

RESEARCH ARTICLE

Resting and action potentials under hypotonic conditions, unlike Na⁺ pump activity, depend only on the alteration of intracellular [Na⁺] and [K⁺] in frog skeletal muscle

Roque A. Venosa

Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 1900 La Plata, Argentina

rvenosa@aetos.med.unlp.edu.ar

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SUMMARY

It is well established that hypotonicity generates a marked and unexpected increase in active Na⁺ efflux in frog muscle fibers as well as in other cells like cardiac myocytes, astrocytes, brain synaptosomes and renal cells. The effect of hypotonicity on the electrical activity of skeletal muscle related to Na⁺ and K⁺ voltage-gated channels, however, has not been specifically addressed. The results of the present investigation show that the changes in resting and action potentials produced by hypotonicity can be fully explained by the reduction of intracellular [Na⁺] and [K⁺] due to the increase in cellular water content.

Key words: muscle, hypotonicity, membrane potentials.

INTRODUCTION

In frog skeletal muscles exposed to hypotonic media the active extrusion of Na⁺, at variance with the expected reduction due to the fall in its intracellular concentration ([Na⁺]_i), increases (Venosa, 1978). In this tissue, the effect is produced, apparently, by the incorporation of spare Na⁺/K⁺-ATPase units into the sarcolemma (Venosa, 1991; Venosa, 2003). The hypotonic stimulation of the sodium pump has also been observed in brain synaptosomes (Mongin et al., 1992; Aksensev, 1994), astrocytes (Mongin et al., 1994), cardiomyocytes (Walley et al., 1993; Bewick et al., 1999) and renal cells (Coutry et al., 1994). In skeletal muscle, at least, the effect is triggered by the stretching of the cell membrane caused by the swelling resulting from exposure to hypotonic media.

It was thought that if, in frog muscle, hypotonicity produces changes in the voltage-gated Na⁺ and K⁺ channels involved in the excitation process, akin to those elicited in Na⁺/K⁺ active transport, then the action potential (AP) and the corresponding extra Na⁺ influx (J_i^{Na}) would also be affected under hypotonic conditions. The results of the present study show that the fall in [Na⁺]_i and intracellular K⁺ concentration ([K⁺]_i), produced by the rise in cell water content when in hypotonic media, does not alter J_i^{Na} and is sufficient to fully account for the observed changes in resting potential (V_m) and AP.

MATERIALS AND METHODS

Experiments were performed on isolated frog (*Leptodactylus ocellatus* L.) paired sartorius muscles. Animals were maintained and the experiments were conducted in accordance with the guidelines of the local Ethics Committee, which are similar to those of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996). Before dissection, the animals were chilled in an ice-water mixture until fully immobile and then double pithed.

The normal Ringer solution had the following composition (mmol l⁻¹): NaCl 115; KCl 2.5; CaCl₂ 1.8; Na₂HPO₄ 2.15; and

NaH₂PO₄ 0.85 (pH 7.18). The reference isotonic medium (Π_1) was similar to the normal saline except that 62 mmol l⁻¹ NaCl, one-half of the total osmolarity, was replaced by an osmotically equivalent concentration of sucrose as determined using a vapor pressure osmometer (Wescor model 5100C, Wescor Inc., Logan, UT, USA). The hypotonic media (no sucrose added) had an osmotic pressure one-half ($\Pi_{0.5}$) that of Π_1 and the same ionic composition. In some experiments Cl⁻ was replaced by SO₄²⁻. The electrical measurements were made using conventional glass microelectrodes filled with 3 mol l⁻¹ KCl with a resistance of 10–15 M Ω and coupled to a high input impedance electrometer (WPI, New Haven, CT, USA), whose output was recorded online by a data acquisition system (Power Lab/410, AD Instruments, Sydney, Australia) connected to a personal computer. The effects of hypotonicity were studied in muscles exposed to $\Pi_{0.5}$ for 1.5 h. During that period, no regulatory volume decrease (RVD) was observed. In fact, in our experience, RVD was not observed during much longer exposures to $\Pi_{0.5}$ either. In some experiments the AP and its time derivative (obtained with a function module; Frederic Haer & Co., Brunswick, ME, USA) were directly recorded in a digitizing oscilloscope with screen memory (Tektronix model 5223, Beaverton, OR, USA). To diminish the twitch movement and the subsequent dislodgment of the microelectrode, muscles with the inner face up were stretched (20%) and a small bundle of fibers were stimulated externally using a thin tungsten electrode. APs were elicited with rectangular pulses lasting 1 ms, delivered by a stimulator (Grass model S48, Quincy, MA, USA) through a Grass isolation unit (model SIU 5).

