 or 120 min after the first sample for cells in isotonic media. Cells in isotonic media required a longer sample period because the Na uptake was too small to measure accurately over 10 min. The values for cells in isotonic media were then normalized to 10 min. $N = 5$.

second flask containing ^{24}Na in amiloride-free medium C_H. Samples were taken 5 and 15 min later. The period before washing will be referred to as the pretreatment period (period I), the wash period as period II, the 5-min period between the addition of cells to the second flask containing ^{24}Na and the first

^{24}Na uptake sample as the initial flux period (period III), and the 10-min period between the first and second ^{24}Na uptake samples as the final flux period (period IV). The period of pretreatment with amiloride (period I) was 5 min. "Pretreated cells" refers to cells exposed to 10^{-3} M amiloride during period I. "Untreated cells" refers to cells not pretreated with amiloride during period I; their response serves as a control.

Fig. 11 shows the relative tracer Na uptake of pretreated cells during the initial and final flux periods. In the initial flux period (period III), the Na uptake of pretreated cells actually exceeded that of untreated cells by ~30% ($P < 0.05$).

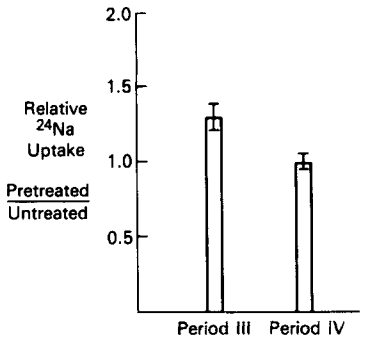

FIGURE 11. Reversibility of amiloride inhibition. Cells were incubated in ouabain-containing (10^{-4} M) solution C_H in the presence or absence of amiloride (10^{-3} M) and the effect of washing the cells in amiloride-free medium C_H on tracer Na uptake was examined. As described in the text, the experiment was divided into four time periods (I–IV). At zero time, cells were added to either amiloride-containing medium C_H (pretreated cells) or amiloride-free medium C_H (untreated cells). At 5 min, both pretreated and untreated cells were centrifuged at 700 *g* and washed once with amiloride-free medium C_H at 25°C. 10 min after zero time, both washed groups of cells were added to new flasks containing amiloride-free medium C_H and tracer Na. Samples for the tracer Na uptake measurements were taken at 15 and 25 min after zero time in five experiments and at 15 and 20 min in one experiment. The left bar of the figure indicates the tracer Na uptake of pretreated cells relative to that of untreated cells during period III (10–15 min), while the right bar indicates the relative Na uptake during period IV (15–25 min). $N = 6$.

This "amiloride pretreatment effect" was consistently observed immediately after the removal of amiloride and will be further demonstrated in Fig. 12. It appears that amiloride inhibition is not only completely reversible, but that a period of inhibition results in enhancement of Na uptake after the removal of amiloride. During the final flux period (period IV), the Na uptake of pretreated cells was no longer enhanced relative to untreated cells (1.01 ± 0.06, $N = 6$).

Fig. 12*A* shows the time course of the changes in cell Na content for a representative experiment performed in the same manner as the experiments of Fig. 11, except that the pretreatment period (period I) was increased to 25 min. This pretreatment time was chosen to be toward the end of the period of maximal Na uptake (Fig. 7) to observe whether amiloride pretreatment results in rates of Na uptake that exceed values observed in the normal VRI response

at this osmolality. Another change in the protocol from that of Fig. 11 was that half the cells not exposed to amiloride before washing were exposed to 10^{-3} M amiloride after washing to determine whether amiloride inhibits volume-stimu-

