### Title

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# Cell Volume Regulation by *Amphiuma* Red Blood Cells

## *The Role of $Ca^{+2}$ as a Modulator of Alkali Metal/$H^+$ Exchange*

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ABSTRACT In response to osmotic perturbation, the *Amphiuma* red blood cell regulates volume back to "normal" levels. After osmotic swelling, the cells lose K, Cl, and osmotically obliged $H_2O$ (regulatory volume decrease [RVD]). After osmotic shrinkage, cell volume is regulated as a result of Na, Cl, and $H_2O$ uptake (regulatory volume increase [RVI]). As previously shown (Cala, 1980*a*), ion fluxes responsible for volume regulation are electroneutral, with alkali metal ions obligatorily counter-coupled to H, whereas net Cl flux is in exchange for $HCO_3$. When they were exposed to the Ca ionophore A23187, *Amphiuma* red blood cells lost K, Cl, and $H_2O$ with kinetics (time course) similar to those observed during RVD. In contrast, when cells were osmotically swollen in Ca-free media, net K loss during RVD was inhibited by ~60%. A role for Ca in the activation of K/H exchange during RVD was suggested from these experiments, but interpretation was complicated by the fact that an increase in cellular Ca resulted in an increase in the membrane conductance to K ($G_K$). To determine the relative contributions of conductive K flux and K/H exchange to total K flux, electrical studies were performed and the correspondence of net K flux to thermodynamic models for conductive vs. K/H exchange was evaluated. These studies led to the conclusion that although Ca activates both conductive and electroneutral K flux pathways, only the latter pathways contribute significantly to net K flux. On the basis of observations that A23187 did not activate K loss from cells during RVI (when the Na/H exchange was functioning) and that amiloride inhibited K/H exchange by swollen cells only when cells had previously been shrunk in the presence of amiloride, I concluded that Na/H and K/H exchange are mediated by the same membrane transport moiety.

### INTRODUCTION

The regulation of cell volume after osmotic perturbation has been an area of much interest in recent years. The initial studies of this process in

vertebrate cells were focused on erythrocytes and other blood components (Fugelli, 1967; Kregenow, 1971*a*, *b*; Poznansky and Solomon, 1972; Roti-Roti and Rothstein, 1973; Parker, 1973*a*, *b*; Weissenberg and Katz, 1975; Cala, 1977, 1980*a*, *b*; Schmidt and McManus, 1977; Siebens and Kregenow, 1978, 1980; Lauf, 1982). Subsequently, cell volume regulation, or processes that can be construed as such, has been demonstrated in renal proximal tubule, Ehrlich ascites cells, mitochondria, cardiac tissue, nervous tissue, and mammalian gall bladder (Dellasega and Grantham, 1973; Grantham et al., 1974; Hendil and Hoffmann, 1974; Garlid, 1978; E. K. Hoffmann, 1978; Vislie, 1980; Kevers et al., 1981; Fisher et al., 1981). Although the details of the volume-regulatory mechanisms characteristic of various cells differ, there are general features that are becoming apparent. It appears that volume regulation by vertebrate cells is the result of $H_2O$ flow, which is secondary to net inorganic ion fluxes, and that these ion fluxes are not directly coupled to cellular metabolism.

On the basis of measurements of ion fluxes as well as direct measurement of the cell membrane potential, this laboratory was able to demonstrate that cell volume regulation by *Amphiuma* red blood cells is the result of ion fluxes by electroneutral pathways (Cala, 1980*a*). Further, studies designed to determine the nature of volume-regulatory ion fluxes in other systems (duck and fish red blood cells and gall bladder) have shown, with a few exceptions (Grinstein et al., 1982*a*, *b*; E. K. Hoffmann, 1978, 1982), that cell volume regulation is secondary to electroneutral ion transport (Siebens and Kregenow, 1980; Kregenow, 1981; Fisher et al., 1981; Haas and McManus, 1982).

The possibility that volume regulation by *Amphiuma* red blood cells was mediated by electroneutral ion flux was first suggested in studies using valinomycin (Val) (Cala, 1980*a*). These studies showed that Val treatment in isotonic media resulted in a 20–30-mV membrane hyperpolarization in response to a Val-induced K flux of 10–20 mmol/kg dry cell solid (dcs) in 1 h. On the basis of the above values, the Val-induced membrane conductance to K ($G_K^{Val}$) was calculated to be $\sim 9 \times 10^{-7}\ \Omega^{-1}\ cm^{-2}$. Since a Val-induced $G_K$ of $9 \times 10^{-7}\ \Omega^{-1}\ cm^{-2}$ resulted in a 20–30-mV membrane hyperpolarization, it was concluded that, in the absence of Val, the total membrane conductance ($G_m$) must be of the order of $10^{-6}\ \Omega^{-1}\ cm^{-2}$ (within an order of magnitude of $G_K^{Val}$; see also Lassen et al., 1978, 1980; cf. Stoner and Kregenow, 1980). In contrast, when cells were osmotically perturbed (in the absence of Val), net alkali metal ion fluxes were nominally one to two orders of magnitude larger than the Val-induced K fluxes, but during volume regulation, the membrane potential ($E_m$) remained unchanged relative to ionophore-free cells in isotonic medium. Upon exposure to Val and anisotonic media, however, the $E_m$ of volume-regulating cells assumed the same value as that of Val-treated cells in isotonic medium ($E_m^{Val} \cong -40$ mV). Since the Val-induced K current ($I_K^{Val}$) and $E_m^{Val}$ are volume insensitive, it was concluded that $G_K^{Val}$ is also independent of volume. Further, since $E_m^{Val}$ is in all cases the same, then $I_K^{Val}/I_m$, and therefore $G_K^{Val}/G_m$, are volume independent. It follows, then, that $I_m$ and $G_m$ are invariant with volume and that the volume-dependent (volume-regulatory) ion fluxes do not contribute to the membrane current.

On the basis of the above and the observation that, during volume regulation, net alkali metal ion fluxes can be much greater than those of Cl, a model was proposed and tested. The salient features of the model are that alkali metal ion fluxes responsible for volume regulation are obligatorily

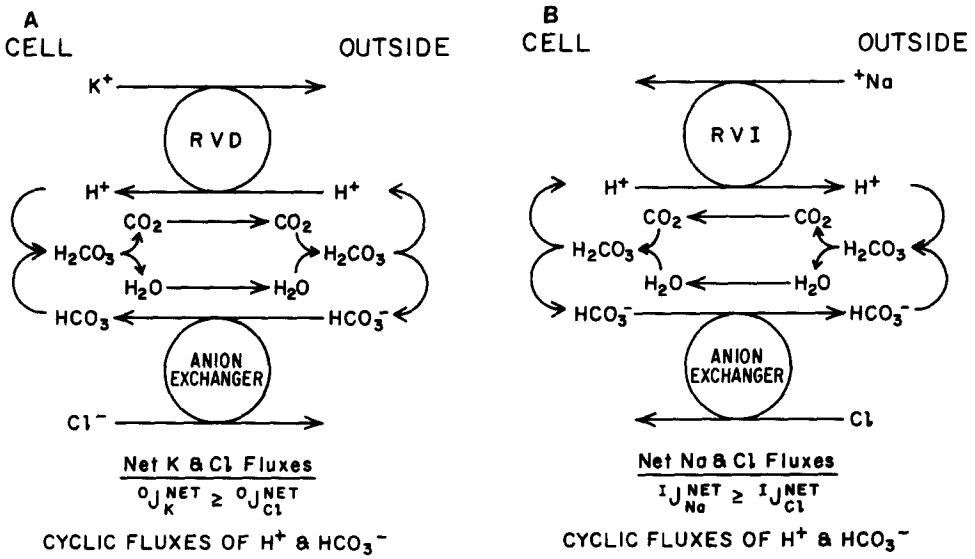


FIGURE 1. Proposed volume-regulatory ion flux pathways in *Amphiuma* red blood cells. The models are based upon ion flux and electrical measurements presented in a previous publication (Cala, 1980*a*). The salient feature of this model is that net alkali metal and $Cl^-$ fluxes are electroneutral by virtue of obligatory counter-coupling with $H^+$ and $HCO_3^-$, respectively. Although $H^+$ and $HCO_3^-$ serve as counter-ions in an electrical sense, they are without osmotic effect because these ions are cycled through the membrane alternately as $H^+$ and $HCO_3^-$ or $CO_2$ and $H_2O$. The only ion fluxes of osmotic consequence are those of the alkali metals and chloride. The electroneutral alkali metal H exchangers and the $Cl^-/HCO_3^-$ exchange are functionally coupled as a result of the flux of $H^+$. This is in contrast to anion and cation fluxes via conductive pathways, where coupling occurs through $E_m$. *A* represents the ion flux pathways responsible for volume regulation after osmotic swelling (regulatory volume decrease [RVD]). Net K flux via K/H exchange is generally greater (by as much as three times) than net $Cl^-$ flux via the $Cl^-/HCO_3^-$ exchanger. *B* represents the ion flux pathways responsible for volume regulation after osmotic shrinkage (regulatory volume increase [RVI]). The net Na and $Cl^-$ fluxes via the Na/H and $Cl^-/HCO_3^-$ exchangers, respectively, are generally in close correspondence (in contrast to K/H and $Cl^-/HCO_3^-$) with net Na flux equal to or slightly (10%) greater than that of $Cl^-$.

counter-coupled to H while net Cl flux occurs in exchange for $HCO_3$. As a result, net alkali metal and Cl fluxes are electroneutral and proceed in the same direction, coupled as a consequence of the flux of H. The proposed pathways responsible for volume regulation after cell swelling (regulatory

volume decrease [RVD]) and shrinkage (regulatory volume increase [RVI]) are depicted in Fig. 1, *A* and *B*, respectively.