Determination of the extra Na⁺ influx per impulse (J_i^{Na}) with ²²Na⁺ (New England Nuclear, Boston, MA, USA) was done using a technique previously described (Venosa, 1974; Kotsias and Venosa, 2001).

Student's *t*-test was used to estimate the statistical significance of differences. Values are expressed as means \pm s.e.m.

RESULTS
The resting potential

In hypotonic medium the equilibrium potential of Na⁺ and K⁺ (E_{Na} , E_K) as well as V_m are altered because of the decrease of $[K^+]_i$ and $[Na^+]_i$. V_m is also strongly dependent on the extracellular K⁺ concentration ($[K^+]_o$), particularly at values greater than 10 mmol l⁻¹. In frog muscle fibers, the relationship between V_m and the concentration of Na⁺ and K⁺ is expressed by Eqn 1 (Hodgkin and Horowicz, 1959):

$$V_m = (RT/F) \ln(([K^+]_o + \alpha[Na^+]_o) / [K^+]_i), \quad (1)$$

where α represents the ratio of Na⁺ and K⁺ permeabilities ($\alpha = P_{Na}/P_K$), which under normal conditions is of the order of 0.01–0.02. R , T and F have their usual meanings. As $[K^+]_o$ increases (>10 mmol l⁻¹), $\alpha[Na^+]_o$ becomes negligible compared with $[K^+]_o$ and can be suppressed in Eqn 1. Moreover, at 20°C, and using log instead of ln, Eqn 1 becomes:

$$V_m = 58 \log[K^+]_o - 58 \log[K^+]_i, \quad (2)$$

which expresses a linear relationship between V_m and $\log[K^+]_o$ where 58 mV is the slope (S) of the line for an ideal K⁺ electrode. The measurement of V_m at different $[K^+]_o$, under isotonic and hypotonic conditions was done in Cl⁻-free media (Cl⁻ replaced by SO₄²⁻) to avoid Cl⁻ transients upon solution changes (Hodgkin and Horowicz, 1959) and assuming $[K^+]_i$ is constant. To keep the osmolarity constant, the increments in $[K^+]_o$ were made by equimolar reductions of extracellular Na⁺ concentration ($[Na^+]_o$). Fig. 1 shows the plot of V_m as a function of $\log[K^+]_o$ in both isotonic (Π_1) and hypotonic medium ($\Pi_{0.5}$). The linear fitting of the data corresponds to a relationship of the form $V_m = S \log[K^+]_o - S \log[K^+]_i$ ($r = 0.999$ and 1.000 for Π_1 and $\Pi_{0.5}$, respectively). Under both conditions the fibers behave similar to K⁺ electrodes with S values of 55.2 and 55.7 mV for Π_1 and $\Pi_{0.5}$, respectively. On the other hand, the extrapolation to V_m of 0 mV, which should occur when $[K^+]_o = [K^+]_i$, yielded values of 151.7 mmol l⁻¹ for Π_1 and 93.0 mmol l⁻¹ for $\Pi_{0.5}$.

Eqn 1 can be rearranged so that:

$$e^{V_m F/RT} = [K^+]_o (1 / [K^+]_i) + \alpha [Na^+]_o / [K^+]_i, \quad (3)$$

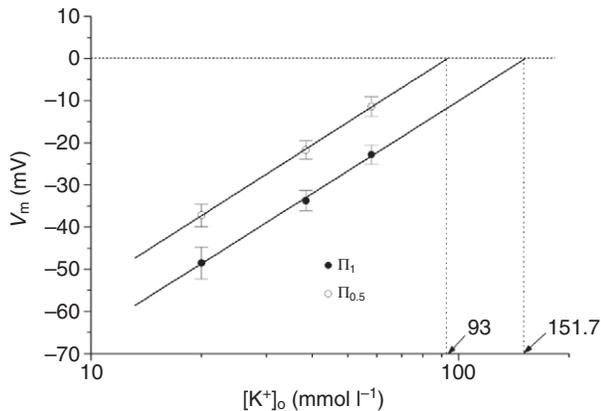


Fig. 1. Resting membrane potential (V_m , in mV) as a function of $\log[K^+]_o$ in muscles equilibrated in reference isotonic medium (Π_1) and hypotonic medium with an osmotic pressure one-half that of Π_1 ($\Pi_{0.5}$). Each experimental point represents the mean (\pm s.e.m.) of between 34 and 39 fibers. The linear fitting yielded slopes of 55.2 mV ($r = 0.999$) for Π_1 and 55.7 mV ($r = 1.000$) for $\Pi_{0.5}$, values which are close to the theoretical 58 mV for a K⁺ electrode. The extrapolation to $V_m = 0$ mV, where $[K^+]_o = [K^+]_i$, indicates $[K^+]_i = 151.7$ mmol l⁻¹ for Π_1 and $[K^+]_i = 93$ mmol l⁻¹ for $\Pi_{0.5}$.

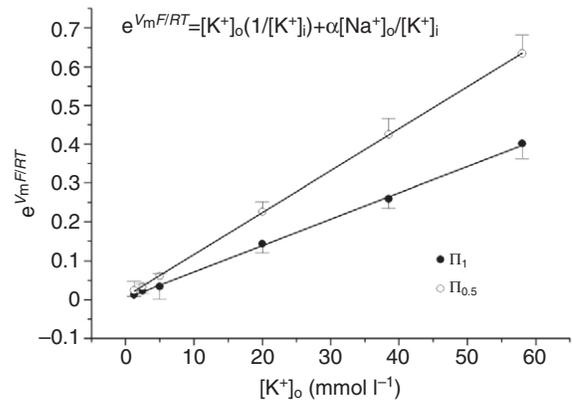


Fig. 2. $e^{V_m F/RT}$ as a function of $[K^+]_o$ in the presence of both Π_1 and $\Pi_{0.5}$. It can be seen that the relationship is fairly linear in accordance with Eqn 3 (see text) in the $[K^+]_o$ range 1.25–58 mmol l⁻¹. The slope of the lines ($1/[K^+]_i$) provides a measure of $[K^+]_i$: 146.4 mmol l⁻¹ for Π_1 and 92.6 mmol l⁻¹ for $\Pi_{0.5}$. Each experimental point represents the mean (\pm s.e.m.) of between 12 and 39 fibers.

where $[K^+]_o$ is the independent variable, $1/[K^+]_i$ is the slope and $\alpha[Na^+]_o/[K^+]_i$ is a constant given by $e^{V_m F/RT}$ when $[K^+]_o = 0$. Fig. 2 shows the plot of the data for $[K^+]_o$ between 1.25 and 58 mmol l⁻¹ for both Π_1 ($r = 0.999$) and $\Pi_{0.5}$ ($r = 0.0994$). The linear fitting of the data yielded $[K^+]_i$ values of 146.4 mmol l⁻¹ (Π_1) and 92.6 mmol l⁻¹ ($\Pi_{0.5}$), which are not significantly different from those calculated from the plot in Fig. 1. On the other hand, for $[K^+]_o = 0$ the first term on the right-hand side of Eqn 3 vanishes and the magnitude of α can be easily calculated. Thus for Π_1 , $\alpha = 0.0063$ and for $\Pi_{0.5}$ $\alpha = 0.0113$. These values are not too far from the value of 0.01 reported by Hodgkin and Horowicz (Hodgkin and Horowicz, 1959).