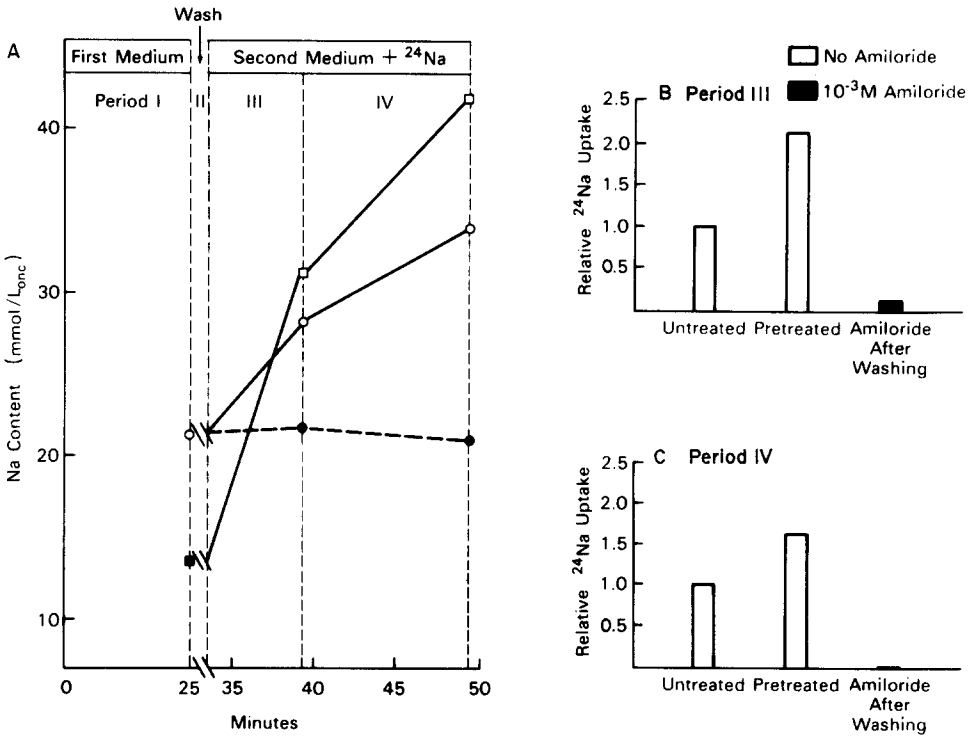

FIGURE 12. Enhancement of Na uptake when amiloride is washed off cells preincubated for 25 min in hypertonic media containing amiloride (10^{-3} M). The media were the same as in Fig. 11 and the protocol was similar, so that the same description of time periods applies. Three modifications were made to the protocol of Fig. 11 in this single representative experiment. First, the duration of the pretreatment period (period I) was increased to 25 min. Second, a sample was taken at the beginning of the wash period to measure the Na content. Third, half the cells not pretreated with amiloride were exposed to amiloride (10^{-4} M) after washing. The times at which the various experimental steps were performed were as follows: at 25 min, the cells were washed and first sample was removed (Na content only); at 33 min, the cells were transferred to the second flask containing ^{24}Na; at 39 and 49 min, the second and third samples were obtained (Na content and ^{24}Na uptake). The Na content of isotonic controls was 9.8 mmol/L$_{onc}$. (*A*) Time course of changes in the Na content of cells under various conditions. Key: closed symbols and dashed line, 10^{-3} M amiloride present; open symbols and solid line, amiloride absent; squares, pretreated cells; circles, untreated cells. (*B*) ^{24}Na uptake during period III (33–39 min) of cells treated with amiloride (10^{-3} M) either before or after washing relative to ^{24}Na uptake of untreated cells. (*C*) ^{24}Na uptake during period IV (39–49 min) of cells treated with amiloride (10^{-3} M) either before or after washing relative to the ^{24}Na uptake of untreated cells. The relative ^{24}Na uptake of untreated cells in panels *B* and *C* is defined as 1.0.

lated Na transport if added late in the response. At 25 min, the Na content of pretreated cells was ~8 mmol/L_{onc} less than that of untreated cells, which indicates that amiloride inhibited the volume-regulatory Na gain during the pretreatment period (period I). After the wash period, the Na gain during both flux periods (periods III and IV) was substantially greater in pretreated cells than in untreated cells.

Fig. 12, *B* and *C*, further demonstrates this amiloride pretreatment effect using ^{24}Na uptake data. During the initial flux period (period III, Fig. 12*B*), the ^{24}Na uptake of pretreated cells was increased over twofold relative to untreated cells. Pretreating cells for 25 rather than 5 min increased the relative Na uptake during the initial flux period (period III) from 1.29 ± 0.09 ($N = 6$) in Fig. 11 to 1.97 ± 0.08 in five experiments in which the protocol of Fig. 12 was used. Fig. 12*C* shows that during the final flux period (period IV), the tracer Na uptake of cells pretreated for 25 min remained enhanced. In five such experiments, the relative Na uptake was 1.48 ± 0.06 ($P < 0.01$), in contrast to the results of Fig. 11, where the relative Na uptake after a 5-min pretreatment period was no longer elevated during period IV. Thus, increasing the amiloride pretreatment time had two effects: (*a*) it increased the magnitude of the relative Na uptake of pretreated vs. untreated cells in the initial flux period, and (*b*) it prolonged the period of enhanced Na uptake so that the pretreatment effect was also observed in the final flux period.

The result of this prolonged enhancement is seen in Fig. 12*A*, where it is clear that the Na content (and, by inference, volume) of pretreated cells did not simply "catch up" to that of untreated cells. By the 39-min sample time, the Na content of the pretreated cells exceeded that of the untreated cells. Thus, pretreated cells, by gaining Na more rapidly than untreated cells, attain their final Na content long before untreated cells, i.e., pretreatment actually decreases the time required for volume regulation. In this experiment, samples taken later in the response indicated that the pretreated cells stopped gaining Na ~55 min after zero time, while the untreated cells continued to gain Na until 75 min after zero time.

Increasing the duration of the pretreatment period beyond 25 min increased the Na uptake still further. Using data from one representative experiment, the maximal ^{24}Na uptake following pretreatment for 25 and 65 min was 16.4 and 25.0 mmol/L_{onc}/5 min, respectively. The 65-min value was >850-fold greater than that of cells in isotonic media, and ~7-fold greater than the maximal ^{24}Na uptake of untreated cells in hypertonic media.

If cells are prevented from gaining (or losing) Na for 25 min by preincubating them in a low-Na (~4 mM) hypertonic choline medium and are then exposed to Na-containing medium C_H, results are obtained that are similar to those shown in Fig. 12 (data not shown; Siebens and Kregenow, 1982). A medium Na concentration of ~4 mM was used to prevent net changes in cell Na and water content: at higher values, the cells gained Na; at lower values, they lost Na. The fact that a similar enhancement of Na uptake was observed after pretreatment with either amiloride or low-Na media is consistent with the prevention of volume regulation, rather than amiloride itself, being the dominant factor in the amiloride-pretreatment effect. However, additional evidence (Siebens and Kre-

genow, 1982) suggests that a portion of the amiloride-pretreatment effect may be attributable to amiloride itself.

Returning to the question of whether amiloride inhibits the Na uptake of cells if added late in the response, Fig. 12*A* (closed circles) indicates that further net

