INDIFFERENCE OF THE RESTING POTENTIAL OF FROG MUSCLE CELLS TO EXTERNAL Mg^{++} IN THE FACE OF HIGH Mg^{++} PERMEABILITY

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• ***Incubation of frog sartorius muscles for 18 hours at constant external concentrations of K^+ and Na^+, but with the external concentration of Mg^{++} varying from 1.2 to 73.2 mM, brought about no change in the resting potential which remained constant at 88 mV. Results were the same in $MgCl_2$ as in $MgSO_4$. However, these cells are nearly as permeable to Mg^{++} as they are to K^+. These results contradict the membrane theory of the cellular potential but are compatible with the surface adsorption model of the cellular potential.***

INTRODUCTION

A number of theories of the nature of the cellular electrical potential exist. Among them are the membrane potential theory of Hodgkin and Katz, first presented in 1949,[1] and the surface adsorption potential theory, a part of the association-induction hypothesis presented first briefly in 1955[2] and later in greater detail in 1960,1962, 1982, and 1983.[3-7] Considerable experimental work has already been reported in the literature aimed at testing the validity of these theories and will be discussed later in this paper. This paper will present the results of yet another investigation designed to evaluate these theories, specifically to test the relation between permeability of the ion, Mg^{++}, and its effect on the resting potential.

THEORY

The Membrane Pump Theory of Hodgkin and Katz. Details of this well-known theory will not be reiterated here. Briefly, this theory of cellular potential is a modification of the membrane potential theory which was first clearly defined by Bernstein.[8] In Bernstein's original version of the theory, the cellular potential is an equilibrium potential; its maintenance does not demand continuous energy expenditure ***per se.*** The discovery that Na^+, long thought to be impermeant, is actually a permeant cation[9,10] renders this version of the membrane theory untenable. Partly to overcome this difficulty and partly to incorporate their important discovery of the key role of Na' in the creation of the action potential,[11] Hodgkin and Katz presented their own conception of the cellular potential. Although it retained the basic characteristics of the membrane potential, it was no longer an equilibrium potential[1] and its maintenance depended on the continuous operation of an energy-consuming outwardly directed Na^+ pump.[12,13]

The Hodgkin-Katz model of the cellular potential is described by the following equation:

$$\psi = \frac{RT}{F} \ln \frac{P_K[K^+]_{in} + P_{Na}[Na^+]_{in} + P_{Cl}[Cl^-]_{ex}}{P_K[K^+]_{ex} + P_{Na}[Na^+]_{ex} + P_{Cl}[Cl^-]_{in}}, \quad (1)$$

where P_K, P_{Na}, and P_{Cl} are the membrane permeability constants of the respective ions; $[K^+]_{in}$, $[Na^+]_{in}$, and $[Cl^-]_{in}$ are their intracellular concentrations; and $[K']_{ex}$, $[Na^+]_{ex}$, and $[Cl^-]_{ex}$ are their extracellular concentra-

tions.' R, F, and T are the gas constant, the Faraday constant, and the absolute temperature, respectively.

The basic tenet of the membrane theory demands that all particles carrying net electrical charges and capable of traversing the cell membrane contribute to the magnitude and polarity of the electrical potential. Thus, ideally, the equation for the cellular potential should incorporate all permeant ions found inside and outside the cells under the condition in which the potential is measured. The Hodgkin-Katz equation, however, deals explicitly with only three ions: K, Na^+, and Cl^-. The ubiquitous divalent Mg'' and Ca'' cations were not included in this equation. Presumably, this omission was justified on the grounds that the intracellular and extracellular concentrations of Mg'' and Ca^{++} were lower than those of K^+, Na', and Cl^- and on the assumption that these alkaline earth ions were also less permeable than K^+, Na^+, and Cl^-. However, unequivocal evidence that the divalent ions have a much lower permeability is not available; indeed, evidence exists showing just the opposite. Thus Tasaki, Teorell, and Spyropoulos demonstrated that in perfused squid axons the time constant of Ca'' efflux was only 25 minutes while that of K' efflux was 8 hours.[14] In other words, the membrane permeability is many times higher for Ca'' than for K! Moreover, the earlier conclusion that Mg^{++} permeability is very low[15] was challenged when Ling et al[16] showed that the gain of Mg^{++} in frog muscle in a high Mg^{++}-Ringer solution was complete within 20 min. even though some of the muscles used were as thick as 2mm in diameter.

In order to test the Hodgkin-Katz model, unequivocal knowledge about Mg^{++} permeability is essential. One purpose of the present investigation is to acquire such information. If Mg^{++} should prove to be as permeable as, for example, K^+, the membrane potential theory would predict a substantial lowering of the resting potential or even reversal of the sign of the potential when there is a large increase in external Mg^{++} concentration.

