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DIFFUSION ACROSS RAT DIAPHRAGM

I. MOVEMENT OF SODIUM AND POTASSIUM; EFFECT OF EXCITATION AND CORRELATION WITH PROTEIN STRUCTURE

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SUMMARY

The diffusion rate of Na^+ and K^+ was measured across surviving rat diaphragm. Stimulation of the muscle through its nerve caused slowing down of Na^+ and acceleration of K^+ diffusion. Similar changes were produced in resting muscle by 2,4-dinitrophenol and other metabolic inhibitors. The effects of stimulation on ion movements were decreased or abolished by some substances, particularly by insulin, iodoacetate, local anesthetics and ouabain. Stimulation, 2,4-dinitrophenol and ouabain also caused changes in the ionizable sulfhydryl groups of the muscle, similar to those previously observed in the brain. The correlation between SH groups and ion movement suggests that at rest the configuration of cell proteins favors K^+ accumulation while during activity it facilitates Na^+ fixation.

INTRODUCTION

Maintenance of a concentration gradient of potassium and sodium between the cell and the extracellular fluid and its breakdown during excitation have been explained either by the permeability properties of the plasma membrane or by the selective binding ability of the cellular matrix. The membrane theory incorporating the subsidiary hypotheses of carrier-operated active transport and of ionic pumps, enjoys at present a wide acceptance. Theories based on selective binding, under various forms have, however, been gaining support in the last few years[1-6].

Our observations on configurational changes of cell proteins during excitation[7,8] have been interpreted as being favorable to the matrix hypothesis. These denaturation-like changes, best designated by the term "transconformation" proposed by LUMRY AND EYRING[9] could alter the matrix in such a way as to shift its affinity from potassium to sodium[10].

The observations reported in the present paper on the diffusion of Na^+ and K^+ across surviving rat diaphragm submitted to various experimental conditions further suggest the possibility of a correlation between protein configuration and the affinity of the cell for the two ions.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

METHODS AND MATERIALS

Diffusion measurements

Diffusion across the diaphragm was measured by means of the device shown in Fig. 1. It consists of two compartments A and B separated by a fragment of rat diaphragm. Compartment A contained 4 ml of bicarbonate–Krebs–Ringer solution with oxygen bubbling through it and compartment B held 40 ml of isotonic sucrose solution.

The diaphragm was removed immediately after decapitation of albino rats (CFN strain) weighing between 80 and 120 g. One hemidiaphragm was stretched across the lower end of tube A attached to it by means of a fine rubber band. The phrenic nerve supplying the muscle was attached to a pair of platinum electrodes. The preparation was kept in a water bath at 37.5° throughout the experiment.

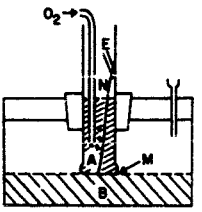

Fig. 1. Diagram of the diffusion system. The membrane (diaphragm or cellophane), M, separates the two compartments A and B. The phrenic nerve, N, is attached to electrodes, E. The needle shown on the right hand side equalizes the atmospheric pressure in the two compartments and is used to collect samples from compartment B. The fluid is stirred before withdrawal of samples.

The area of diaphragm in contact with the solutions was 40 mm². The mean weight of muscle was 12.9 mg (± 2.5 S.D.). The average thickness of the slightly stretched diaphragm was 300 μ and its total volume 12.2 mm³. Using the values given by CREESE[11], the extracellular space was calculated to be 3.0 mm³ (24.6 %) and the total cellular surface 18 cm². In the absence of inhibitors, the muscle conserved its viability, —as judged by its response to electrical stimulation— for the duration of the experiment, usually 1 h.

Samples of 2 ml were collected from compartment B at 15-min intervals and sodium and potassium were estimated by means of a flame photometer, using internal standards (Baird Associates, Inc., Cambridge, Mass.). From these values the amount of material passing from A to B was calculated, taking into account the amount of fluid removed when collecting the previous samples and also the small amounts of Na^+ (0.036 mM) and K^+ (0.608 mM) contained in the sucrose solution.

According to FICK's law, the rate of diffusion of a solute (dQ/dt) is determined by the area of diffusion (A), the gradient (dc/dx) and the diffusion coefficient of the solute (D)

$$\frac{dQ}{dt} = DA\frac{dc}{dx} \tag{1}$$

In the diffusion system described above, it was found empirically that for the first 60–100 min at least, the passage of solute could be expressed by the following equations

$$\log m = \log a + b \log t \tag{2}$$

$$m = at^b \tag{3}$$

In these equations m is the fraction of the solute contained in A which passed through the boundary in time t (equivalent to dQ/dt) and a is a rate constant in-

corporating D, characteristic for the diffusing solute, as well as A and other properties of the system. The exponent b in many systems is usually assumed to be 0.5 (see ref. 12) but this is probably only its lower limiting value. It increases with the degree of interaction between solute particles, between solute and solvent and in the present case, with the degree of a restriction imposed upon the diffusion of the solute by the presence of a barrier. It is proposed to call b a "binding index", the term binding being taken in its broadest sense of limitation of movement and without necessarily implying a chemical bond between the solute and the barrier*.