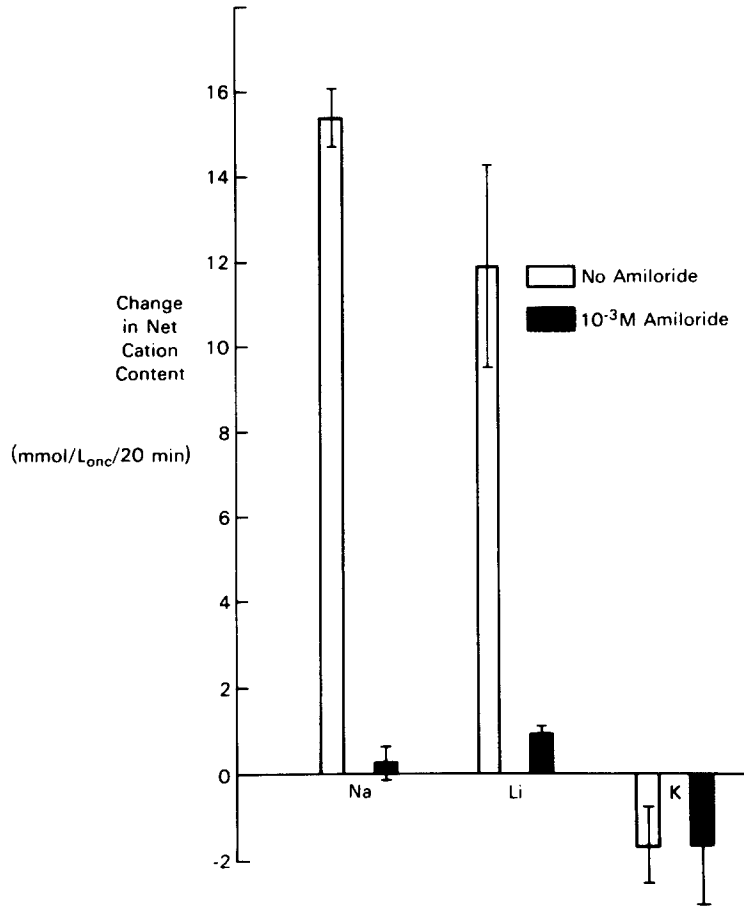

FIGURE 13. 20-min net Na, Li, or K uptake during the VRI response: the effect of amiloride. Cells were incubated in modified hypertonic media (~325 mosmol), which contained 10^{-4} M ouabain, 150 mM of either Na, Li, or K, and either 10^{-3} M amiloride or no amiloride. The remaining constituents of the media were the same as medium C with choline Cl used to match medium osmolalities. Samples were taken at 7 and 27 min. $N = 3$. The response of cells to amiloride-free isotonic media containing 10^{-4} M ouabain and 90 mM of either Na, Li, or K was also examined as a control. The mean cation change (in mmol/L_{onc}/20 min) was −0.02 in the Na medium, 0.06 in the Li medium, and −1.4 in the K medium.

Na gain is blocked when cells not pretreated with amiloride are exposed to amiloride after washing. This is further demonstrated in Fig. 12, *B* and *C*, which shows that under these conditions, amiloride inhibited ~90% of the ^{24}Na uptake during period III and >99% during period IV.

Fig. 13 presents the results of experiments designed to test whether Li or K can substitute for Na in the volume-stimulated uptake process. Li can substitute for Na, whereas K cannot. As with the gain of Na, the gain of Li was blocked by 10^{-3} M amiloride. Although there were small decreases in the K content of cells in both isotonic (see legend) and hypertonic high-K media, these decreases were not statistically significant and represented very small (<2%) changes in total K content.

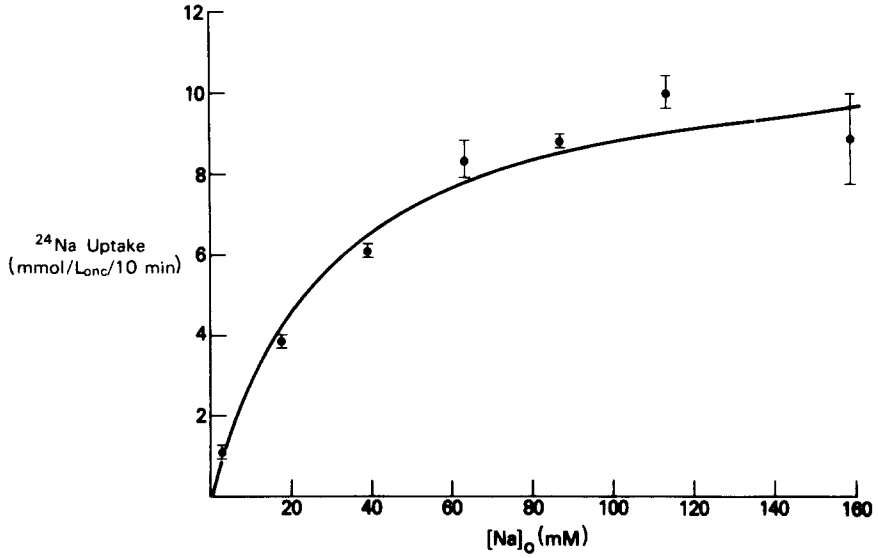

FIGURE 14. ^{24}Na uptake as a function of extracellular [Na] during the VRI response. Cells were incubated in hypertonic media (~325 mosmol) containing different Na concentrations. Media contained ^{24}Na, 10^{-4} M ouabain, and the same constituents as medium C except that the medium [Na] was varied by replacement with choline. $N = 3$. Samples were taken at 3 and 13 min. Since the Na transport rate varied among the experiments, the data from each experiment were normalized by expressing the Na uptake at each Na concentration as a fraction of the projected maximum transport rate (V_{max}) estimated from an Eadie-Hofstee plot of the data from that experiment. The fractional transport rates were then multiplied by the mean V_{max} (11.5 mmol/L$_{onc}$/10 min). The means and SEMs of these values at each Na concentration are shown in the figure. The line represents the curve generated from an Eadie-Hofstee plot of the mean normalized data.

Experiments were performed to determine whether the volume-regulatory Na uptake is a saturable function of external Na. Medium Na was varied at constant osmolality by replacing Na with choline. Fig. 14 depicts the normalized (see legend) ^{24}Na uptake of cells incubated in hypertonic media plotted as a function of the extracellular [Na]. Na uptake is a saturable function of extracellular [Na], which is well described by the Michaelis-Menten equation. The apparent K_m derived from a Hofstee plot of these data was 28.5 mM Na. The apparent K_m's for the individual experiments used in Fig. 14 varied by only 14% (from 26.0 to 29.7 mM Na), whereas the V_{max}'s varied by nearly 250% (from 7.6 to

18.6 mmol/L_{onc}/10 min). Thus, the value for V_{max} was variable, while the value for K_m was relatively constant. This variability in V_{max} appears to be biological. When V_{max} was increased further to ~28 mmol/L_{onc}/10 min by increasing the osmolality of the media, K_m remained at ~31 mM Na (data from Fig. 15).