During the above studies, the Ca ionophore A23187 was used to activate the Ca-gated K conductance of *Amphiuma* red cells and thereby alter $E_m$. As previously described by others (Lassen et al., 1974, 1976; Gardos et al., 1976), such treatment resulted in membrane hyperpolarization with $E_m$ actually assuming the value of $E_K$ ($\cong -90$ mV) in some cases. In parallel ion flux studies performed on A23187-treated cells, net cellular K losses as large as 200 mmol/kg dcs in 15 min were observed. The magnitude of the net K flux induced by A23187 was difficult to rationalize with previous estimates of $G_m$ ($\sim 10^{-6}\ \Omega^{-1}\ cm^{-2}$). Thus, it appeared that either A23187 exposure resulted in a large (two orders of magnitude) increase in $G_m$ or a substantial fraction of the ionophore-induced K flux is electroneutral. In similar studies using *Amphiuma* red blood cells, Lassen et al. (1976) reported that increasing $Ca_o$ to 15 mM caused the membranes to hyperpolarize transiently and that, in association with hyperpolarization, the cells lost K at a rate two orders of magnitude greater than could be explained on the basis of estimates of $G_m$.

The above observations of large Ca-induced net K fluxes, taken together with demonstrations by this laboratory that cell K loss during RVD is electroneutral, suggested the possibility that Ca might activate both conductive and electroneutral K loss. That is, if a large fraction of the Ca-induced net K loss were electroneutral, then $zFJ_K^{A23187} \gg I_K^{A23187}$ and calculations of $G_K^{A23187}$ based upon $J_K^{A23187}$ would be overestimates. The possibility that Ca may serve as an activator of electroneutral alkali metal/H exchange was also of interest as it might elucidate the underlying control of volume-regulatory ion flux pathways. Previous studies by this laboratory (Cala, 1980*a*, *b*), which are consistent with this notion, suggested that changes in pH may be involved in the activation and control of volume-regulatory ion fluxes. Given the suggestion of Ca involvement, the previously reported pH effects upon volume-regulatory ion fluxes may have been referable to pH-dependent Ca binding rather than a more direct effect of pH on the alkali metal/H exchange mechanism. This hypothesis is made more attractive in light of the increasingly recognized role of intracellular Ca as a second messenger; the normally low, free intracellular [Ca] ($\sim 1\ \mu M$) would make Ca a particularly sensitive transducer for osmotic phenomena.

The data presented in this study support a role for Ca as a modulator of the K/H exchange responsible for RVD. The arguments presented are based upon inferences drawn from ion flux and electrical and thermodynamic considerations. The flux studies (*a*) demonstrate similarities between volume- and A23187-stimulated cell K loss, (*b*) illustrate a large disparity between net cell K and Cl loss, an observation consistent with the notion that a substantial fraction of Ca-induced K loss is electroneutral, and (*c*) establish that K/H exchange during RVD is sensitive to media Ca in the absence of A23187. The electrical studies provide information regarding membrane voltage and, when evaluated in terms of evidence demonstrating that $G_m$ is volume independent, they permit an upper-limit estimate of the Ca-induced conduc-

tive K loss. The thermodynamic studies performed under conditions where the driving forces for A23187-induced K flux via conductive and electroneutral pathways differ in both magnitude and direction permit a clear distinction between the two modes of transport. These latter studies establish not only that the A23187-induced K flux is electroneutral but that it is electroneutral by virtue of coupling with H (OH). Finally, evidence will be presented supporting the hypothesis that K/H exchange and Na/H exchange are different transport modes mediated by the same membrane component(s).

Preliminary reports of some of these data were presented to the Red Blood Cell Club, Houston, TX (1981), and have appeared in abstract form (Cala, 1982).

## MATERIALS AND METHODS

### *General*

Blood was obtained from healthy adult *Amphiuma* by cardiac puncture. Blood was drawn into a heparinized syringe, the cells were separated from plasma by centrifugation for 1 min at 1,000 *g*, and the plasma was removed by aspiration. The cells were then suspended in control Ringer (23°C) and centrifuged, the supernatant was removed by aspiration, and the cells were resuspended for a total of three washes in 40 vol each of Ringer. The control (isotonic) Ringer contained: 110 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$, 20 mM imidazole, and 10 mM glucose. This medium was gassed with water-saturated room air and adjusted to pH 7.65 (23°C) by titration with HCl. The washed cells were finally suspended in isotonic (control) medium at 10% hematocrit and incubated for 1.5 h with gentle agitation to assure that the cells had reached steady state with respect to the ion and $H_2O$ content before experimental treatment. To begin an experiment, cells were removed from suspension in control medium by centrifugation and resuspended (10% hematocrit) in the appropriate experimental medium (at time zero). The experimental suspension medium of all cells intended for analysis of cell ion and water content contained [$^{14}$C]polyethyleneglycol (6,000 mol wt) as an extracellular space marker. Samples (375 μl) were then removed at predetermined intervals (15, 30, or 45 min), placed in 400-μl polyethylene tubes (Stockwell Scientific, Monterey Park, CA), and centrifuged in a microcentrifuge (model 3200; Brinkman, Westbury, NJ) at 12,800 *g* for 2 min. After centrifugation the supernatant was removed and saved for analysis. The 400-μl polyethylene tube and wet cell pellet were weighed (24–48 mg wet pellet), dried in an oven at 80°C for 48 h, cooled in a desiccator, and reweighed to determine the dry pellet weight (8–12 mg). Finally, the dry pellet was extracted by addition of 250 μl of glass-distilled $H_2O$ to the tube containing the dry pellet. After a 3-d extraction, aliquots of the extract were removed for ion analysis and liquid scintillation counting. All cell ion and water contents were corrected for extracellular contribution.

### *Cell Ion and $H_2O$ Content*

The procedure for determining cellular content of ions (Na, K, Cl) and water was described previously (Cala, 1980*a*). Briefly, after extraction the following aliquots were removed: 100 μl for chloridometry (Buchler Instruments Div., Searle Diagnostics, Inc., Fort Lee, NJ), 50 μl for Na and K flame photometry (model 343; Instrumentation Laboratories, Lexington, MA), and 40 μl for scintillation counting

(Searle Mark II, liquid scintillation system; Searle Diagnostics, Inc., Des Plaines, IL). The raw data thus obtained, as well as the pellet wet and dry weights, were then used to calculate the cellular ion and $H_2O$ content corrected for extracellular contribution. The cell ion and $H_2O$ content determined by this procedure is given in Table I.

### *Experimental Media*

All experimental media were variants of the control media in that $Mg^{+2}$, $Ca^{+2}$, imidazole buffer, and glucose were present in the same concentrations as described for control media, unless otherwise specified. Media osmolarity was altered by changing the concentration of NaCl. Media was buffered with imidazole at 20 mM and gassed with room air. Experiments using the $Ca^{+2}$ ionophore A23187 (Calbiochem-Behring Corp., San Diego, CA) were performed in a dimly lighted room and the cell suspensions were kept from light except during sampling. A23187 was present throughout the experimental period and was freshly prepared for each experiment. These precautions improved the reproducibility of experiments using the ionophore (G. Plishker, personal communication).

TABLE I

*Ion and $H_2O$ Content of Amphiuma Red Blood Cells in Isotonic, Hypotonic, and Hypertonic Media*

| Medium | $H_2O$ | Na | K | Cl |
|---|---|---|---|---|
| | *l/kg dcs* | | *mmol/kg dcs* | |
| Isotonic (232 mosmol) | 2.1±0.1 | 30±2 | 241±4 | 90±5 |
| Hypotonic (158 mosmol)* | 2.83±0.04 | 30±2 | 237±6 | 84±5 |
| Hypertonic (340 mosmol)* | 1.59±0.02 | 32±3 | 239±4 | 78±4 |

The values presented are means ± SEM for determinations using cells from 20 animals.

* Although the ion and $H_2O$ content of cells in isotonic medium is invariant with time, the values obtained from cells in anisotonic media are not, as a result of volume regulation. As such, the ion and $H_2O$ contents associated with cells in hypo- and hypertonic media were obtained from samples taken within 1.5 min of suspension in anisotonic media.

### *Electrical Measurements*

MEMBRANE POTENTIAL Measurements of the *Amphiuma* red blood cell membrane potential ($E_m$) were obtained as previously described (see Cala, 1980*a*).