The Adsorption Potential Theory According to the Association-Induction Hypothesis. According to the association-induction hypothesis, the bulk of intracellular K' is adsorbed on the β- and γ-carboxyl groups of intracellular proteins[4,7,17] and is not involved in the generation of the electrical potential. Instead, the potential is determined by the β-and γ-carboxyl groups found on a microscopically thin layer of the cell surface and by their preferentially adsorbed ions.[2-4,6,7]

The Resting Potential. In this theory, the cellular resting potential is an equilibrium potential, the maintenance of which does not demand a continuous expenditure of energy. Written in the simplest form possible, the cellular electrical potential, ψ, is described by the equation:

$$\Psi = \frac{RT}{F} \ln [f^-] - \frac{RT}{F} \ln \{ \tilde{K}_K [K^+]_{ex} + \tilde{K}_{Na} [Na^+]_{ex} \}, \quad (2)$$

where [f] is the concentration of fixed anions on the microscopic layer of the cell surface and $\tilde{K}_K$ and $\tilde{K}_{Na}$ are the adsorption constants for K^+ and Na^+, respectively, on the β- and γ-carboxyl groups on the cell surface.[2-4] As mentioned earlier, this equation was first presented in 1955. A further theoretical development of this model was given in 1979,[18] and its experimental support presented in 1982 and 1983.[6,7,19]

Characteristics and Predictions.

1. There is no causal relationship between ionic permeabilities and cellular electrical potentials.

2. No macroscopic interface separating the membrane from the cytoplasm has been recognized. Instead, only one discrete interface that separates the cell surface from the surrounding medium is considered to exist. However, this is not to say that another, artificial, interface may not be created, such as by the removal of cytoplasm; in this case, little or no potential difference at this artificial interface is expected if the exposed surface is amphoteric, containing roughly equivalent concentrations of fixed cations and fixed anions, and if the pH is near the isoionic point.[6,7]

3. The major determinants of the potential are the nature and density of fixed anionic sites on the cell surface and the concentrations of ions in the surrounding medium that can adsorb to these fixed anionic sites.

Experimental evidence exists showing that the predominant fixed charges on the surface of frog muscle cells are indeed the β- and γ-carboxyl groups carried by the aspartic and glutamic residues of proteins:

a. The K' adsorbing sites of the muscle cell surface have a pK value of 4.6, which is characteristic of the β- and γ-carboxyl groups.[20]

b. Recently, with the aid of transmission electron microscopy[21] autoradiography,[22,23] dispersive x-ray microprobe analysis,[24-26] and laser mass spectrometer microprobe analysis (LAMMA),[27] evidence has been presented that cytoplasm K' adsorption sites are β- and γ-carboxyl groups. These are the same sites that in fixed cell preparations bind uranium ions (see below), and we suggest that uranium staining at the cell surface mirrors surface β- and γ-carboxyl groups as well.

There is reason to believe that ionic preference in adsorption varies not only with the nature of the fixed anionic groups and their electron density (see reference 4), but also with their spatial distribution in relation to other fixed anions. Thus, isolated carboxyl groups seem to prefer alkali-metal ions (Cs^+, Rb^+, K^+, Na^+, Li^+) over the divalent ions Mg^{++}, Ca^{++}, and Sr^{++}. As an example, oxidized collodion, which possesses a relatively low concentration of carboxyl groups,[28] shows little or no tendency to adsorb alkaline earth ions; it does adsorb alkali-metal ions avidly.[29]

On the other hand, when fixed carboxyl groups occur in closely placed pairs or clusters, increasing the probability of chelation, preference of the carboxyl groups for the alkaline earth cations supersedes that for the alkali-metal ions. As an example, ion-exchange resins with a very high density of carboxyl groups prefer Ca^{++} and Mg^{++} over the alkali-metal ions.[30]

We may expect therefore that a high external concentration of Mg^{++} may or may not have an effect on the muscle cell resting potential, depending on whether the β- and y-carboxyl groups on the muscle cell surface exist singly or in pairs or clusters. Fortunately, this uncertainty was resolved by the earlier experimental finding that the pK value of the anionic sites of the frog muscle surface is 4.6, the same as that of "solitary" β- and γ-carboxyl groups.[19] If a significant fraction were in pairs or clusters, the pK would have been correspondingly higher.[31,32] These considerations led us to conclude that the surface β- and γ-carboxyl groups are indeed solitary and show little preference for Mg^{++}. As a result, the prediction of the association-induction hypothesis is diametrically opposed to that of the membrane pump theory of Hodgkin and Katz, i.e., the resting potential is expected to be insensitive to variations of external Mg^{++} concentration.