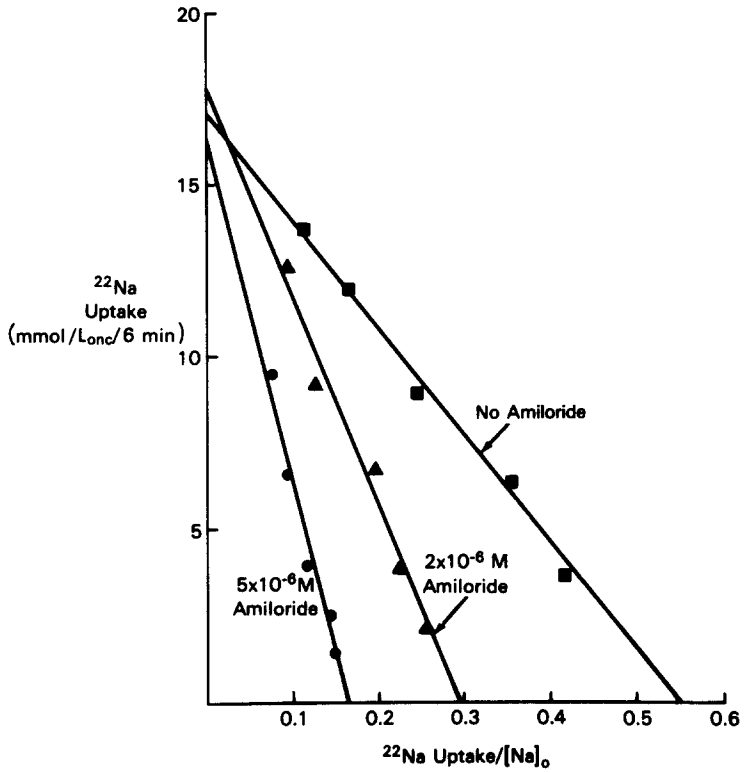

FIGURE 15. Eadie-Hofstee plot of the inhibitory effect of amiloride as a function of extracellular [Na]. Media contained the same constituents as medium C_H except that (*a*) Na was varied by replacement with choline, and (*b*) the osmolality of all media was increased to 365 mosmol by the addition of choline Cl. Cells were incubated in three groups of flasks that contained hypertonic media and 10^{-4} M ouabain. Each group included five flasks with Na concentrations of 9, 17, 35, 71, and 129 mM. One group served as control and contained no amiloride (■). The other two groups contained 2×10^{-6} (▲) and 5×10^{-6} (●) M amiloride, respectively. ^{22}Na was added 4.5 min after the addition of cells. Samples were taken at 12, 15, and 18 min; the ^{22}Na uptakes between 12 and 18 min are presented in the figure. The fact that the rate of Na uptake was linear during this period is indicated by the finding that the V_{max} for cells not exposed to amiloride was 8.2 ± 0.8 mmol/L_{onc} during the 12–15-min flux period and 8.4 ± 0.8 mmol/L_{onc} during the 15–18-min flux period. The data from the three groups of flasks are shown on an Eadie-Hofstee plot. The estimated "V_{max}" and the standard error of the estimate was 17.0 ± 0.4 mmol/L_{onc} with no amiloride, 18.0 ± 1.2 mmol/L_{onc} with 2×10^{-6} M amiloride, and 16.3 ± 1.4 mmol/L_{onc} with 5×10^{-6} M amiloride. A nonlinear least-squares analysis yielded similar results. ●: $r = 0.981$, $K_m = 100.7 \pm 11.5$ mM; ▲: $r = 0.984$, $K_m = 61.7 \pm 6.3$ mM; ■: $r = 0.996$, $K_m = 31.2 \pm 1.4$ mM.

Because the kinetics of Na uptake as a function of extracellular Na can be described by the Michaelis-Menten equation, we used the analytic methods of enzyme kinetics to investigate the mechanism of action of amiloride in these cells. Tracer Na uptake was measured for cells in hypertonic media containing various concentrations of Na and amiloride, and the data were analyzed as indicated in the Methods. The medium osmolality (365 mosmol) and sample times (12, 15, and 18 min after addition of cells) were selected to obtain ^{22}Na uptake values that were linear with respect to time during the sampling period (see the legend to Fig. 15). Fig. 15 shows a Hofstee plot of the data from a representative experiment. Amiloride caused no statistically significant change in the estimated V_{max} (Y intercept), while in this experiment the apparent K_m (slope) was increased from 31 to 101 mM Na by 5×10^{-6} M amiloride. The results are consistent with the interpretation that amiloride acts as a competitive inhibitor of Na uptake in these cells. The apparent K_i for amiloride derived from the experiment of Fig. 15 and another experiment using the same protocol was $1-3 \times 10^{-6}$ M. K_i values obtained by using media with the osmolality of medium C (~325 mosmol) were also in this range.

DISCUSSION

Ouabain-insensitive salt transport mechanisms that enable cells to regulate their volume in anisotonic media were initially described in nonmammalian red cells (reviewed in Kregenow, 1981; Cala, 1983); other cells besides nucleated red cells also possess similar transport mechanisms (reviewed in Hoffmann, 1977; Spring and Ericson, 1982; Grinstein et al., 1984; Siebens, 1985). The results of the present investigation demonstrate that *Amphiuma* red cells can regulate their cell volume in both hypotonic and hypertonic media by using ouabain-insensitive salt transport mechanisms (see also Cala, 1980, 1983).

Comparison with Volume Regulation in Duck, Flounder, and Dog Red Cells

VRD RESPONSE The VRD responses of *Amphiuma*, duck, and flounder red cells all involve losses of K. The K loss is associated with a Cl loss in the *Amphiuma* (Table I) and duck responses (Kregenow, 1971*a*, 1973), but not in the flounder response (Cala, 1977). The increase in net Na uptake seen with ouabain inhibition in the *Amphiuma* response is also observed in the flounder response (Cala, 1977) but not in the duck response (Kregenow, 1971*a*). It has been proposed that the K loss of *Amphiuma* red cells takes place though a K/H exchange mechanism (Cala, 1980). The evidence for this hypothesis is that the mechanism is apparently electrically silent, and that the medium pH increases during the response (Cala, 1980). However, both these characteristics are shared by the duck red cell VRD response, which involves Cl-dependent K loss, probably K/Cl cotransport (Kregenow and Caryk, 1979; Kregenow, 1981). Although the model of a K/H exchanger for the VRD response may be valid, the evidence for it is not yet conclusive in our view. The VRD response of dog red cells is very different from the *Amphiuma*, duck, and flounder VRD responses inasmuch as dog red cells are high-Na, low-K cells. The VRD response of dog red cells appears to involve a Ca-dependent Na extrusion mechanism, possibly a Na/Ca exchanger (Parker, 1973; Parker et al., 1975).