DETERMINATION OF K TRANSFERENCE ($t_K$) The K transference of *Amphiuma* red blood cells was determined graphically from the maximum slope region of a plot of $E_m$ vs. $\log[K]_o$ using the expression

$$\left[\frac{\partial E_m}{\partial \log[K]_o}\right]_{E_i} = 58\ t_K$$

(Brown et al., 1970; Christoffersen, 1973). The maximum slope of $E_m$ vs. $\log[K]_o$ is at $[K]_o > 20$ mM (Cala, 1980*a*) for *Amphiuma* red blood cells exposed to A23187 and/or valinomycin; therefore, measurements of $E_m$ were performed between 20 and 40 mM $[K]_o$. This expression assumes (*a*) a linear relation between $E_m$ and $\log[K]_o$; (*b*) that the total membrane current equals 0 ($dE_m/dt = 0$); and (*c*) that the equilibrium

potentials of the primary charge-carrying species ($E_i$) are constant. Between $[K]_o$ 20 and 40 mM, the relationship between $\log[K]_o$ and $E_m$ is linear; therefore, assumption *a* is met. Since $E_m$ is invariant with time, assumption *b* is appropriately met. With regard to assumption *c*, the equilibrium potential for the primary charge carrier, Cl (Lassen et al., 1978), is unaltered, but as $[K]_o$ is increased at the expense of $[Na]_o$, $E_{Na}$ is decreased by 15%. Since the change in $E_{Na}$ is slight and the fraction of the membrane current carried by Na is small (<10% of $I_m$), the changes in $E_{Na}$ are insignificant and have no consequence.

## RESULTS

### *Effects of A23187 on K and Cl Loss and $E_m$*

Fig. 2*A* depicts changes in the cell K and Cl content of *Amphiuma* red blood cells exposed to the Ca ionophore A23187 in isotonic medium. Measurements of $E_m$ performed in parallel experiments reveal that the membranes of the A23187-treated cells are hyperpolarized ($E_m^{A23187} \cong -55$ mV) relative to the ionophore-free controls ($E_m = -23$ mV). The hyperpolarization exhibited by ionophore-treated cells is due to increased $G_K$ (Lassen et al., 1974, 1976; Gardos et al., 1976) with $E_m$ varying by as much as 40 mV/decade $\Delta[K]_o$, at $[K]_o > 20$ mM. The data in Fig. 2*B* were obtained from parallel studies performed on cells in hypotonic medium in the absence and presence of A23187. As was the case for cells in isotonic medium, exposure to A23187 resulted in cellular K loss and caused membrane hyperpolarization ($E_m^{A23187} \cong -55$ mV) relative to cells swollen in ionophore-free medium ($E_m = -23$ mV). Fig. 3, *A* and *B*, shows that at 7 $\mu$M A23187 (the highest concentration used in this study), cellular K loss in isotonic (Fig. 3*A*) and hypotonic (Fig. 3*B*) media is a function of media [Ca]. These data establish that the effects of A23187 upon cell K loss are referable to its ionophoretic activity and not to a Ca-independent disruption of membrane structure.

### *Effect of Ca Removal on K/H Exchange Flux in Osmotically Swollen Cells*

On the basis of the observations that (*a*) exposure to A23187 results in membrane hyperpolarization, and (*b*) although in the absence of A23187 $E_m$ is virtually independent of $[K]_o$, and after ionophore treatment $E_m$ varies by 35–40 mV/decade $\Delta[K]_o$, it is clear that addition of A23187 results in an increased $G_K$. However, as stated in the Introduction, I hypothesized that Ca may also play a role as an activator of the electroneutral K flux associated with volume regulation after cell swelling. If indeed the volume-regulatory K loss is activated by Ca, it should be possible to inhibit such K loss by swelling cells in Ca-free medium. Table II presents data obtained from cells osmotically swollen in normal (0.5 mM) $Ca^{+2}$ medium and in $Ca^{+2}$-free, EGTA-containing medium. These data illustrate that in the absence of external $Ca^{+2}$ and in the presence of EGTA (1 mM), net K loss during RVD is inhibited by 56%. Since all K loss during RVD is electroneutral (Cala, 1980*a*), it follows that Ca is able to modulate the electroneutral, volume-regulatory K flux pathway.

*Separation of Conductive and Electroneutral Components of A23187-induced Net K Flux: Attempts Based on Ion Flux and Membrane Voltage*

Although the above studies are consistent with a role for $Ca^{+2}$ in activating K/H exchange, they do not resolve the problem that exposure of cells to

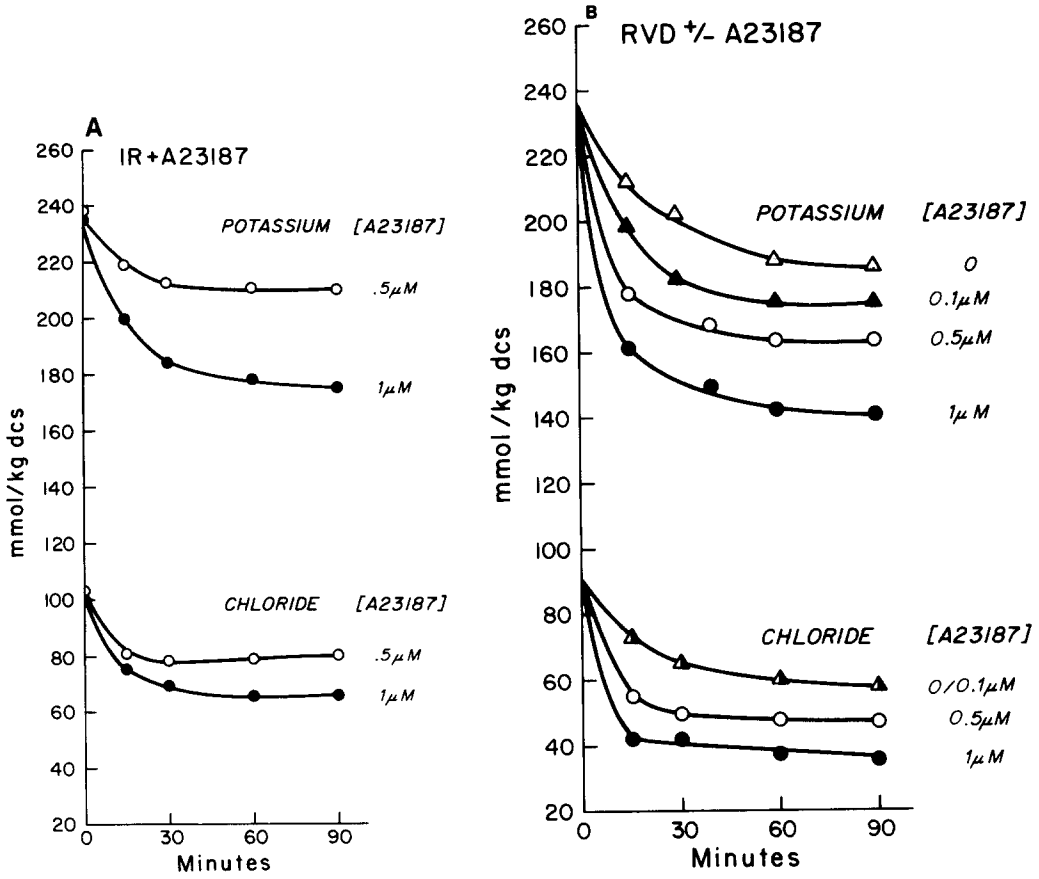


FIGURE 2. Cell K content as a function of time following transfer of cells from isotonic ionophore-free medium to (*A*) media of the same osmolarity containing 0.5 (○) or 1 μM (●) A23187. The cell K contents in *B* were obtained from cells transferred from isotonic ionophore-free medium to hypotonic media containing 0, (△), 0.1 (▲), 0.5 (○), or 1 μM (●) A23187. These data represent seven such paired experiments performed in iso- and hypotonic media. Cell $H_2O$ content (not shown) changes in parallel with ion content (see Cala, 1980*a*).

A23187 results in net cellular K loss, at least some of which, based upon measurements of $E_m$, is conductive. Thus, it was necessary to attempt to distinguish between $Ca^{+2}$-activated conductive ($J_K^G$) and electroneutral ($J_K^e$) K flux.