The AP

In skeletal muscle, as in most excitable cells, the peak of the AP reaches a value not too far from that of E_{Na} because of the marked and transient increase of P_{Na} during its rising phase. It seems reasonable to assume that the ratio ($[K^+]_i$ in Π_1)/($[K^+]_i$ in $\Pi_{0.5}$) provides an estimate of the increment in fiber water content in $\Pi_{0.5}$. Taking the averaged values of $[K^+]_i$ from Eqns 1 and 2, i.e. 149 mmol l⁻¹ in Π_1 and 92.8 mmol l⁻¹ in $\Pi_{0.5}$, and assuming $[Na^+]_i = 15$ mmol l⁻¹ in Π_1 , similar to that in NR (Venosa and Horowicz, 1973), a $[Na^+]_i$ of 9.3 mmol l⁻¹ [=15(92.8/149)] can be estimated for fibers equilibrated in $\Pi_{0.5}$. This further indicates an increase in cell water of about 60% in $\Pi_{0.5}$ relative to Π_1 (149/92.8=1.61), which is similar to that found previously in the same preparation (Venosa, 2003). With these values of $[Na^+]_i$, the calculated E_{Na} , would be 34.1 mV [=58 log(58.2/15)] in Π_1 and 46.2 mV [=58 log(58.2/9.3)] in $\Pi_{0.5}$. The mean peak AP (pAP) in Π_1 was 19.9 mV while in $\Pi_{0.5}$ it was 27.9 mV, close to the value of 29.4 mV measured in the presence of NR where the ratio $[Na^+]_o/[Na^+]_i$, and therefore E_{Na} , is close to that in fibers equilibrated in $\Pi_{0.5}$ (see Table 1). It is known that the maximum value of dV_m/dt ($dV_{m,max}/dt$) during the upstroke of the AP is an expression of the inward Na⁺ current (I_{Na}) during that period. As can be seen in Table 1, it amounted to 417 V s⁻¹ in NR while in Π_1 and $\Pi_{0.5}$ it was significantly lower. It can also be appreciated that the difference between the values of this parameter in Π_1 and $\Pi_{0.5}$ is not significant. This is not surprising because I_{Na} , and therefore $dV_{m,max}/dt$, is directly proportional to $E_{Na} - V_m$, the driving force (DF) on Na⁺. Although, as a result of intracellular Na⁺ dilution, E_{Na} is greater in $\Pi_{0.5}$ than

Table 1. Action potential data

	V_m (mV)	E_{Na} (mV)	pAP (mV)	dV_m/dt ($V s^{-1}$)	DF* (mV)	f/m
NR	-91.4 ± 6.9	52.4	29.4 ± 5.8	417 ± 5.6	143.8	37/4
Π_1	-89.7 ± 6.8	34.1	19.9 ± 6.1	306 ± 7.2	123.8	102/7
$\Pi_{0.5}$	-76.9 ± 7.0	46.2	27.9 ± 6.6	286 ± 6.3	123.1	35/5

*To calculate $DF = E_{Na} - V_m$ it was assumed that $[Na^+]_i = 15 \text{ mmol l}^{-1}$ for Π_1 and 9.3 mmol l^{-1} for $\Pi_{0.5}$ (see text).

V_m , membrane potential; E_{Na} , equilibrium potential of Na^+ ; pAP, peak action potential; DF, driving force; f/m , number of fibers/number of muscles; NR, normal Ringer solution; Π_1 , reference isotonic medium; $\Pi_{0.5}$, hypotonic medium.

in Π_i , it is also true that V_m , because of intracellular K^+ dilution, is less negative, so that the DF is virtually the same under the two conditions (Table 1). Fig. 3 shows representative APs and their time derivative of fibers equilibrated in NR, Π_1 and $\Pi_{0.5}$.

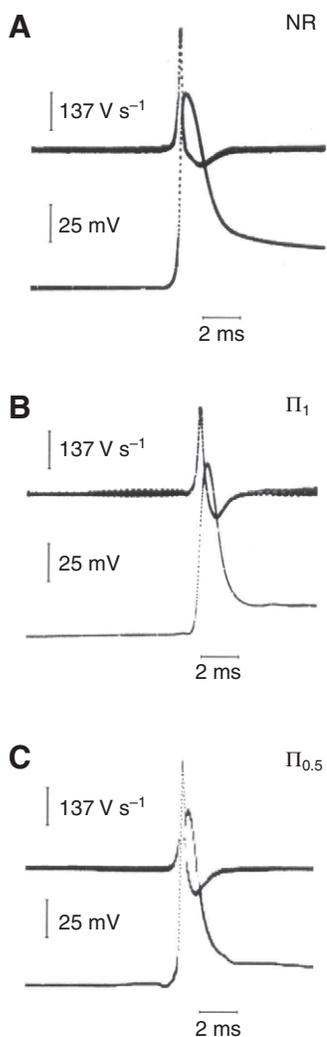


Fig. 3. Representative records of action potentials (APs) and their time derivatives in three different fibers equilibrated in normal Ringer solution (NR) (A), Π_1 (B) and $\Pi_{0.5}$ (C). In each record the lower curve corresponds to the AP and the upper one to its time derivative. The base line of the derivative record ($dV_m/dt=0$) coincides with the 0 mV of the membrane potential record ($V_m=0$). The upper calibration bar corresponds to dV_m/dt and the lower one to V_m .