VRI RESPONSE The ionic mechanism of the *Amphiuma* VRI response differs substantially from that of avian red cells. The avian response involves a furosemide-sensitive cotransport of Na, K, and Cl (Kregenow, 1978, 1981; Schmidt and McManus, 1977*a*, *b*; McManus and Schmidt, 1978; Palfrey and Greengard, 1981; Haas et al., 1982). A sufficient decrease in the medium concentration of any of these three ions prevents duck cells from enlarging (Kregenow, 1977, 1978, 1981; Schmidt and McManus, 1977*a*, *b*; Kregenow and Caryk, 1979). In contrast, the Na uptake in the *Amphiuma* VRI response is amiloride-sensitive (Fig. 10) and does not require K (Fig. 9) or Cl (Kregenow et al., 1985). As discussed in the companion paper (Kregenow et al., 1985) and elsewhere (Siebens and Kregenow, 1980; Cala, 1980), the *Amphiuma* VRI response appears to use H (or OH) ions rather than Cl ions to maintain electroneutrality, behaving as a Na/H antiport (or Na/OH symport). Another difference is that the "delay" period of the *Amphiuma* VRI response (Fig. 7, present study; Cala, 1980) is not observed in the duck response, where maximal rates of electrolyte uptake are observed within a minute after exposure of cells to hypertonic media (Kregenow, F. M., unpublished data). The possible inhibition of the *Amphiuma* VRI response by furosemide was examined in two experiments. Furosemide (10^{-3} M) inhibited ^{22}Na uptake by only 15 and 21% in these experiments, which suggests that furosemide does not specifically inhibit volume-regulatory Na uptake in these cells.

It should be noted that after our report of amiloride-sensitive, volume-regulatory Na uptake (Siebens and Kregenow, 1978), Palfrey et al. (1980) reported that amiloride-sensitive (furosemide-insensitive) Na transport can be elicited in frog red cells by catecholamines or cAMP, as well as by hypertonicity. Previous reports in avian red cells indicated that catecholamines stimulate a cAMP-dependent transport mechanism similar to that of the avian VRI response (Riddick et al., 1971; Kregenow, 1973; Kregenow et al., 1976). The possibility that hypertonicity and catecholamine-stimulated salt transport in avian and amphibian red cells may have common characteristics was anticipated by the work of Orskov (1954, 1956*a*, *b*).

The present results may explain some of the findings of Orskov's study of frog red cells incubated in plasma made hypertonic with NaCl (Orskov, 1956*a*). Orskov found that over a period of hours, the cells gradually gained K, but that the K gain was much smaller than the increase in cellular osmotic content required to explain the cell volume. Unfortunately, he did not report initial volume changes or changes in cell Na. Extrapolating from Figs. 5 and 6 of the present study, Orskov's findings can be explained by a primary increase in cell Na content, resulting in volume recovery, followed by a secondary uptake of K brought about by increased Na/K pump activity.

The flounder red cell VRI response is similar to the *Amphiuma* response in that both involve increases in cell Na and Cl content, but the flounder response lacks the "delay" period (Cala, 1977). Studies of the flounder and *Amphiuma* VRI responses may not be directly comparable, however, because experimental conditions differed in the two studies. Flounder cells, rather than being shrunk in hypertonic media as in the present study, were preincubated in a hypotonic medium and allowed to complete the VRD response. These osmotically depleted

cells were then shrunken by reintroducing them to isotonic media to study their VRI response.

Although dog red cells are very different from *Amphiuma* red cells, lacking a nucleus and being high-Na, low-K cells, the mechanism of the VRI response of both cells may be similar. Evidence presented by Parker (1983) indicates that the increase in Na fluxes observed in shrunken dog red cells (Parker et al., 1975) is almost completely blocked by 0.5 mM amiloride or 0.5 mM quinidine. (Possible effects of quinidine on *Amphiuma* red cells have not been examined.)

COMPARISON WITH ANOTHER STUDY OF VOLUME REGULATION IN *AMPHIUMA* RED CELLS Cala (1980) used single-cell micropuncture to demonstrate the absence of large changes in membrane potential during volume regulation after confirming that *Amphiuma* red cells regulate their volume in anisotonic media (Siebens and Kregenów, 1978). The basic observations concerning ion movements in both studies are similar: the VRD response involves cellular loss of K, Cl, and H_2O, whereas the VRI response involves cellular gains of Na, Cl, and H_2O. There are, however, several noteworthy differences between the findings of Cala and those reported here. First, Cala observed no increase in Na uptake in ouabain-treated cells undergoing the VRD response (at pH 7.65), in contrast to the increase in net Na uptake shown in Figs. 2 and 4. Second, the net Cl change relative to the Na + K change ($\Delta Cl/\Delta Na + K$) was about half as great in the present study for both the VRD and VRI responses. We do not have an adequate explanation for these differences, although differences in methods may be involved. For example, the effect of ouabain on cells undergoing the VRD response was examined using net Na and K changes during the initial 90 min in the Cala study. This incubation period may have been too brief to detect the increase in Na uptake we observed using tracers in the initial hour (Fig. 4), and chemical Na measurements over longer periods (Fig. 2). In addition, most of Cala's experiments were performed in low-bicarbonate (<1 mM) imidazole- (20 mM) buffered media, rather than bicarbonate- (9.2 mM) buffered media as in the present study. Also, cells in the Cala study were preincubated in a 245-mosmol medium before experimentation, an osmolality hypertonic to the usual plasma osmolality of our animals (217 ± 0.6 mosmol, $N = 17$). Preincubation at this osmolality may result in cell volume regulation before experimentation. In support of this premise, we found that when we tested the effect of 245 mosmol media under the conditions of Fig. 7 (with choline Cl used to vary osmolality with medium Na ~94 mM), the ^{22}Na uptake during the initial 35 min in three experiments was increased 5- to 16-fold relative to that of cells in media matched to the plasma osmolality of the animal.