MATERIALS AND METHODS

All experiments were carried out on the isolated sartorius muscles of the North American leopard frog (Rana *pipiens,*

pipiens, Schreber) from Vermont and occasionally from New Jersey. ^{28}Mg was obtained from Brookhaven Laboratory, New York (Lot 121779-7).

Resting Potential Measurements

Resting potentials of the sartorius muscles were measured using the method described by Ling and Gerard.[33]

Composition of Mg^{++}-Ringer Solutions

To prepare a functionally "isotonic" high Mg^{++}-Ringer solution with varying Mg^{++} concentrations but a constant concentration of K^+ (2.5 mM) and of Na^+ (28.5 mM), two stock solutions were prepared: the high-Mg^{++} stock solution contained 24.6 mM $MgCl_2$, 36.6 mM $MgSO_4$, 2.5 mM KCl, 1.0 mM $CaCl_2$, 15.7 mM $NaHCO_3$, 2.0 mM NaH_2PO_4, and 11.6 mM D-glucose in addition to 10% K'-free GIB medium, penicillin (0.1 mg/ml), and streptomycin (0.1 mg/ml);[34] the low-Mg^{++} stock solution contained 147.5 mM sucrose, 23.2 mM D-glucose in addition to the same concentrations of KCl, $CaCl_2$, $NaHCO_3$, NaH_2PO_4, GIB medium, and antibiotics as in the high-Mg^{++} solution. By mixing these stock solutions in different proportions, a series of Ringer's solutions containing different Mg^{++} concentrations ranging from 1.2 to 73.2 mM, but constant concentrations of K^+ (2.5 mM) and of Na^+ (28.5 mM), were prepared (for further details, see reference 16).

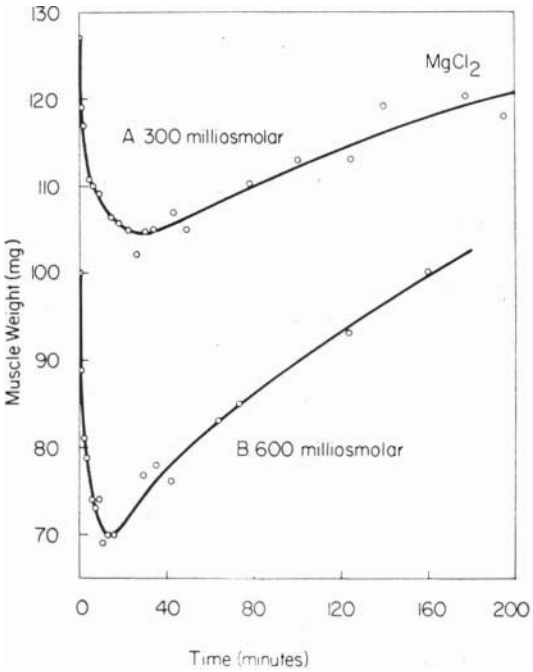

FIGURE I. The time course of weight change of a frog sartorius' muscle after immersion in a Ringer-GIB medium containing. in addition to the normal constituents at their respective normal concentrations, 100 mM $MgCl_2$ (*top* curve) and 200 mM $MgCl_2$ (*bottom* curve). The muscle was weighed at intervals after blotting on moist filter paper.

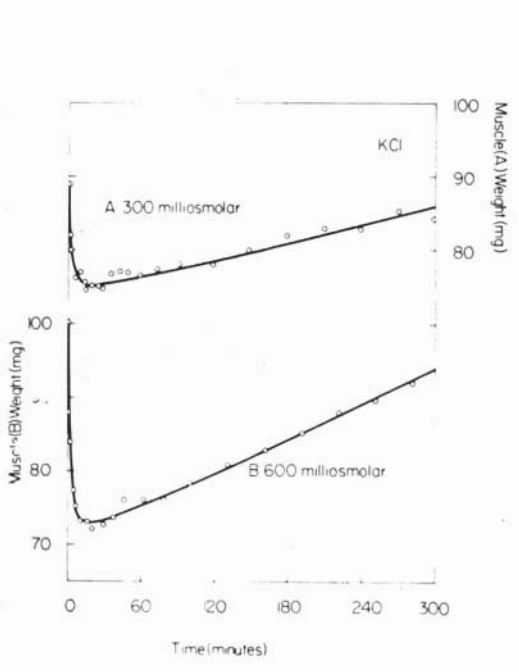

FIGURE 2. The time course of weight change of a frog sartorius muscle after immersion in a Ringer-GIB medium containing. in addition to the normal constituents at their respective normal concentrations, 150 mM KCl (*top* curve) and 300 mM KCl (*bottom* curve). The muscle was weighed at intervals after blotting on moist wetted filter paper.