Using cells from the same animal, exposure to a given concentration of A23187 results in the same degree of membrane hyperpolarization regardless

of cell volume (see discussion of Figs. 2 and 3 and Table V). I have demonstrated in a previous publication (Cala, 1980*a*) that membrane conductance is volume insensitive, and barring some peculiar effect of volume upon A23187-induced K conductance, the ionophore-induced K current required to produce a given $\Delta E_m$ should be the same regardless of cell volume. In the most simple case, if the ionophore-induced K flux is all

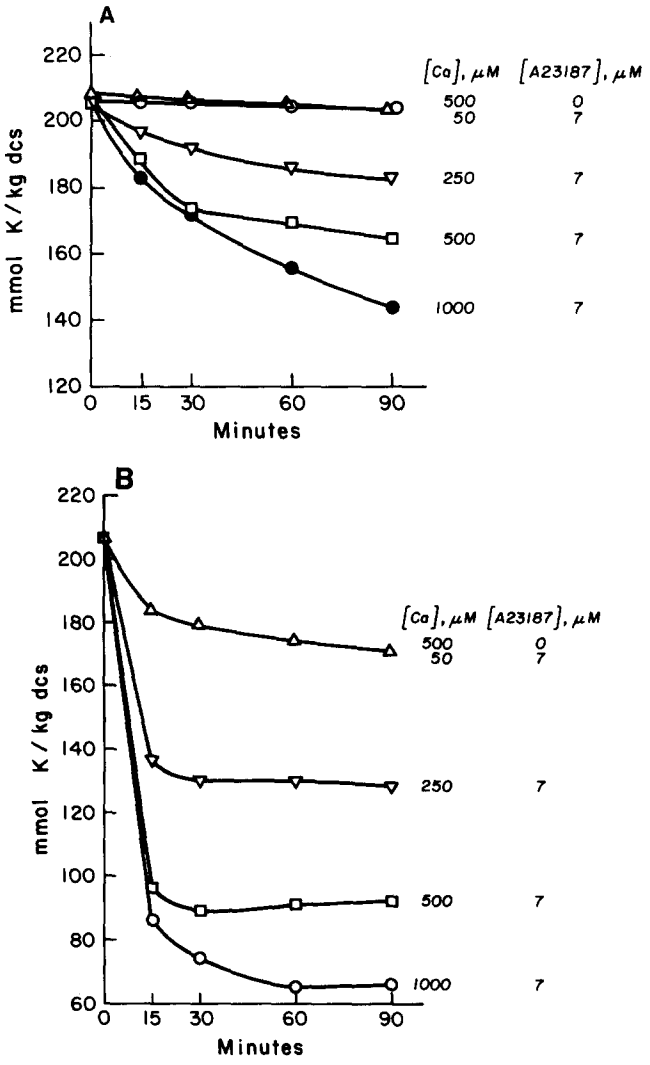


FIGURE 3. Effects of varied Ca (50, △; 250, ▽; 500, □; 1,000 μM, ○) upon K content of cells in isotonic (*A*) and hypotonic (*B*) media. The points obtained at no ionophore and 500 μM Ca (△) represent controls in iso- and hypotonic media and were in all cases indistinguishable from K contents of cells exposed to 7 μM A23187 and 50 μM Ca (○). All other cell subsamples were exposed to 7 μM A23187. The data illustrated are typical of 10 such experiments.

conductive, given that the volume-induced K loss is all electroneutral, then net K loss from cells swollen in the presence of A23187 should be due to the parallel operation of conductive and electroneutral K flux pathways. As such, Ca-induced conductive ($J_K^C$) and electroneutral ($J_K^e$) K flux should be additive: in paired experiments performed using cells from the same animals, net K loss from swollen cells ($J_K^e$) plus that from cells in isotonic A23187-containing medium ($J_K^C$) should equal net K loss from cells swollen in the presence of A23187 ($J_K^e + J_K^C$). The data in Fig. 4 were obtained using cells from the same animal suspended in isotonic medium in the presence of A23187 and in hypotonic media in the presence and absence of A23187. These data show that the sum of net K loss from ionophore-treated cells in isotonic medium and net K loss from swollen cells is not equal to net K loss from cells swollen in the presence of A23187. The non-additive nature of K fluxes, while not conclusive, is consistent with the notion that a fraction of the ionophore-induced K loss is non-conductive.

TABLE II

*Net K Loss from Amphiuma Red Blood Cells Swollen in the Absence and Presence of External $Ca^{+2}$*

| Treatment | $J_K^{net}$ in 90 min | Percent inhibition |
|---|---|---|
| | *mmol/kg dcs* | |
| RVD | 55±5 (5)* | — |
| RVD + 1 mM EGTA (Ca-free) | 24±4 (5) | 56 |

* Mean ± SEM ($n$ = animals).

Studies performed on cells in media containing 1 mM EGTA and 1.5 mM $[Ca]_o$ were indistinguishable from those performed at 0.5 mM $[Ca]_o$. As such, any inhibitory effects of EGTA upon K loss during RVD appear unlikely. Simple Ca removal in the absence of EGTA provided similar results to those obtained in Ca-free EGTA-containing media, but the results were more variable, presumably because of Ca contamination.

### *The Nature of A23187-induced K Flux*

INFERENCE BASED ON IONIC TRANSFERENCE AND ESTIMATES OF MEMBRANE CONDUCTANCE In the presence of 0.5 mM $Ca_o$ and 7 μM A23187, net K loss in osmotically swollen *Amphiuma* red blood cells can be as great as 200 mmol/kg dcs per 15 min. If all of the ionophore-induced K loss is conductive ($zFJ_K^{A23187} = I_K^{A23187}$), given the measured $E_m = -55$ mV, then $G_K^{A23187}$ [$G_K^{A23187} = zFJ_K^{A23187}/(E_m - E_K)$] is of the order of $5 \times 10^{-5}\ \Omega^{-1}\ cm^{-2}$. This value is in sharp contrast to $G_K^{Val}$ [$zFJ_K^{Val}/(E_m - E_K)$], which is calculated to be $9.3 \times 10^{-7}\ \Omega^{-1}\ cm^{-2}$. Given the disparity in the calculated values of $G_K^{Val}$ and $G_K^{A23187}$, cells exposed to Val and A23187 should be indistinguishable (electrically) from those exposed to A23187 alone; the fraction of the membrane current carried by the Val-K complex will be small relative to that carried by the Ca-gated K conductance ($G_K^{A23187}$). However, exposure of cells to 1 μM Val ($K_o$ = 3) and 7 μM A23187 resulted in a 12 ± 3-mV hyperpolarization relative to cells exposed only to A23187. This observation suggests that

$G_K^{A23187}$, calculated assuming that $zFJ_K^{A23187} = I_K^{A23187}$, is an overestimate and inferentially that $zFJ_K^{A23187} > I_K^{A23187}$. The values for K transference obtained from cells treated with A23187 and/or Val (Table III) also support the notion that $G_K^{A23187}$, calculated assuming that $zFJ_K^{A23187} = I_K^{A23187}$, is an overestimate. Since the partial ionic transference ($t$) of a membrane to an ion (i) is equal to

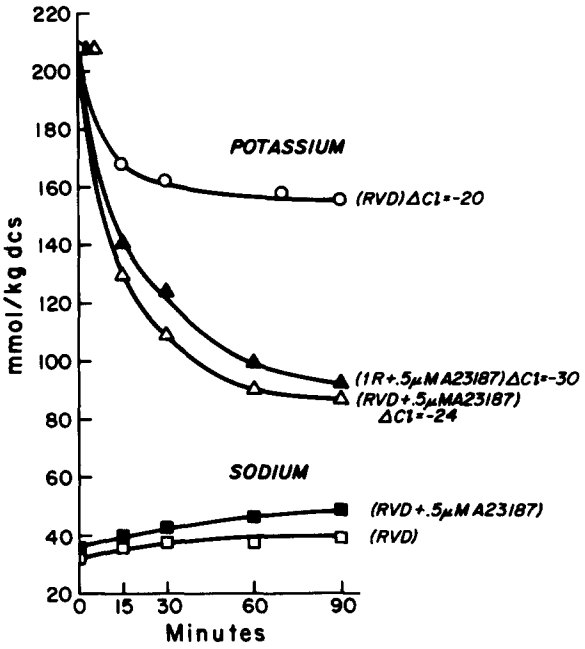


FIGURE 4. *Amphiuma* red blood cell K and Na content as a function of time after osmotic swelling in the presence (△) and absence (○) of 0.5 μM A23187 or exposure of cells in isotonic medium to 0.5 μM A23187 (▲). These data were obtained from paired experiments performed upon subsamples of cells obtained from the same animal. The cell suspension media contained 1 mM Ca and differences in osmolarity are due to differences in media [NaCl]. These results are typical of those from five identical treatments.

$G_i/G_m$, where $G_i$ is the conductance to ion i and $G_m$ is the sum of the conductances to all charge carriers including i, then:

$$t_K^{Val} = \frac{G_K^{Val}}{G_K^{Val} + G_{other}}; \quad (1)$$

$$t_K^{A23187} = \frac{G_K^{A23187}}{G_K^{A23187} + G_{other}}; \quad (2)$$

$$t_K^{A23187+Val} = \frac{G_K^{A23187} + G_K^{Val'}}{G_K^{A23187} + G_K^{Val'} + G_{other}}, \quad (3)$$

where $G_K^{Val'}$ is the Val-induced K conductance minus the K conductance ($G_K$) of the unmodified membrane (included in $G_K^{A23187}$), and $G_{other}$ is the sum of

the membrane conductance to all charge carriers other than K (i.e., $G_{Cl}$ + $G_{Na}$ + $G_{OH}$ + $G_{HCO_3}$ + $G_H$). If $G_K^{A23187} = 5 \times 10^{-5}\ \Omega^{-1}\ cm^{-2}$, and $G_K^{Val}$ is $9.3 \times 10^{-7}\ \Omega^{-1}\ cm^{-2}$, then $t_K^{A23187}$ should be unaffected by the addition of Val (see Eq. 3). However, the addition of Val to cells treated with A23187 (Table III) results in an increase in $t_K$ from 0.62 to 0.86. Thus, it appears that $G_K^{A23187}$ (calculated assuming $zFJ_K^{A23187} = I_K^{A23187}$) is overestimated; therefore, $zFJ_K^{A23187} > I_K^{A23187}$ and a substantial fraction of $J_K^{A23187}$ is electroneutral.