DO MOVEMENTS OF NA, K, CL, AND HCO_3 ACCOUNT FOR THE OBSERVED WATER MOVEMENTS? If one assumes that (*a*) there is no transmembrane osmotic gradient, (*b*) the HCO_3 concentration ratio equals the Cl concentration ratio, and (*c*) the osmotic coefficient of each solute is constant throughout the experiment and is the same in the cell and medium, one can determine whether the measured movements of Na, K, and Cl and the inferred small movements of HCO_3 account for water movements observed during volume recovery. The calculated apparent osmolality of the fluid that moves during volume regulation, π_{app}, should equal that of the medium. Pooling values for ouabain-treated and untreated cells from

each experiment, the value of π_{app} for the VRD response was 142 ± 5 mosmol compared with a medium osmolality of 140 ± 3 mosmol in these experiments (N = 5). The value of π_{app} for the VRI response analyzed in this manner was 323 ± 20 mosmol compared with a medium osmolality of 326 ± 5 mosmol (N = 4). Thus, the movements of Na, K, Cl, and HCO_3 appear to account for the water movements in both responses. Similarly, volume-regulatory movements of Na, K, and Cl in duck red cells account for the observed water movements (Kregenow, 1971*a*, *b*; Schmidt and McManus, 1977*a*). In contrast, movements of K, Na, and Cl during the VRD and VRI responses of flounder red cells are insufficient to account for changes in cell water, with changes in cellular amino acids thought to make up the difference (Fugelli, 1967; Cala, 1977; Fugelli and Reierson, 1978).

Because the osmotic coefficient of mammalian hemoglobin is known to change as a function of concentration (Adair, 1929; Dick and Lowenstein, 1958; McConaghey and Maizels, 1961; Freedman and Hoffman, 1979), the predicted value of π_{app} is actually expected to be slightly greater than the medium osmolality in the VRD and VRI responses because of changes in the osmotic coefficient of hemoglobin during volume recovery (Siebens, 1985). In the *Amphiuma* red cell, this slight effect predicts that π_{app} will be ~6 and 13 mosmol hypertonic to the medium in the VRD and VRI responses, respectively. This small effect, although of theoretical interest, is below the resolution of our methods since the calculation of π_{app} in each experiment requires an initial and final determination of four parameters: Na, K, Cl, and H_2O.

Cl Movements and Distributions

Three findings from Tables I and II concerning Cl movements during volume regulation are noteworthy. First, net Cl movements are associated with net Na + K movements in both the VRD and the VRI responses. Second, the changes in cell Cl content are substantially smaller than the changes in Na + K content, which implies that electroneutrality is maintained by movements of ions other than Cl. Third, the Cl concentration ratio and, by inference, the membrane potential change slightly during volume regulation despite strong evidence that the volume-regulatory transport mechanisms are electroneutral (Cala, 1980). These findings can all be explained if one makes the classical red cell assumption that:

$$\frac{[Cl]_i}{[Cl]_o} = \frac{[HCO_3]_i}{[HCO_3]_o} = \frac{[OH]_i}{[OH]_o} = \frac{[H]_o}{[H]_i} \tag{8}$$

(Warburg, 1922; Van Slyke et al., 1923; Funder and Wieth, 1966). As discussed by Jacobs and Stewart (1942), the rapid (<1 min) equilibration of anions and H^+ is dependent on bicarbonate and what was later identified as the anion exchanger (pH equilibration reviewed in Hladky and Rink, 1977). Jacobs and Stewart (1947) also addressed the complex relationship between factors such as hemoglobin and the medium and cell concentrations of "nonpenetrating" cations, such as Na and K, on the equilibrium distributions of "penetrating ions," such as Cl, HCO_3, OH, and H. Formula 1*a* of Jacobs and Stewart (1947) describes what is intuitively clear, namely, that as cell Na + K content increases and electroneu-

trality is maintained, the contribution of nonpenetrating anions, such as hemoglobin, to the total cell anion content decreases, while the contribution of penetrating anions, such as Cl, increases. As Cl makes up a greater fraction of the cell anion content, rCl moves toward unity. The cell buffers, such as hemoglobin, play a critical role in the equilibration of anions and H, since they tend to minimize changes in $[H]_i$ and, by Eq. 8, minimize changes in rH, rCl, $rHCO_3$, and rOH. The fixed anion content (i.e., charge of hemoglobin) therefore shifts as a function of cell pH, which, in turn, is a function of the cell Na + K content.

Let us first consider why the changes in cell Cl in Tables I and II are smaller than the Na + K changes. Inasmuch as evidence has been presented for Na entering by Na/H exchange (or Na/OH cotransport) in the VRI response of *Amphiuma* (Siebens and Kregenow, 1980; Cala, 1980; Kregenow et al., 1985) and dog (Parker, 1983; Parker and Castranova, 1984) red cells, we will use this as an example. It is easiest to describe if one envisions the process as Na/OH cotransport. In step one, Na enters the cell with OH, increasing the osmotic content and volume of the cell but driving OH out of its former equilibrium with Cl and HCO_3. In step two, part of this OH re-equilibrates with Cl and HCO_3 through the anion exchanger (the "Jacobs-Stewart" cycle), while part is buffered by cellular buffers such as hemoglobin. When the NaOH gain ceases, the final result is a new equilibrium distribution according to Eq. 8. Thus, the sum of the uptakes of Cl, HCO_3, and OH will equal the Na uptake, but the Cl uptake will be smaller than the Na uptake, with most of the difference between the Na and Cl uptakes being accounted for by cellular buffering of H and OH. Another aspect of this re-equilibration is that rCl, $rHCO_3$, rOH, and rH will all move toward unity as $[Cl]_i$, $[HCO_3]_i$, and $[OH]_i$ increase. This explains why rCl increases during the VRI response (Table II). The finding that the cell interior becomes alkaline during the *Amphiuma* VRI response (Kregenow et al., 1985) is also consistent with this interpretation. Additional support for the concept that Cl enters by equilibration through the anion exchanger is that the net Cl gain shown in Table II is entirely blocked by inhibition of the anion exchanger with SITS (4-acetamide-4′-isothiocyano-2,2′-stilbene disulfonic acid) (Kregenow et al., 1985) or DIDS (4,4′-diisothiocyano-2,2′-stilbene disulfonic acid) (Cala, 1980). Note that the same final Cl and H distribution would result regardless of the transport mechanism involved in changing the cell cation content. Indeed, similar changes in rCl are observed in duck red cells in hypertonic media when the cell cation content is changed by volume-regulatory Na/K/Cl cotransport (Kregenow, 1971*b*, 1981), and in human red cells in hypertonic media when the cell cation content is experimentally altered using the ionophore nystatin (Dalmark, 1975).