Measurements of Mg" Permeability

We determined Mg^{++} permeability of frog muscles in two ways.

Volume changes in isotonic $MgCl_2$ and $MgSO_4$ solutions. This is a very old method:" cells are immersed in a Ringer's solution containing a high concentration of a solute, the permeability of which is being investigated. The cells promptly and rapidly shrink. If the cells are "impermeable" to the solute, the cell will, theoretically at least, remain shrunken indefinitely. On the other hand, if the cells are "permeable" to the solute, the cell will take up the solute and, in doing so, will regain its lost water. The rate at which the cell's weight is regained provides the basis for measuring the rate at which the solute enters the cell.

Radioactive ^{28}Mg isotope efflux analysis. The basic method here is an extension of the technique extensively used in studying the efflux rates of Na^+, sugars, etc. developed in this laboratory. Isolated and weighed sartorius muscles were equilibrated in Ringer solutions either at 25°C or 0°C in which the Mg^{++} has been labelled with ^{28}Mg. Connective tissues from areas adjacent to the sartorius muscle were isolated and similarly exposed to the labeled Ringer solutions and for the same lengths of time. At the conclusion of incubation in the radioactively labeled Ringer solution, the muscles and connective tissues were placed between decks of partially wet filter paper, sealed hermetically in parafilm and centrifuged for 4 min. at 1000 g. Removed from the filter paper, the tissues were weighed in a torsion balance kept in a humidity chamber. Previous work has shown this centrifugation removes all the extracellular fluids and no significant amount of intracellular fluid of the sartorius muscle.[35]

The time courses of labeled Mg^{++} efflux from the muscle and connective tissues were separately studied by washing the tissues in successive portions of Ringer solution having similar chemical composition but no radioactivity. After about 2½ hours of washing, the muscles and connective tissues were blotted dry, reweighed, and their labeled Mg^{++} content analyzed. From the remaining labeled Mg^{++} found in the tissues, the efflux curves were constructed. The efflux-curves of the connective tissues from the same animals

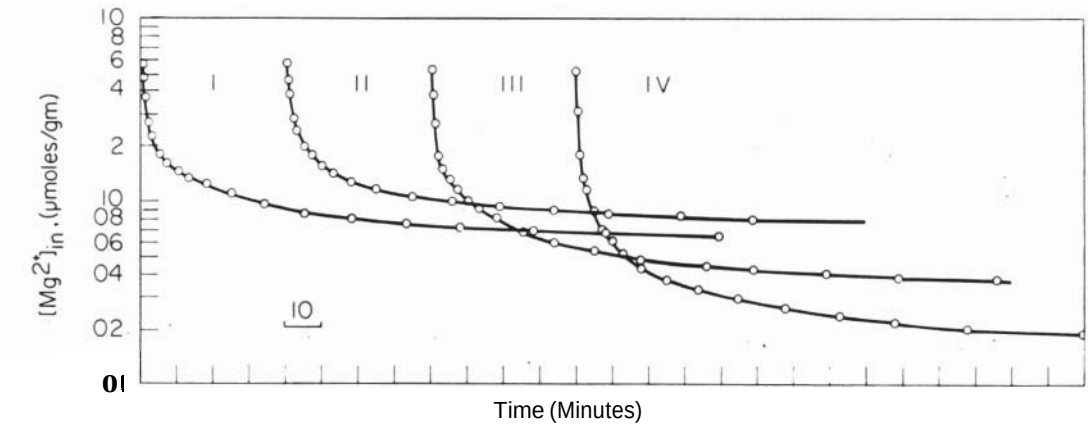

FIGURE 3. Efflux of labeled Mg^{++} from isolated frog "connective tissues." Connective tissues from 4 different frogs were incubated and washed in the same manner as frog sartorius muscles and these curves are used to make corrections for contribution of similar connective tissues in the sartorius muscle efflux curves (see legend of Figure 4). Connective tissues represent loose connective tissues and small nerves, blood vessels, and other non-muscle cell tissues.

were then used to make corrections for the labeled Mg^{++} in the connective tissues of the sartorius muscles using the average volume of the centrifuged connective tissue weights. The details of this technique were described elsewhere.[36] The corrected efflux curves plotted semilogarithmically are then resolved into components by peeling off the slower exponential first, subtracting from the total and replotting, and so on. From the slopes of each fraction, its half-time of exchange and its permeability constant were obtained.