### *The Nature of A23187-induced K Flux*

**DISTINCTION BETWEEN CONDUCTIVE AND ELECTRONEUTRAL K FLUX BASED ON THERMODYNAMIC CRITERIA** If net K flux in A23187-treated cells is electroneutral K/H exchange, it should be possible to drive net conservative

TABLE III

*Amphiuma Red Blood Cell Membrane K Transference ($t_K$) in the Presence of A23187 (7 μM) and/or Valinomycin (1 μM)*

| $t_K^{A23187}$ | $t_K^{val}$ | $t_K^{A23187+Val}$ |
|---|---|---|
| 0.62±0.04 (8)* | 0.5±0.03 (10) | 0.86±0.05 (6) |

* Mean ± SEM (number of animals).

The data presented were calculated from measured values of $E_m$ ($[K]_o$ = 20–40 mM) obtained from cells in isotonic medium (see below) containing 500 μM Ca. The value of $E_m$ at any given $[K]_o$ was taken as the mean of at least 10 measurements. All measurements of $E_m$ were made within the first 15 min because this is the period during which A23187-induced K loss is greatest. Values of $t_K$ were calculated using the expression:

$$58\ \text{mV}\ t_K = \left(\frac{\partial E_m}{\partial \log[K]_o}\right) E_{other},$$

where $E_{other}$ signifies the equilibrium potential of all charge carriers other than K (Brown, 1970; Christofferson, 1973; Lassen et al., 1978). The measurements of $E_m$ were obtained at $[K]_o$ 20–40 mM because (*a*) the slope of $E_m$ vs. $\log[K]_o$ is maximal and linear in this region and (*b*) $E_{ion}$ for all ions other than Na is unchanged, whereas changes in $E_{Na}$ are small. Although the data reported were obtained from cells in isotonic medium, indistinguishable results were obtained from swollen and shrunken cells (thus, $G_m$ must be the same regardless of cell volume). The cells in isotonic medium were preferred because $[K]_i$ is more stable than that of swollen cells and the cells are more easily impaled than those in hyperosmotic media (possibly because of the relatively high surface to volume of the latter). The value of $t_K^{A23187}$ obtained in the present studies is in remarkably good agreement with that obtained by Lassen et al. (1974) ($t_K$ = 0.6), who used repeated cell puncture to introduce Ca into the cell interior.

K flux with the H gradient. Although net conductive K flux is responsive to the electrochemical potential difference for K ($[\Delta\tilde{\mu}_K/zF = (E_m - E_K)]$, where $E_K$ is the potassium equilibrium potential), K flux via K/H exchange depends upon the difference in the K and H chemical potential differences ($\Delta\mu_K - \Delta\mu_H$, where $\Delta\mu_K = RT \ln[K]_i/[K]_o$ and $\Delta\mu_H = RT \ln[H]_i/[H]_o$). To determine $\Delta\tilde{\mu}_K$ and $\Delta\mu_K - \Delta\mu_H$, it is necessary to obtain values for $E_m$, $[K]_o/[K]_i$, and $[H]_o/[H]_i$. With the exception of $[H]_o/[H]_i$, all of the above can easily be measured directly. The transmembrane [H] distribution, while not directly measured, can be calculated from the chloride distribution, if, as is the case for the human red blood cell, $[Cl]_i/[Cl]_o = [H]_o/[H]_i$ (Funder and Wieth, 1966). To test the applicability of the above relative to *Amphiuma* red blood

cells, I made use of the fact if $[H]_o/[H]_i = [Cl]_i/[Cl]_o$, then $[H]_o \times [Cl]_o = [H]_i \times [Cl]_i$. The above relations predict (*a*) that independent changes in [H] or [Cl] should result in flux of both H and Cl until $[H]_o/[H]_i = [Cl]_i/[Cl]_o$, and (*b*) that alteration of [H] or [Cl] at constant product should result in no net H or Cl flux. In agreement with the above predictions, increasing $[H]_o$ from $2.2 \times 10^{-8}$ (pH 7.65) to $8.9 \times 10^{-8}$ (pH 7.05) resulted in net cellular uptake of 46 ± 5 mmol Cl/kg dcs. In paired experiments where $[H]_o$ and $[Cl]_o$ were changed at constant product ($\Delta[H]_o$, $2.2 \times 10^{-8}$ mM to $8.9 \times 10^{-8}$ mM; $\Delta[Cl]_o$, 110–28 mM), there was no measurable net Cl flux. Given the above, it appears that $[H]_o/[H]_i = [Cl]_i/[Cl]_o$ in the *Amphiuma* red blood cell and as such $[H]_i$ can be determined from a knowledge of $[H]_o$, $[Cl]_i$, and $[Cl]_o$.

Thus, it is possible, by altering external K in the medium bathing A23187-treated cells, to change both $\Delta\tilde{\mu}_K$ and $\Delta\mu_K - \Delta\mu_H$. The relevant driving force for net K flux ($J_K^{A23187}$) can be determined from a knowledge of the magnitude and direction of $J_K^{A23187}$ and its correspondence to either $\Delta\tilde{\mu}_K$ or $\Delta\mu_K - \Delta\mu_H$.

Because $[Cl]_i/[Cl]_o = [H]_o/[H]_i$ in the *Amphiuma* red blood cells, the driving force for K/H exchange ($\Delta\mu_K - \Delta\mu_H$) is equal in magnitude and direction (sign) to that for K + Cl cotransport ($\Delta\mu_K + \Delta\mu_{Cl}$) (see Cala, 1983). Consequently, the observation that K flux corresponds to predictions of one of the above expressions does not in the absence of additional information establish the mode of transport. Since osmotically perturbed and A23187-treated *Amphiuma* red blood cells transport alkali metal ions with no net Cl transport (Figs. 2 and 4; Cala, 1980*a*), the notion that K flux is due to K + Cl cotransport is rejected in favor of a K/H exchange model.

To evaluate the correspondence between net K flux and $\Delta\tilde{\mu}_K$ or $\Delta\mu_K - \Delta\mu_H$, cells were osmotically swollen in medium containing 70 mM $[K]_o$ and 7 μM A23187 (Table IV). Under these conditions, $E_m = -4$ mV, whereas $E_K = -5$ mV ($[K]_i = 85$ mM) and the net force ($\Delta\tilde{\mu}_K$) driving K out of the cell via conductive routes is 1 mV (96 J $mol^{-1}$). In contrast to the above situation regarding conductive flux, given a chloride distribution ratio of 0.34, $[K]_o =$ 70 mM and $[K]_i = 85$ mM; the driving force acting upon an electroneutral K/H exchanger ($\Delta\mu_K - \Delta\mu_H$) is directed into the cell and equals −2,150 J $mol^{-1}$ (see Table IV). Measurements of the K content of cells treated as described above reveal a net (K) uptake of 96 ± 4(3) mmol K/kg dcs per 15 min (Table IV).

### *Separation of Conductive and Electroneutral Components of Ca-activated K Flux: Inference Based on the Effects of Cell Volume*

In a previous study (Cala, 1980*a*), cells in anisotonic and isotonic media were exposed to the K ionophore valinomycin. This study showed that volume-induced ion fluxes were without effect upon Val-induced membrane hyperpolarization and lead to the conclusion that the Val-induced K current (and therefore $G_K^{Val}$) was the same fraction of total membrane current in all cases. Given the above, it follows that $G_m$ is volume insensitive. Further, since the Val-induced net K flux was one to two orders of magnitude smaller than the volume-induced ion fluxes, I concluded that fluxes of the latter type are

TABLE IV

*Net K Flux Associated with Osmotically Swollen Amphiuma Red Blood Cells Exposed to 7 μM A23187 at External [K] = 3 or 70 mM: Correspondence Between Flux and Driving Force*

| Condition | $[K]_o$ | $J_K^{net}$ | Net driving force | |
|---|---|---|---|---|
| | | | $\Delta\tilde{\mu}_K$* | $\Delta\mu_K - \Delta\mu_H$‡ |
| | *mM* | *mmol/kg dcs × 15 min* | *J mol*$^{-1}$ | |
| RVD + A23187 | 3 | +101±6 (5)§ | +2,824 | +5,509 |
| RVD + A23187 | 70 | −96±4 (3) | +97 | −2,150 |

The data were obtained from cells osmotically swollen in the presence of 7 μM A23187 at external [K] = 3 or 70 mM. The intracellular [K] was equal to 85 ± 2 mM for both treatments, whereas $H_i/H_o$ was assumed to be equal to $[Cl]_o/[Cl]_i \cong 2.94$. At $[K]_o$ = 3 mM, $E_m$ was measured at −55.4 ± 3 (60), whereas at $[K]_o$ = 70 mM, $E_m$ was −4 mV ± 1.4 (40).

* Relevant driving force for conductive flux.

‡ Relevant driving force for K/H exchanger.