The same considerations concerning anion and H re-equilibration apply to the *Amphiuma* VRD response (Table I) except that rCl, $rHCO_3$, rOH, and rH are expected to move away from unity. Again, the Cl loss is expected to be smaller than the cation loss. Changes in rCl similar to those of Table I are also observed in the duck (Kregenow, 1971*a*) and dog (Parker, 1973) VRD responses.

With respect to the implications of changes in rCl for V_m, it is highly likely that V_m does in fact change slightly, particularly in the *Amphiuma* VRI response. As discussed by Lassen (1977) concerning *Amphiuma* red cells, ". . .chloride does

not solely *determine* the membrane potential in non-equilibrium situations (but the statement holds that it *reflects* the potential when at equilibrium)." The cells in hypertonic media at 6 h in Table II are almost undoubtedly at equilibrium inasmuch as all values are nearly identical to those at 1 h. In addition, if the V_m values during nonequilibrium conditions in Table II are at least qualitatively correct, they can be easily reconciled with the finding of Cala (1980) that there was no statistically significant change in V_m in cells undergoing the VRI response. Cala found that the mean value of V_m under hypertonic conditions was -19.1 ± 1.9 mV ($N > 100$), 4.5 mV less than that of isotonic controls (-23.6 ± 1.8 mV, $N > 100$). Because the major issue in the Cala study was whether or not there were large changes in V_m resulting from conductive Na movements, data from the initial 90 min of the response were pooled. This pooling may have obscured the apparently small but statistically significant ($P < 0.01$) decrease seen at the 0.5-, 1.0-, and 6.0-h sample times in Table II. The finding that the V_m data derived from Cl ratios (Tables I and II) are in qualitative agreement with the microelectrode data of Cala (1980), together with the observation (Kregenow et al., 1985) that anion and H^+ equilibration requires <1 min in these cells, suggests that small changes in V_m occur during volume recovery. As discussed above and elsewhere (Siebens, 1985), these changes in V_m can be accounted for by changes in the relative contribution of the impermeant anions to the membrane potential. If this is true, it should be possible in future studies to use nystatin to make *Amphiuma* red cells permeable to cations and anions (Dalmark, 1975; Freedman and Hoffman, 1979) and to obtain values of rCl as a function of volume similar to those of the present study.

In summary, re-equilibration of Cl, HCO_3, OH, and H according to Eq. 8 accounts for the findings that Cl movements are large, but smaller than cation movements, as well as the finding that rCl changes during volume recovery. This re-equilibration is expected to result in small changes in V_m despite electroneutral volume-regulatory ion movements.

Pump Stimulation During Volume Regulation

In both the VRD and VRI responses, Na/K pump activity is greater than predicted from intracellular Na concentrations by extrapolating from the human red cell data of Garay and Garrahan (1973). In the VRI response, pump activity increases ~10-fold, while a 2-fold increase is expected from the increase in $[Na]_i$. In the VRD response, a threefold increase is observed despite a decrease in $[Na]_i$ in the osmotically swollen cells (Fig. 2). This latter finding is noteworthy because it indicates that it is not always valid to infer that $[Na]_i$ increases when an increase in pump activity is measured. Increased Na/K pump activity following an osmotically induced decrease in $[Na]_i$ has also been reported in flounder red cells (Cala, 1977) and toad urinary bladder (Lipton, 1972).

The mechanism of pump stimulation during the VRD and VRI responses is unknown. Possible explanations include the following: (*a*) the number of Na/K pumps increases; (*b*) the activity of existing pumps is somehow modulated; (*c*) the pump rate is limited by substrate (e.g., Na, K) levels different from estimated values: for example, $[Na]_i$ at the pump site may actually increase in the VRD

response when $[Na]_i$ estimated from Eq. 5 decreases. We favor the first two possibilities. An increase in pump number has been observed under certain conditions in HeLa cells (Pollack et al., 1981). However, the full response requires ~24 h (Pollack et al., 1981), whereas the increases in pump activity in the present study were observed in the initial 1 or 2 h. With regard to the second possibility, a change in the relative affinity for $[K]_i$ and $[Na]_i$ rather than an increase in pump number appears to be the explanation for an increase in pump activity in LK goat red cells induced by an anti-L antibody (Ellory, 1977).

Pump Considerations in the Post–Volume-regulatory Period

One of the main conclusions of this paper and that of Cala (1980) is that *Amphiuma* red cells are able to regulate their volume despite inhibition of the Na/K pump with ouabain. However, early studies of human red cells indicated that if the cells are loaded with Na, active extrusion of Na exceeds active uptake of K (Flynn and Maizels, 1950; Post and Jolly, 1957), resulting in a decrease in cell volume (Flynn and Maizels, 1950). Indeed, this was known before it was demonstrated that cardiac glycosides inhibit active Na and K transport (Schatzmann, 1953). In the present study, the results in the post–volume-regulatory period (i.e., after 8 h in the VRD response, after 1 h in the VRI response) suggest that a small "dehydrating" effect of the pump may be present during this period of elevated pump activity. The cell Na + K content after 12 h in hypotonic media is 3.2 $mmol/L_{onc}$ smaller in untreated cells than in ouabain-treated cells (Table I), while this difference is 4.8 $mmol/L_{onc}$ after 6 h in the hypertonic media (Fig. 6).

The cell Na and K contents at the above sample times were also used to calculate the ratio of the ouabain-induced net cation changes, $\Delta Na_{ouab}/\Delta K_{ouab}$. The 95% confidence interval for this ratio was 1.07–1.34 in the VRD response ($N = 6$) and 1.16–1.33 in the VRI response ($N = 5$). Thus, in both responses, the apparent Na/K pump ratio calculated in this manner is statistically >1.0, but <1.5, the value proposed for the pump ratio of human red cells (Post and Jolly, 1957). A possible explanation for these findings is that volume-regulatory movements of Na and/or K may partially compensate for the dehydrating effect of the stimulated pump; i.e., ouabain-insensitive Na and K movements may not be the same in the presence and absence of ouabain (Siebens, 1985). If such volume-regulatory movements were perfectly balanced with the pump fluxes, they would result in an apparent Na/K pump ratio of unity. Our finding that $\Delta Na_{ouab}/\Delta K_{ouab}$ exceeds unity is consistent with a previous report that the Na/K pump in these cells is electrogenic, at least under high-$[Na]_i$ conditions (Hoffman et al., 1979).