RESULTS

Time Course of Swelling and Recovery of Frog Sartorius Muscles in Concentrated Solutions of $MgCl_2$ and KCl.

Using the volume change method, we studied the rate of entry of $MgCl_2$ into isolated sartorius muscle cells. Figure 1 shows the typical pattern of initial rapid loss of weight followed by a slower regain. Curve B represents a muscle immersed in a Ringer's solution that contained an excess of 600 milliosmoles of $MgCl_2$ per liter; Curve A represents muscle immersed in a solution containing an excess of only 300 milliosmoles of $MgCl_2$ per liter. The half-time ($t_{1/2}$) of weight gain is about 70 min. for Curve B, while that for Curve A appears longer, owing in part at least to the merging of the shrinkage process with the initial re-gain in weight.

For comparison, Figure 2 represents a shrinkage-regain curve of a comparable experiment in which an excess of KCl instead of $MgCl_2$ was added. In this case, the half-time, ($t_{1/2}$) of regain of weight in Curve B is approximately 180 minutes — more than twice as long as the $t_{1/2}$ of weight gain in $MgCl_2$.

We elected to use the swelling-recovery method in order to leave little doubt that by any reliable method, high Mg^{++} salt permeability is demonstrable. However, there is doubt that the rates of weight changes really reflect a permeability-limited process.'" [37] Thus, for more accurate and quantitative data we used the radioactive tracer method to be described next.

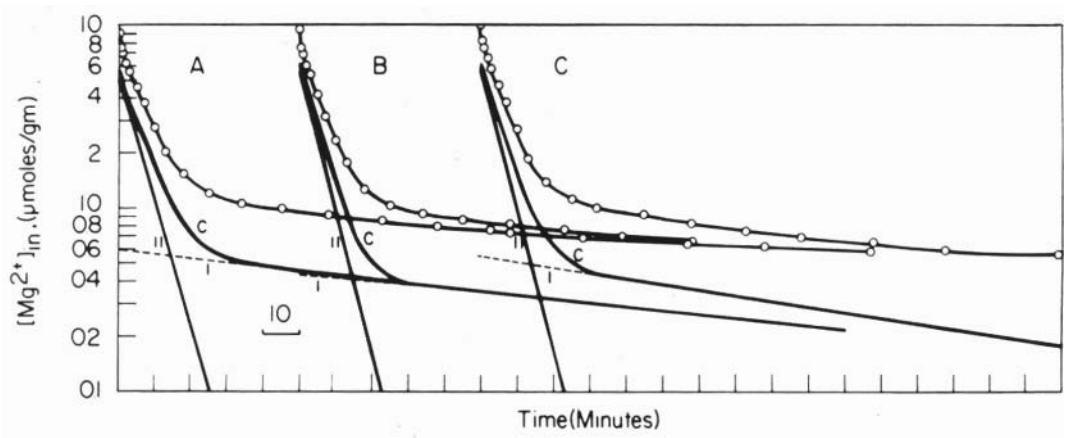

FIGURE 4. The efflux curves of labeled Mg^{++} from 3 frog sartorius muscles. Muscles were incubated in a Ringer solution containing 78 mM labeled Mg^{++} for 21 min at 25° C and centrifuged to remove extracellular space. Washout was carried out in a similar Ringer solution without ^{28}Mg and also at 25° C. Curves marked C were obtained after correcting for connective tissue contribution but without further calculation to change the unit of concentration to the base of pure cell weights. The corrected curves were resolved into two fractions (I, and II). The $t_{1/2}$'s of the fast fraction (II) correspond to the time it took for radioactively labeled Mg^{++} belonging to that fraction to fall to ½ of its initial value.

Labeled Mg^{++} Efflux Studies

Figures 3 and 4 show the Mg^{**} efflux from the centrifuged connective tissues, and the centrifuged sartorius muscles, respectively. The curves marked C were obtained after correction had been made for the connective tissue contributions and they are resolved into two fractions marked I and II. The slow fraction I has a half-time of exchange of 100 minutes or longer (A: 136; B: 148; C: 97 min.); its small intercepts (0.4 to 0.5 μmole/g) suggest that this fraction had only exchanged with the labeled Mg^{**} to a small extent. Of much greater interest here is the fraction II which represents the bulk of labeled Mg^{++} that has exchanged. Indeed the $t_{1/2}$'s from the three sets of data are 4.5 (A), 4 (B) and 4 (C) minutes. These and three other sets of data are given in Table I.

The fast exchanging fraction, with a $t_{1/2}$ of 4.7 min., is rate-limited by permeation through the cell surface in agreement with the earlier demonstration that a new equilibrium of Mg^{**} distribution is reached within 20 min.[16] The present findings contradict the conclusion that Mg^{++} permeability is low in frog muscle.[15] On the contrary, it is very rapid, as Tasaki et al[14] demonstrated for Ca^{++} permeability in squid axons.