§ A positive value for flux denotes a net efflux, whereas a negative value denotes cellular uptake. Similarly, a negative value for driving force denotes a gradient directed from medium to cell, whereas a positive value signifies the cell-to-medium direction. Thus, under conditions where the net driving force for conductive K loss ($\Delta\tilde{\mu}_K$) is decreased by 97% ($K_o$ = 70 mM), net K flux is high and in the opposite direction of the electrochemical driving force. In contrast, the driving force for K/H exchange, ($\Delta\mu_K - \Delta\mu_H$) at $K_o$ = 70 mM, is relatively large and in the direction of the observed flux. The above data support the hypothesis that the bulk of Ca-induced K flux is electroneutral K/H exchange. Mean ± SEM (*n*).

TABLE V

*Membrane Potentials of Amphiuma Red Blood Cells in Isotonic and Anisotonic Ca-containing (0.5 mM) Media in the Presence of A23187 (1–7 μM)*

| Medium | $E_m$ |
|---|---|
| | *mV* |
| Isotonic | −55.1±2.2 (80)* |
| RVD (hypotonic) | −53.4±3 (62) |
| RVI (hypertonic) | −57.1±2.5 (67) |

* Mean ± SEM (*n*); *n* is the number of individual measurement based upon using cells from at least four different animals.

Although the ionophore concentration required to produce a K flux of a given magnitude was reproducible from one batch of cells to the next, this was not true with respect to the membrane voltage. In some batches of cells, 1 μM A23187 resulted in membrane hyperpolarization to −55 mV, whereas 7 μM A23187 was required in others. Within any given batch of cells (obtained from the same animal), however, the ionophore concentration required to produce a given $\Delta E_m$ was independent of cell volume. Thus, the membrane voltages presented were obtained by performing paired experiments upon cells from the same animals and exposing the cells to progressively higher concentrations of A23187 (in isotonic medium) until $E_m \cong -55$ mV. After the concentration of A23187 required to cause the membrane to hyperpolarize to −55 mV was found, the samples of the same batch of cells were suspended in hypo- and hypertonic media at the same ionophore concentration and $E_m$ measurements were performed. As shown in the table, A23187-induced membrane hypopolarization is volume independent.

electroneutral. The experiments presented in this section are similar to those described above in that they investigate the effects of A23187 upon cell membrane potential and ion fluxes across cells in isotonic and anisotonic media. As with the studies with valinomycin, exposure of cells to A23187 (1–7 $\mu$M) resulted in the same degree of membrane hyperpolarization, regardless of volume status (Table V). As shown in Figs. 2–4 and Table IV, the addition of A23187 to cells in isotonic and hypotonic media results in net K fluxes as large as 130 mmol/kg dcs per 15 min. In contrast, cells exposed to 7 $\mu$M A23187 at $Ca_o$ = 500 $\mu$M in hypertonic media lose only 9.3 ± 2 mmol K/kg dcs per 30 min (16 trials). The data in Table V show that the $E_m$ of A23187-treated cells is volume independent, whereas the data presented in Table III (discussed in the legend) indicate that the $G_m$ of A23187-treated cells is also volume insensitive. Since membrane voltage and conductance are the same for all cells exposed to A23187, regardless of volume, it must be concluded that membrane current is the same in all cases. More precisely, since A23187-induced membrane hyperpolarization is due to increased K current, $I_K$ must be the same in all cases. As such, the smallest measured net K flux associated with a given level of membrane hyperpolarization is the upper limit for conductive K loss; $zFJ_K^{smallest} \geq I_K$.

The results obtained in studies of cells exposed to A23187 during RVI support the notion that a large fraction of the ionophore-induced K flux is electroneutral. The data in Table IV show that the electroneutral K loss activated by A23187 is K/H exchange.

*The Relationship Between Na/H and K/H Exchange: Studies Using Amiloride*

The inability of A23187 to stimulate K loss from shrunken cells when Na/H exchange is operative raises the question of why activation of Na/H exchange during RVI should prevent ionophore-induced K/H exchange. One explanation is that both Na/H and K/H exchange are mediated by the same transport entity, which in response to events associated with osmotic perturbation and/or [Ca] is committed to function in either an Na/H or K/H exchange mode. If this hypothetical case is correct, amiloride may be useful in establishing the validity of such a relationship. As first shown by Siebens and Kregenow (1978), amiloride is a potent inhibitor of Na uptake during RVI, but it is without effect upon K loss during RVD. If indeed Na/H and K/H exchange are carried out by the same membrane transport component(s), it may be possible to "lock" the transporter into the Na/H mode by shrinking the cells in the presence of amiloride and thereby prevent K loss from cells subsequently swollen still in the presence of amiloride. The data obtained from this experiment are presented in Fig. 5. These data show that swelling cells in the presence of amiloride, after preincubation in isotonic amiloride-containing media, is without effect upon cell K loss (lower line, closed circles), as is swelling cells in amiloride-free medium after preincubation in hypertonic amiloride-containing medium (lower line, open circles). In contrast, if cells are preincubated in hypertonic amiloride-containing medium, followed by swelling in amiloride-containing medium, net cell K loss

(K/H exchange) is inhibited (upper line, open triangles). The hypertonic medium used in the above studies was made hypertonic by increasing [NaCl] to 1.5 times ($1.5R$) the values of control medium. Similar studies performed in progressively more hypertonic media (1.7 and $1.9R$) show that net cell K loss after resuspension in hypotonic amiloride-containing media was progressively inhibited (data not shown).

Taken together, the data associated with Fig. 5 and the observation that A23187 is not able to stimulate K/H exchange in cells where Na/H exchange is functioning support the hypothesis that Na/H and K/H exchange are alternative modes of the same transport moiety (Fig. 6).

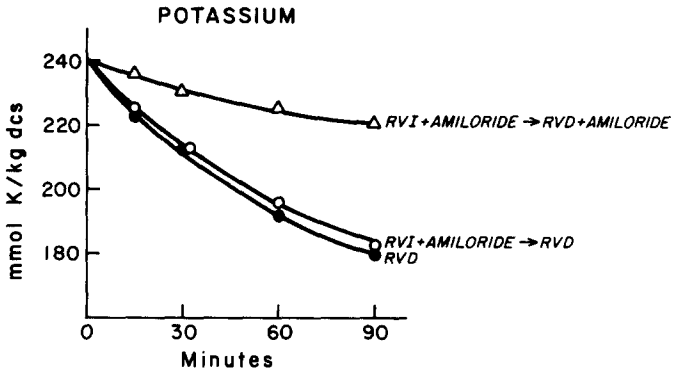


FIGURE 5. The K content of cells osmotically swollen by transfer (at time zero) from iso- to hypotonic medium (●); from hypertonic amiloride- ($10^{-4}$ M) containing medium to hypotonic medium (○); and from hypertonic amiloride- ($10^{-4}$ M) containing medium to hypotonic amiloride- ($10^{-4}$ M) containing medium (△). When cells are shrunken in amiloride-free medium followed by swelling in the absence of amiloride (not shown), net K loss proceeds normally during RVD.

## DISCUSSION

The data presented show that $Ca_i$ has a profound stimulatory effect upon net K flux. As initially shown by Gardos (1956, 1958) in his studies of metabolically depleted human red blood cells, Ca activates a K conductance pathway. Previous studies (Gardos et al., 1976; Lassen et al., 1976) have shown that *Amphiuma* red blood cells respond to increased cell Ca by exhibiting a K-dependent membrane hyperpolarization (increased $G_K$). Ion flux studies performed upon *Amphiuma* red cells exposed to high $[Ca]_o$ (Lassen et al., 1976) or to A23187, as in the present study, reveal K fluxes one to two orders of magnitude larger than expected based upon estimates of $G_m$. Since a previous work (Cala, 1980*a*) showed that the *Amphiuma* red blood cell is capable of electroneutral K loss during RVD, it seemed that the disparity between measured net K flux and predictions based upon $G_m$ might reflect K loss by both conductive and electroneutral routes. The most direct means of separating conductive and electroneutral components would be to measure

$G_m$ in the presence and absence of A23187 and determine whether A23187 induces an increase in $G_m$ large enough to support the observed K flux. Since this is not possible because of poor electrode-membrane sealing, less direct means were necessary (see Lassen and Rasmussen, 1977; Lassen, 1977; Cala, 1980*a*).