The determination of the extra Na^+ influx per AP (J_i^{Na}) yielded mean values of $3.31 \pm 0.88 \text{ nmol g}^{-1} \text{ AP}^{-1}$ ($N=8$) in Π_1 and $3.47 \pm 0.69 \text{ nmol g}^{-1} \text{ AP}^{-1}$ ($N=6$) in $\Pi_{0.5}$, which is in good agreement with the measurements of $dV_{m,max}/dt$.

DISCUSSION

In frog muscle (Venosa, 1978; Venosa, 1991; Venosa, 2003) as well as in several other cell types, hypotonicity produces a marked and unexpected increase in the active extrusion of Na^+ . The aim of the present experiments was to find out how the basic electrical properties of muscle fibers are affected under similar conditions. As described above, hypotonicity depolarizes the resting potential in a predictable fashion, mainly due to the fall in $[K^+]_i$ and practically no change in the value of the P_{Na}/P_K ratio in $\Pi_{0.5}$ with respect to that in Π_1 .

The increase in the activity of the Na^+ pump, which is electrogenic, does not measurably affect V_m . The reason for this is as follows. The increase in active K^+ influx produced by the Π_1 to $\Pi_{0.5}$ transfer is of the order of $0.7 \text{ pmol cm}^{-2} \text{ s}^{-1}$ (Venosa, 1991). Given the stoichiometry of the pump is $3 Na^+/2 K^+$, the corresponding increase in active Na^+ transport would be $0.7 \times 3/2 = 1.05 \text{ pmol cm}^{-2} \text{ s}^{-1}$; that is, a net outward transfer of $1.05 - 0.70 = 0.35 \text{ pmol cm}^{-2} \text{ s}^{-1}$ or a current density of $0.35 \times 10^{-12} \text{ mol cm}^{-2} \text{ s}^{-1} \times 96500 \text{ C mol}^{-1} = 3.4 \times 10^{-8} \text{ A cm}^{-2}$. Assuming a membrane resistance of $4000 \Omega \text{ cm}^2$ (Katz, 1966), this current density would produce a hyperpolarization of only $3.6 \times 10^{-8} \text{ A cm}^{-2} \times 4000 \Omega \text{ cm}^2 = 0.14 \text{ mV}$.

With regard to the AP parameters, the magnitude of pAP, which strongly depends on E_{Na} , increased in $\Pi_{0.5}$ with respect to its value in Π_1 , in a predictable manner according to the fall in $[Na^+]_i$ in $\Pi_{0.5}$. On the other hand, the magnitude of $dV_{m,max}/dt$, an expression of the inward I_{Na} during the upstroke of the AP, in $\Pi_{0.5}$ was not different from that in Π_1 , because the swelling in $\Pi_{0.5}$, and the consequent fall of both $[Na^+]_i$ and $[K^+]_i$, produced virtually no change in the DF. This is supported by the fact that J_i^{Na} in $\Pi_{0.5}$ ($3.47 \pm 0.69 \text{ nmol g}^{-1} \text{ AP}^{-1}$) was not different from that in Π_1 ($3.31 \pm 0.88 \text{ nmol g}^{-1} \text{ AP}^{-1}$). In this regard it is worth mentioning that when J_i^{Na} is expressed in terms of the superficial sarcolemma [$430 \text{ cm}^2 \text{ g}^{-1}$ (Venosa, 1991)], we have 7.70 and $8.07 \text{ pmol cm}^{-2} \text{ AP}^{-1}$ for Π_1 and $\Pi_{0.5}$, respectively. These values, in what might be the result of a species difference, are about one-third of those previously determined in sartorii from *Rana pipiens* ($552 \text{ cm}^2 \text{ g}^{-1}$) in the presence of $60 \text{ mmol l}^{-1} [Na^+]_i$ (Venosa, 1974).