The outward rate constant of Mg^{++} efflux, k_{outw}, is equal to $\ell n2/t_{1/2}$. The outward permeability constant, κ_{outw} is equal to k_{outw} divided by the surface-volume ratio (A/V), which equals 550 cm^2/g for frog muscle.[4,p.208] From the $t_{1/2}$'s given in Table I, one obtains an average κ_{outw} equal to $0.693/(4.7 \times 60 \times 550) = 4.5 \times 10^{-6}$ cm sec^{-1}. At equilibrium the inward flux (Mi) and outward flux (M_o), defined as the number of moles per cm^2 per second must be equal.

Since $$M_o = M_i, \tag{3}$$

$$M_o = \kappa_{outw}[Mg^{++}]_{int}, \tag{4}$$

$$Mi = \kappa_{inw}[Mg^{++}]_{ex}, \tag{5}$$

where $[Mg^{++}]_{int}$ is the intracellular free Mg^{++} concentration, or interstitial Mg^{**} concentration, and $[Mg^{++}]_{ex}$ is the extracellular Mg^{++} concentration; hence

$$\kappa_{inw} = \frac{[Mg^{++}]_{int}}{[Mg^{++}]_{ex}} \kappa_{outw}. \tag{6}$$

From an earlier publication,[16] we know that at equilibrium (25°C), the Mg^{++} equilibrium distribution coefficient, is

$$q_{Mg} = \frac{[Mg^{++}]_{int}}{[Mg^{++}]_{ex}} = 0.206. \tag{7}$$

Thus $\kappa_{inw} = 0.206 \times 4.5 \times 10^{-6}$ cm/sec $= 0.93 \times 10^{-6}$ cm/sec. Next we shall calculate the permeability constant for Mg^{**}, P_{Mg}, accord-

TABLE I. The half time of exchange of labeled Mg^{++} from isolated frog sartorius muscle at 25°C. Isolated sartorius muscles were incubated for diferent lengths of time at 25°C as indicated. The $t_{1/2}$ of exchange was estimated from the fast fraction, i.e., Fraction II as in Figure 4.

Date	Experiment No.	Incubation Duration (min)	$t_{1/2}$ of Fast Fraction (min)
12-18-79	A	21	4.5
	B	21	4.0
	C	21	4.0
	E	10	5.0
	F	10	4.8
	H	10	7.0
	M	11	5.0
	N	11	4.8
	O	11	4.8
3-18-80	I	62	3.5
	J	62	3.6
	K	62	5.0
mean ± S.E.			4.7 ± 0.25

ing to the model of Hodgkin-Katz-Goldman.[38,p.60]

$$P_{Mg} = \frac{\kappa_{inw}}{\phi} , \quad (8)$$

where the factor

$$\phi = \frac{Z\psi F/RT}{1 - \frac{1}{\exp(Z\psi F/RT)}} , \quad (9)$$

where Z, the valency, is 2; ψ, the resting potential, is equal to 86 mV (see below) and positive. R and T are the gas constant and absolute temperature, respectively. At 25°C, RT is equal to $8.614 \times 10^{-5} \times 298 = 2.567 \times$ volt-Faraday or roughly 26 mV-Faraday.

$$\phi = \frac{2 \times 86/26}{\left(\frac{1}{\exp(\frac{2 \times 86}{26})}\right)} = 6.62. \quad (10)$$

From Equation 8, we find

$$P_{Mg} = \frac{0.93 \times 10^{-6}}{6.62} = 1.4 \times 10^{-7} \text{ cm sec}^{-1} \quad (11)$$

The Mg^{++} permeability constant P_{Mg} calculated according to Hodgkin-Katz-Goldman is then compared with similarly calculated values of P_K in Table II. Note that P_{Mg} is not vastly lower than P_K.

TABLE II. A comparison of the Hodgkin-Katz permeability constant (P,) of K' and Mg^{++} in frog muscles.