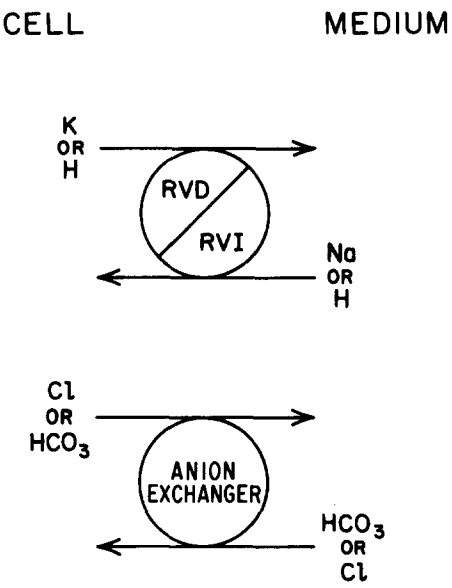


FIGURE 6. Current view of the alkali metal/H exchanger responsible for cell volume regulation. In a previous study (Cala, 1980*a*), Na/H and K/H exchange were assumed to occur by separate and distinct pathways; the data in the present study indicate that Na/H and K/H exchange are mediated by the same membrane transport component(s). The above conclusion is based upon the observation that (*a*) A23187 is unable to activate K/H exchange by shrunken cells when Na/H exchange is operative, and (*b*) amiloride, although unable to inhibit K/H exchange by swollen cells, is inhibitory to K/H exchange by cells previously shrunken in, and continuously exposed to, amiloride. Selectivity of the alkali metal/H exchanger for K or Na would appear to be determined by cytosolic reactions that occur as a result of swelling or shrinkage. The present studies suggest a central role for $Ca^{+2}$ in the activation of K/H exchange.

*Separation of Conductive and Electroneutral K Fluxes: Estimates of the Magnitude of $G_K^{A23187}$ Based on a Determination of $t_K$*

Although flux studies suggest that the Ca-activated K flux by *Amphiuma* red cells occurs primarily via electroneutral K/H exchange, more quantitative arguments can be made based upon electrophysiological studies. A previous study (Cala, 1980*a*) investigating valinomycin-induced $\Delta E_m$ and K flux established that the *Amphiuma* red blood cell $G_m \cong 10^6\ \Omega^{-1}\ cm^{-2}$ and that $G_m$ was invariant with volume (see also Lassen et al., 1974, 1976, 1978; J. F. Hoffman

et al., 1979). Since $t_K^{Val} = [G_K^{Val}/(G_K^{Val} + G_{other})]$, where $G_{other}$ is the membrane conductance to all other charge carriers and $t_K^{Val} = 0.5$ (Table III), $G_K^{Val} = G_{other}$. On the basis of measurements of $J_K^{Val}$ and $E_m^{Val}$, $G_K^{Val}$ is calculated to be $9.3 \times 10^{-7}$ $\Omega^{-1}$ cm$^{-2}$ and $G_m^{Val}$ $(G_K^{Val} + G_{other}) \cong 1.8 \times 10^{-6}$. Given estimates of $G_K$ for untreated membranes of $2.5 \times 10^{-7}$ $\Omega^{-1}$ cm$^{-2}$ (see also Lassen et al., 1978), the normal membrane conductance of the *Amphiuma* red blood cell membrane is of the order of $1.1 \times 10^{-6}$ $\Omega^{-1}$ cm$^{-2}$. On exposure to A23187 in Ca-containing medium, net K fluxes as large as 200 mmol/kg dcs in 15 min, which corresponds to a calculated K conductance of $5 \times 10^{-5}$ (assuming $zFJ_K^{A23187} = I_K^{A23187}$), have been observed. Thus, the calculated value of $G_K^{A23187}$ is over an order of magnitude greater than the $G_m$ of untreated cells. As a result, unless the conductance to some other ionic species is also increased such that $G_m^{A23187} \cong 10^{-4}$ $\Omega^{-1}$ cm$^{-2}$, the membranes will not support K flux (via conductive routes) of the magnitude observed in the presence of A23187.

If, as calculated from $J_K^{A23187}$, $G_K^{A23187} = 5 \times 10^{-5}$ $\Omega^{-1}$ cm$^{-2}$, and if we assume that $G_{other}$ is unchanged from the value calculated in experiments performed using Val ($9.3 \times 10^{-7}$ $\Omega^{-1}$ cm$^{-2}$), then, from Eq. 2, $t_K^{A23187}$ should equal 0.98. However, as seen in Table III, $t_K^{A23187} = 0.62$, as calculated from the maximum slope region ($[K]_o > 20$ $\mu$M) of the curve relating $E_m$ to $\log[K]_o$ (see also Lassen et al., 1974). This value of 0.62 suggests that $G_K^{A23187}$ is $<5 \times 10^{-5}$ and/or $G_{other}$ is $>9.3 \times 10^{-7}$. If in fact $G_K^{A23187} = 5 \times 10^{-5}$ $\Omega^{-1}$ cm$^{-2}$ (and/or $G_{other}$ is $>9.3 \times 10^{-7}$ $\Omega^{-1}$ cm$^{-2}$), then the addition of Val would be without electrical consequence since the additional K conductance ($G_K^{Val} - G_K$, where $G_K^{Val} = 9.3 \times 10^{-7}$ and $G_K = 2.5 \times 10^{-7}$) would be insignificant. Exposure of cells to both Val and A23187 caused $t_K$ (measured over the same range of $K_o$ and $E_m$) to increase from 0.62 to 0.86. Clearly, then, $G_K^{A23187}$ is $\ll 5 \times 10^{-5}$ $\Omega^{-1}$ cm$^{-2}$. Using $G_{other} = 9.3 \times 10^{-7}$ $\Omega^{-1}$ cm$^{-2}$ and $t_K^{A23187} = 0.62$, Eq. 2 can be solved for $G_K^{A23187}$. This solution is independent of assumptions regarding $J_K^{A23187}$ and yields a value of $1.5 \times 10^{-6}$ $\Omega^{-1}$ cm$^{-2}$ for $G_K^{A23187}$. Given $E_m^{A23187} \cong -55$ mV (Table V) and $G_K^{A23187} = 1.5 \times 10^{-6}$ $\Omega^{-1}$ cm$^{-2}$, the maximum value for A23187-induced, conductive K flux is $\cong 7$ mmol/kg dcs in 15 min. This value is consistent with (*a*) measured values of $E_m$ in the presence of Val and A23187, (*b*) inferences based upon studies evaluating $J_K^{A23187}$ in terms of $\Delta\tilde{\mu}_K$ and $\Delta\mu_K - \Delta\mu_H$, and (*c*) measured values of $E_m$ associated with and net K loss from osmotically shrunken, A23187-treated cells.

Although the above calculation of $G_K^{A23187}$ avoids assumptions regarding $J_K^{A23187}$, it relies upon the assumption that $G_{other}$ is not increased in the presence of A23187 (if $G_{other}$ is decreased, the arguments are strengthened). In regard to assumptions concerning the Ca insensitivity of $G_{other}$, if $G_K^{A23187} = 1.5 \times 10^{-6}$ $\Omega^{-1}$ cm$^{-2}$ and $G_{other} = G_K^{Val} = 9.3 \times 10^{-7}$ $\Omega^{-1}$ cm$^{-2}$, then $t^{A23187+Val}$ should equal 0.7. Since $t_K^{A23187+Val} = 0.86$, either the presence of both A23187 and Val leads to an augmentation of A23187 and Val effects upon $G_K$ or $G_{other}$ is decreased by A23187. Of the two possibilities, the latter seems to be the most probable explanation since addition of both Val and A23187 to *Amphiuma* red blood cells does not result in a significant increase in K flux, and, as reported by Low (1978), increased $[Ca]_i$ results in inhibition of anion exchange. Since the anion exchange and conductance appear to be intimately

related, the notion that Ca inhibits $G_{anion}$, and therefore $G_{other}$, is consistent with the present observation. If indeed this is the case, then the calculated value of $G_K^{A23187}$ ($1.5 \times 10^{-6}$ $\Omega^{-1}$ $cm^{-2}$) is an overestimate and the argument that conductive K loss contributes minimally to total A23187-induced K flux is strengthened.

Given the uncertainties regarding the Ca insensitivity of $G_{other}$, the calculated value of $G_K^{A23187}$ ($1.5 \times 10^{-6}$ $\Omega^{-1}$ $cm^{-2}$) should be viewed as an order of magnitude estimate. Since the arguments being made are aimed at distinguishing between possible values of $G_K^{A23187}$ that are separated by more than an order of magnitude, the uncertainty associated with calculated values of $G_K^{A23187}$ does not detract from the validity of the conclusions. Taken together, the data obtained in ion flux and electrophysiological studies are internally consistent. Using $1.5 \times 10^{-6}$ $\Omega^{-1}$ $cm^{-2}$ as an upper limit for A23187-induced K conductance, 7 mmol/kg dcs in 15 min is an upper-limit estimate for $J_K^C$. Inferentially, then, the bulk of the A23187-induced K flux is electroneutral.

*Separation of Electroneutral and Conductive Components of A23187-induced K Flux: A23187-treated, Osmotically Shrunken Cells*

Consistent with the above estimates of Ca-induced conductive K loss is the net K flux associated with osmotically shrunken cells during RVI. From the fact that Val is able to produce the same degree of membrane hyperpolarization (and Val-induced K flux) regardless of cell volume, I concluded that $G_m$ was volume insensitive (Cala, 1980*a*). This conclusion is confirmed and extended to include cells exposed to A23187 (Tables III and V, see legends). Given that $G_m$ is independent of cell volume and that $E_m$ in the presence of A23187 is invariant with volume, the A23187-induced potassium current must be the same for all cells regardless of volume. Since the net cell K loss from osmotically shrunken, A23187-treated cells is 9.3 ± 2 mmol K/kg dcs in 30 min, $I_K^{A23187}$ has as its maximum value $zF$ $9.3 \times 10^{-3}$ mol/$1.2 \times 10^7$ $cm^2$ × 1,800 s. That is, since $E_m^{A23187}$ and $G_m^{A23187}$ are independent of volume, $I_K^{A23187}$ is the same for all cells regardless of volume. As such, the smallest A23187-induced net K flux required to produce a given $\Delta E_m$ (in this case, the $\Delta E_m$ during RVI) is the upper limit for conductive K flux ($I_K^{A23187} \leq zFJ_K^{A23187}$). On the basis of estimates of $G_K^{A23187}$ from transference experiments (Table III), conductive K flux is estimated at 7 mmol/kg dcs in 15 min, whereas the estimates obtained using shrunken cells set $J_K^C$ as ≤9.3 mmol/kg dcs in 30 min.