In conclusion, it is interesting to note that, in frog muscle, while hypotonicity generates a series of changes in active Na^+ transport, involving an increase in the membrane density of Na^+ pumps, apparently through the insertion of spare pumps in the sarcolemma mediated by actin filaments of the cytoskeleton (Venosa, 2003), no changes of that sort seem to occur to the voltage-gated Na^+ channels. Instead, the observed changes in V_m and AP promoted by

hypotonicity can be fully explained by the reduction of $[\text{Na}^+]_i$ and $[\text{K}^+]_i$ due to the increase in cell water content.

LIST OF SYMBOLS AND ABBREVIATIONS

AP	action potential
DF	driving force on ion x ($E_x - V_m$)
E_x	equilibrium potential of ion x
J_1^{Na}	extra Na^+ influx per AP
NR	normal Ringer solution
P_x	permeability of ion x
pAP	peak AP
V_m	resting potential
$\Pi_{0.5}$	hypotonic medium ($\Pi_1/2$)
Π_1	isotonic medium

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REFERENCES

- Aksensev, S. T., Mongin, A. A., Orlov, S. N., Racovich, A. A., Kaler, G. V. and Konev, S. V. (1994). Osmotic regulation of sodium pump in the rat brain synaptosomes: the role of the cytoplasmic sodium. *Brain Res.* **644**, 1-6.
- Bewick, N. L., Fernández, C., Pitt, A. D., Rasmussen, H. H. and Whalley, D. W. (1999). Mechanisms of $\text{Na}^+\text{-K}^+$ pump regulation in cardiac myocytes during hyposmolar swelling. *Am. J. Physiol.* **276**, C1091-C1099.
- Coutry, N., Farman, N., Bonvalet, J. P. and Blot-Chabaud, M. (1994). Role of cell volume variations in $\text{Na}^+\text{-K}^+\text{-ATPase}$ recruitment and/or activation in cortical collecting duct. *Am. J. Physiol.* **266**, C1342-C1349.
- Hodgkin, A. L. and Horowitz, P. (1959). The influence of potassium and chloride ions on the membrane potential on single muscles fibres. *J. Physiol.* **148**, 127-160.
- Katz, B. (1966). *Nerve, Muscle and Synapse*. New York: McGraw-Hill, Inc.
- Kotsias, B. A. and Venosa, R. A. (2001). Sodium influx during action potential in innervated and denervated rat skeletal muscles. *Muscle Nerve* **24**, 1026-1033.
- Mongin, A. A., Aksensev, S. L., Rakovich, A. A., Okun, I. M., Konev, S. V. and Orlov, S. N. (1992). Osmotic regulation of the sodium pump in rat brain synaptosomes. *Biophysics* **37**, 847-852.
- Mongin, A. A., Aksensev, S. L., Orlov, S. N., Slepko, N. G., Kozlova, M. V., Maximov, G. V. and Konev, S. V. (1994). Swelling-induced K^+ influx in cultured primary astrocytes. *Brain Res.* **655**, 110-114.
- Venosa, R. A. (1974). Inward movement of sodium ions in resting and stimulated frog's sartorius muscle. *J. Physiol.* **241**, 155-173.
- Venosa, R. A. (1978). Stimulation of the Na^+ pump by hypotonic solutions in skeletal muscle. *Biochim. Biophys. Acta* **510**, 378-383.
- Venosa, R. A. (1991). Hypo-osmotic stimulation of active Na^+ transport in frog muscle: Apparent upregulation of Na^+ pumps. *J. Membr. Biol.* **120**, 97-104.
- Venosa, R. A. (2003). Hypotonic stimulation of the Na^+ active transport in frog skeletal muscle: role of the cytoskeleton. *J. Physiol.* **548**, 451-459.
- Venosa, R. A. and Horowitz, P. (1973). Effects on sodium efflux of treating frog's sartorius muscles with hypertonic glycerol solutions. *J. Membrane Biol.* **14**, 33-56.
- Whalley, D. W., Hool, L. C., Ten Eick, R. E. and Rasmussen, H. H. (1993). Effect of osmotic swelling and shrinkage on $\text{Na}^+\text{-K}^+$ pump activity in mammalian cardiac myocytes. *Am. J. Physiol.* **265**, 1201-1210.