Ion	Author	Pi (cm/sec.)
K^+	Mullins (52)	5.8×10^{-7}
	Ling (4)	4.2×10^{-7}
	Katz (38)	5.8×10^{-7}
Mg^{++}	Present Paper	1.4×10^{-7}

Effect of Varying External Mg^{++} Concentration upon the Resting Potential of Frog Sartorius Muscle

Two sartorius muscles were introduced into each one of several flasks containing 25 ml of each of the solutions with different Mg^{++} concentrations. They were incubated at 4°C, and the flasks were shaken gently for 18 hours. At the end of this period, the flasks were warmed to room temperature before the resting potentials of at least 8 single muscle fibers (four from each muscle) were measured while the muscles were immersed in an aliquot of their incubation solutions. The

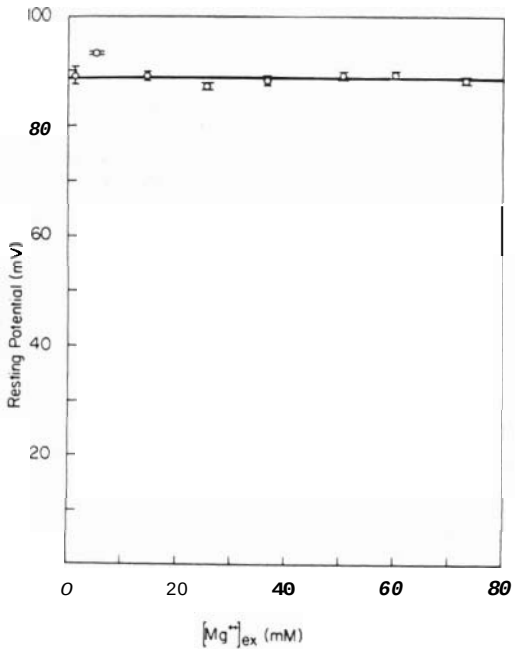

FIGURE 5. The effect of external Mg* concentration upon the resting potential of isolated frog sartorius muscles. The muscles were exposed to Ringer's solution with varying concentrations for 18 hours at 4°C followed by warming to 25°C before measurements of resting potential were made. The 4°C incubation permitted Mg^{++} to fully equilibrate between cell and external medium.[16]

result is shown in Figure 5. No significant depolarization of the resting potential was observed in response to increasing external Mg^{++} concentration from a concentration of 1.2 mM to 73.2 mM.

Effect of Varying Duration of Exposure to High External Mg^{++} Concentration at 25° C upon the Resting Potential

The data of Figure 5 were obtained after prolonged exposure of frog muscles to the experimental solutions at 4° C followed by warming to 25° C. Figure 6 shows the indifference of the resting potential to the high external Mg^{++} concentration was the same when the temperature was maintained at 25° C throughout the incubation period of over 15 hours.

The promptness with which the potential of sartorius muscles responds to high external K^+ concentrations has long been known.[3,4,p.277] In this case, a new reduced level of potential was reached in the high K^+ solution within a matter of 1 or 2 minutes and remained there for at least 10 hours without further change. The difference between the effectiveness of high external K^+ in reducing the potential and the total ineffectiveness of similar high concentrations of external Mg^{++} in reducing the potential, could not be more striking.

DISCUSSION

The demonstration that the Mg^{++} permeability constant calculated according, to Hodgkin and Katz is not far lower than the K' permeability constant, while at high concentrations Mg^{++} produces no effect on the resting potential, offers strong evidence against the membrane-pump model of the cellular potential. The same findings also offer evidence in support of the surface adsorption model of the association-induction hypothesis.

The sensitivity of the potential to external alkali-metal ions and insensitivity to external Mg^{++} correlates the behavior of the frog muscle cell surface to a model that was introduced years ago — oxidized collodion-coated glass electrodes.[29] This model exhibits a similar electrical behavior (sensitivity to K', lesser sensitivity to Na^+ and insensitivity to Mg^{++})

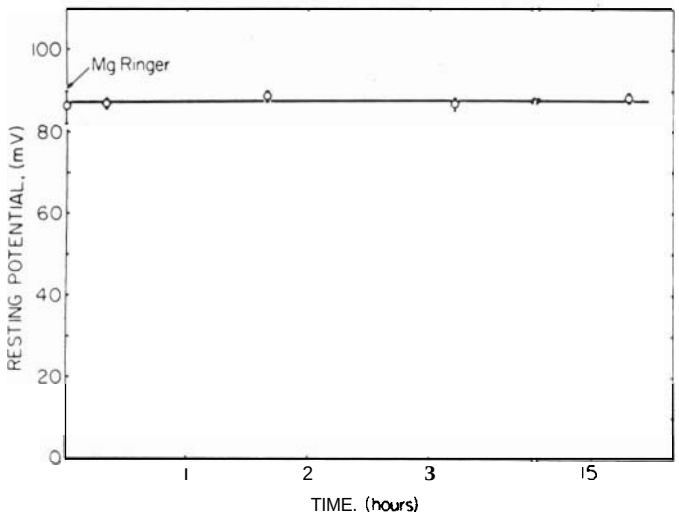

FIGURE 6. The graph shows that the resting potential is independent of the prolonged exposure to a high Mg^{++} Ringer solution (73.2 mM Mg^{++}). The entire experiment was carried out at 25° C.

as a result of the introduction of carboxyl groups onto its surface. These findings support the theory that the muscle cell surface is endowed with primarily solitary β- and γ-carboxyl groups and that these fixed anionic groups are responsible for both the polarity and the magnitude of the resting potential of frog muscle cells.