The above estimates of conductive flux rate and magnitude are at the lower limit of K fluxes measured studying A23187-treated cells. It is reasonable to conclude that although A23187-induced K loss by *Amphiuma* red blood cells is due to flux via both conductive and electroneutral pathways, the latter is the primary contributor to measurable net flux.

*The Nature of A23187-induced K Flux: Identification of the Major Component as K/H Exchange, Based upon Thermodynamic Criteria*

When *Amphiuma* red blood cells are osmotically swollen in the presence of 7 μM A23187 at external [K] = 70 mM, $E_m = -4$ mV and $E_K = -5$ mV. The

driving force for conductive K flux ($E_m - E_K$) is small (1 mV) and is directed out of the cell. The magnitude (96 mmol K/kg dcs in 15 min) and direction (medium to cell) of net K flux under such conditions are clearly in opposition to predictions based upon the assumption that A23187-induced K flux is conductive (Table IV). The net K flux is, however, consistent with the predicted behavior of a K/H exchanger since the driving force for K/H exchange ($\Delta\mu_K - \Delta\mu_H$) is large (2,200 J $mol^{-1}$; 23 mV) and directed into the cell. In this regard, at $[K]_o = 70$ mM the ratio of $[K]_i/[K]_o$ is ~1.2, but the ratio $[H]_i/[H]_o$ is ~3. As such, the H gradient drives the secondary active, cellular uptake of K. That the dissipative transport of H is able to drive the conservative transport of K is strongly supportive of the notion that Ca-activated net K flux occurs via K/H exchange. It should be noted that the absolute magnitude of the driving force for K/H exchange ($\Delta\mu_K - \Delta\mu_H$) is decreased by 50% relative to conditions at $[K]_o = 3$, but the magnitude of the flux is not substantially decreased relative to cells suspended in medium where $[K]_o = 3$. Possible explanations for this are that (*a*) the K/H exchange is saturated at $|\Delta\mu_K - \Delta\mu_H| \geq 2{,}200$ J $mol^{-1}$, and/or (*b*) since as cells gain K and volume at $[K]_o = 70$ mM, the K/H exchanger is stimulated (kinetically) to transport K at higher rates.

The data presently available suggest that both explanations may apply because when external K is reduced to 1 mM, $\Delta\mu_K - \Delta\mu_H$ is increased from 5,000 to 8,000 J $mol^{-1}$ with no discernible effect upon the rate or magnitude of cell K loss. Clearly then, the K flux pathway is saturable at driving forces of >5,000 J $mol^{-1}$. With regard to the second point, as cells are swollen to progressively greater volume, $J_K^{net}$ is increased, even though, because of dilution of $[K]_i$, $\Delta\mu_K - \Delta\mu_H$ is decreased. Since at $[K]_o = 70$ mM the cells (already osmotically swollen and/or exposed to A23187) rapidly gain K and swell, the apparent insensitivity of K flux to the decreased magnitude of $\Delta\mu_K - \Delta\mu_H$ may be referable to superimposition of volume-related stimulation. In this regard, when cells are swollen in high K medium, but in the absence of A23187, the rate of net K uptake increases as cells gain K and therefore volume. Thus, the observed K uptake by cells exposed to 7 μM A23187 and 70 mM $[K]_o$ supports the notion that K uptake is a result of K/H exchange and is consistent with known responses of the volume-regulatory flux pathway (K/H) to changes in driving force and volume.

Finally, it is important to note that, on the basis of thermodynamic arguments, the distinction between conductive and electroneutral transport is complicated if one of the co/counter-ions participating in an electroneutral transport process is distributed at electrochemical equilibrium; for any and all ions i distributed at electrochemical equilibrium, $E_m = E_i$. Thus, although the force (in joules $mole^{-1}$) driving the flux of ion J by conductive routes = $\Delta\tilde{\mu}_J$ ($zFE_m - zFE_J$), the force (joules $mole^{-1}$) driving the obligatorily coupled electroneutral exchange flux of J for some equilibrium-distributed species i is $\Delta\mu_J - \Delta\mu_i = (zFE_i - zFE_J) = (zFE_m - zFE_J)$. As such, it is necessary to move the co/counter species away from electrochemical equilibrium ($E_m \neq E_i$) in order to distinguish between electroneutral coupled flux of species J and i

(for all species i that are normally distributed at electrochemical equilibrium) and the conductive flux of J. In the present study this criterion has been satisfied since the A23187-induced K conductance causes the membrane voltage to change away from its resting value so that $E_H \neq E_m$ and therefore $\Delta\tilde{\mu}_K \neq \Delta\mu_K - \Delta\mu_H$ (for a more detailed discussion see Cala, 1983).

*The Relationship Between Na/H and K/H Exchange: Studies Using Amiloride*

An intimate relationship between Na/H and K/H exchange is suggested by the fact that exposure of osmotically swollen cells and those in isotonic medium to A23187 results in large net K loss, although the ionophore produces only modest K loss from (shrunken) cells when the Na/H exchange is operative (Figs. 2 and 3 and Table V). That the failure of A23187 to stimulate K flux is not some peculiarity of ionophore/Ca interaction with osmotically shrunken cells is shown by the fact that although the ionophore-induced K flux is modest, the membrane hyperpolarization is not different from that of cells at normal or expanded volume. Since $G_m$ is volume insensitive, as shown previously (Cala, 1980*a*) and in the present study (see the legend to Table III), the decrease in ionophore-induced K flux is attributable to that by electroneutral (K/H) routes. The decreased rate of ionophore-induced K/H exchange in shrunken cells when the Na/H exchange is operating suggested that Na/H and K/H exchange might represent different operating modes of the same transport entity. Since amiloride is a potent inhibitor of Na/H exchange by *Amphiuma* red cells but has no effect upon K/H exchange (Cala, 1980*a*), this compound was used to evaluate the relationship between Na/H and K/H exchange. Further, support for the notion that Na/H and K/H exchange are mediated by the same transport pathway was obtained from studies of cells shrunken in amiloride-containing medium and subsequently swollen in the presence of amiloride (Fig. 5). It appears that by activating Na/H exchange, while binding it with amiloride, the transporter is prevented from exiting the Na/H mode and affecting K/H exchange upon swelling (if amiloride is continually present). In this regard, if the cells are shrunken in amiloride, washed (2 min), and immediately swollen in amiloride-containing medium, amiloride is without effect upon cell K loss. Similarly, preincubation in isotonic amiloride-containing medium followed by swelling in amiloride has no effect upon K loss. Amiloride inhibition of K loss by swollen cells requires (*a*) previous interaction between amiloride and the Na/H exchanger (shrunken cells) and (*b*) the continued presence of amiloride in the medium in which cells are swollen. Taken together, the observations that A23187 is unable to stimulate K/H exchange in shrunken cells when Na/H exchange is operative, and that amiloride is inhibitory to K/H exchange by swollen cells only when cells have previously been shrunk in amiloride, support the notion that Na/H and K/H exchange represent different modes of the same transport moiety.

In summary, the data presented show that Ca has a profound stimulatory effect upon K loss by *Amphiuma* red blood cells. Since Ca causes K-dependent changes in $E_m$, a component of K loss is conductive. However, on the basis

of kinetic similarities and the fact that electroneutral, volume-regulatory K loss is Ca dependent (Table II), a substantial fraction of Ca-activated K flux was thought to be electroneutral. Independent attempts to evaluate the contribution of conductive to total Ca-activated net K flux, based upon electrical, thermodynamic, and kinetic criteria, established 5–7 mmol K/kg dcs in 15 min as an upper limit for flux via conductive routes. Since Ca-activated net K flux can be as great as 200 mmol/kg dcs in 15 min, the major component is electroneutral. The correspondence of the Ca-activated K flux to expressions for the driving force relevant to K/H exchange (or K/OH cotransport) leads to the conclusion that the electroneutral K flux is referable to K/H exchange (K + OH cotransport). The data demonstrating that Ca is an activator of K/H exchange, taken together with the observation that removal of external Ca is inhibitory to K flux during RVD, suggest that Ca plays a role in activation and/or control of K/H exchange responsible for RVD. Finally, the inability of A23187 to activate K/H exchange by shrunken cells when Na/H exchange is operative, and the ability of amiloride to inhibit K/H exchange during RVD only when cells are previously shrunken in and continually exposed to amiloride, suggest a common pathway for both Na and K/H exchange.

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