Amiloride-sensitive Na Transport

The findings concerning amiloride-sensitive Na transport in the VRI response are noteworthy in two senses. In a qualitative sense, these studies and others (Cala, 1980; Spring and Ericson, 1982; Parker, 1983; Parker and Castranova, 1984; Grinstein et al., 1983) indicate that amiloride-sensitive Na uptake can be induced or increased by cell shrinkage, resulting in a return of cell volume toward control levels. In a quantitative sense, the relative increase in Na uptake

in the *Amphiuma* red cell VRI response of over two orders of magnitude (Fig. 7) is much larger than other volume-regulatory mechanisms, most of which involve transport increases of roughly one order of magnitude (VRD data, present study; Kregenow, 1971*a*, *b*; Cala, 1977; Parker, 1983). To our knowledge, the rate of Na uptake after pretreatment with amiloride (as much as 25 mmol/L_{onc}/5 min, i.e., 300 mmol/L_{onc}/h) is the largest Na uptake rate observed to date in a red cell. The *Amphiuma* red cell transport rates are even more dramatic, given that the membrane surface of a liter of *Amphiuma* red cells is only about one-fifth that of human red cells (Dunham and Hoffman, 1978).

Several characteristics of amiloride-sensitive Na transporters in epithelia are also shared by the Na transporter of the *Amphiuma* red cell VRI response. First, inhibition by amiloride is readily reversible (Bentley, 1968; Ehrlich and Crabbé, 1968). Second, Na transport is enhanced beyond control levels after exposure of pretreated cells to control media when the cells have been pretreated with either amiloride-containing normal media or low-Na media (all solutions isotonic; Ehrlich and Crabbé, 1968; Turnheim et al., 1978). Third, Na transport is a saturable function of external Na (Ussing, 1948; Frazier et al., 1962; Kinsella and Aronson, 1981). Fourth, Li can substitute for Na, whereas K cannot (Zerahn, 1955; Lindley and Hoshiko, 1964; Herrera, 1972; Kinsella and Aronson, 1981). In addition, amiloride appears to act as a competitive inhibitor of Na uptake in the *Amphiuma* red cell VRI response. Evidence that amiloride acts as a competitive inhibitor of Na transport has been presented in frog skin (Cuthbert and Shum, 1974; Fuchs et al., 1977), toad bladder (Sudou and Hoshi, 1977), renal microvillus vesicles (Kinsella and Aronson, 1981), and trout gill (Greenwald and Kirshner, 1976). However, noncompetitive or mixed inhibition by amiloride has also been reported in epithelia (Benos et al., 1979) and cultured kidney cells (Rindler et al., 1979). The data of Figs. 14 and 15 indicate that the K_m of Na uptake as a function of $[Na]_o$ is nearly constant (~29 mM) despite a nearly fourfold variation in V_{max}. These data suggest that the VRI response involves an increase in the number and/or the Na turnover rate of amiloride-sensitive transporters, rather than a change in the affinity of these transporters for Na.

Despite the many similarities between amiloride-sensitive Na transport in *Amphiuma* red cells and conductive Na transport in "tight" epithelia, such as toad urinary bladder (Sudou and Hoshi, 1977), frog skin (Fuchs et al., 1977; Helman and Fisher, 1977), and rabbit colon (Schultz et al., 1977), we have noted that the *Amphiuma* red cell transporter acts as a Na/H antiport (or a Na/OH or Na/HCO_3 symport) (Cala, 1980; Siebens and Kregenow, 1980; Kregenow et al., 1985). This mechanism appears to be similar to that in various cells including many "leaky" epithelia, such as gallbladder (Weinman and Reuss, 1982) and proximal renal tubule (Kinsella and Aronson, 1981; Boron and Boulpaep, 1983). It is noteworthy that the apparent amiloride-sensitive Na/H exchange mechanism of *Necturus* gallbladder (Weinman and Reuss, 1982) is postulated to be involved in the VRI response of these cells, with amiloride blocking volume recovery by osmotically shrunken cells (Ericson and Spring, 1982; Spring and Ericson, 1982).

Intracellular Na concentration has been postulated to have a regulatory role in transepithelial Na transport through feedback inhibition of amiloride-sensitive

Na entry in frog skin (Erlij and Smith, 1973; Cuthbert and Shum, 1977), rabbit urinary bladder (Lewis et al., 1976), and rabbit colon (Turnheim et al., 1978). Several findings indicate that $[Na]_i$ does not regulate amiloride-sensitive Na entry in the *Amphiuma* red cell VRI response. First, the large increase in Na uptake of the VRI response develops despite an initial 35% increase in the calculated $[Na]_i$ when cells shrink osmotically in ~325-mosmol media. Second, as $[Na]_i$ increases during the VRI response, the rate of Na entry into cells in ~325-mosmol media continues to increase for ~10 min (Fig. 7); a decrease would be expected if there were negative feedback of $[Na]_i$ on Na entry. Finally, the calculated $[Na]_i$ of cells that have completed the VRI response increases with increased medium hypertonicity. In the experiment shown in Fig. 7, the calculated $[Na]_i$ values at 10 h for cells in the 292-, 326-, and 383-mosmol media were 64, 91, and 125 mM, respectively. The final $[Na]_i$ of cells in different hypertonic media would presumably be similar if $[Na]_i$ regulated Na entry.

Na entry in the VRI response, on the other hand, appears to respond to some factor(s) related to cell volume. First, decreasing the cell volume initially by increasing the osmolality of the hypertonic incubation medium enhances Na entry (Fig. 7). Second, cells shrunken in hypertonic media to different degrees gain different amounts of Na, depending on the medium osmolality, and swell until they approach their original volume (Figs. 5–7).

Four variables described in this paper affect the rate of volume-regulatory Na uptake in the VRI response when external Na is held constant: medium osmolality (Fig. 7), duration of exposure to a given osmolality (the delay period of Fig. 7), pretreatment with amiloride (Figs. 11 and 12), and duration of pretreatment with amiloride (Figs. 11 and 12). Further studies of how these factors affect Na uptake may yield important information concerning the mechanisms involved in the regulation of amiloride-sensitive Na/H exchange.

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