This set of experimental findings and conclusions should be viewed along with a number of other developments which are almost unanimously in harmony with the surface adsorption model, and it is timely to review them briefly.

There are seven variables in the Hodgkin-Katz equation: T, $[K']_{ex}$, $[Na']_{ex}$, $[Cl^-]_{ex}$, $[K^+]_{in}$, $[Na^+]_{in}$, and $[Cl^-]_{ex}$. The predicted relationship between ψ and the first three variables has been repeatedly confirmed. However, this cannot be said for the predicted relationship between ψ and the last four variables. A lasting change of the potential with variations in $[Cl^-]_{ex}$ was not observed;[39] and although a confirmation of the predicted relationship between $[K^+]_{in}$ in perfused squid axon and ψ was reported,[40] the slope of the plot of ψ vs. $\ell n\,[K^+]_{in}$ has a much lower value than required. Furthermore, this relation was directly contradicted by a subsequent report from another laboratory in which no change of potential was observed in perfused squid axons when a perfusant solution of K_2SO_4 was changed to one of Na_2SO_4.[41]

In addition, no less than six other sets of independent experimental observations contradict the theoretical prediction of a relation between $[K]_{in}$ and ψ.[42-48] Two of these papers also report failure to detect the predicted relationship between $[Na^+]_{in}$ and ψ during nerve or muscle activity.[42,44,45]

If an equation is derived rationally it must have an internal coherence. When experimental studies fail to confirm the predicted relationship between four of the seven variables and ψ, the basic theory is probably incorrect. However, the association-induction hypothesis offers equations for ψ (Equations 2 and 3) that contain no more than the three variables whose relationships to ψ have already been verified.

It is to be noted that the association-induction hypothesis agrees with both the data that support the membrane theory and the data which do not support the membrane theory. Thus, the association-induction hypothesis does not predict a dependence of the potential upon external anions (e.g., chloride) and, in fact, none was found. The association-induction hypothesis also does not predict a mandatory dependence on intracellular ions, and, again, none was consistently found (for further discussion, see 6,7).

Two other new findings bear on the critical subject of a choice between the two alternate models; in both, the young German scientist, Ludwig Edelmann, made a major contribution.[45] If one keeps $[Cl^-]_{ex}$, $[Cl^-]_{in}$, $[K^+]_{in}$, and $[Na^+]_{in}$ essentially constant, say, in a short-term experiment, Equation 1 can be written as

$$\psi = \text{constant} - \frac{RT}{F}\,\ell n\{P_K[K^+]_{ex} + P_{Na}[Na^+]_{ex}\}. \quad (12)$$

Equation 12 is formally identical to Equation 2 except that the coefficients of the external concentrations have different meanings. The P's are permeability constants for K' and Na', and the $\widetilde{K}$'s are adsorption constants of these ions on surface anionic sites. Edelmann, having noted this fundamental difference, designed an experiment to test the two alternate theories. He concluded that the cellular electrical potential has no relation to permeability but represents adsorption constants, as described by Equation 2.[45]

An even more recent finding is that the bulk of intracellular K' is not freely distributed in the cell water.[21-26] In skeletal muscles, intracellular K' is localized primarily in the A-bands and the Z-line, in agreement with the association-induction hypothesis, which has long contended that intracellular K' is selectively adsorbed on the β- and y-carboxyl groups of cellular proteins. This follows from the facts that most muscle β- and y-carboxyl groups are carried by the myosin that makes up the A-band,[22] that β- and y-carboxyl groups bind uranium in EM plates of fixed cell sections,[49] and that uranium is concentrated on the A-band, Z-line, etc.[50]

The demonstration of the adsorbed state of intracellular K', on the one hand, makes the Hodgkin-Katz equation untenable, since this equation was based on the assumption that virtually all intracellular K^+ exists in the free state. On the other hand, this demonstration affirms the association-induction hypothesis in a most basic manner, since in this hypothesis the electrical potential should have no direct causal relationship to the bulk-phase intracellular K', its concentration, or its state of adsorption.

Therefore, the finding presented here of the independence of ψ and external Mg^{++} is not an isolated observation but is one of many findings contradicting the membrane theory and supporting the association-induction hypothesis.

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