Both a and b can be determined graphically by plotting the experimental results results according to Eqn. 2. The values given below were determined by the least squares method. Fig. 2 illustrates the procedure and shows, as an example, the diffusion of NaCl and KCl across a cellophane membrane.

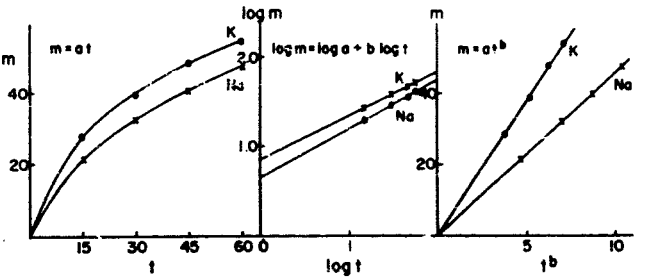

Fig. 2. Diffusion of Na^+ and K^+ across cellophane. Abscissa: time in minutes; ordinate: fractions of solute diffusing from A to B (m). From left to right, the results are plotted according to the equations indicated on top of each graph.

Evaluation of protein structure

Among the many methods available for the study of protein configuration few are applicable to non-purified material. In the present studies, as in the previous ones[7,8], structural changes in proteins were characterized in terms of the unmasking of reactive side groups. This was done either by measuring the shift in the ultraviolet spectrum characteristic for the ionization of the phenolic hydroxyl of tyrosine and sulfhydryl of cysteine[7] or by amperometric titration of the latter[8].

Muscle extracts prepared according to the procedure previously described for brain were diluted in Tris buffer and titrated with silver nitrate in the presence of nitric acid[14]. The results are expressed in terms of μmoles of SH/g of wet weight of tissue.

Metabolic inhibitors and other agents

The following metabolic inhibitors were used: 2,4-dinitrophenol (Nutritional Biochemicals), iodoacetic acid (Matheson, Coleman and Bell), Na p-chloromercuribenzoate (Nutritional Biochemicals) all at 1 mM concentration and Na fluoroacetate (K & K Laboratories) at 5 mM. The other agents studied were insulin (Eli Lilly and Co.) at 0.1 U/ml, procaine at 15 mM, ouabain (Nutritional Biochemicals) at 0.07 mM

* In preliminary reports of this work[10,13] instead of b the value of $(b-1)$ was used as the exponent of t and the results were plotted according to the equation: $\log m/t = \log a - (b-1)\log t$. Both treatments give essentially the same results but some confusion may arise because the symbol $(b-1)$ was used to designate b.

l-epinephrine (Eastman Chemicals) at 0.05 mM and eserine sulfate (Nutritional Biochemicals) at 1 mM concentration. All substances were added to compartment A. Anoxia was produced by bubbling nitrogen through both compartments.

RESULTS

Rat diaphragm representing a thin layer of muscle fibers, appeared an appropriate preparation to measure diffusion across living tissues and its changes under various conditions. The most important result obtained with this method is shown in Fig. 3 which summarizes the data obtained on 12 resting and 12 stimulated diaphragms. Stimulation was done through the phrenic nerve at double the threshold voltage and 6 cycles for 60 min. It is seen that the diffusion rate (designated in the previous sections as *a* and which represents the slope of the lines shown in the figures) of both Na^+ and K^+ is significantly altered by stimulation. At rest, Na^+ diffuses faster than K^+ but during stimulation the ratio reverses and K^+ passes across the muscle much more rapidly than Na^+.

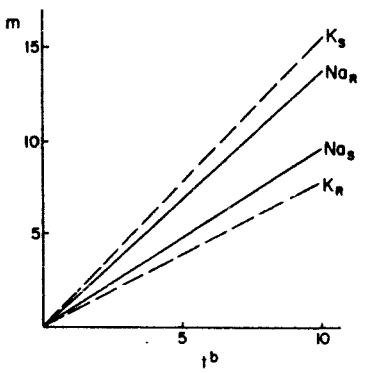

Fig. 3. Diffusion of Na^+ and K^+ across resting and stimulated rat diaphragm. Abscissa: t^b (min); ordinate: fraction of solute diffusing across diaphragm (m). It is seen that across the resting muscle Na^+ (Na_R) moves faster than K^+ (K_R) while during stimulation K^+ (K_S) is faster than Na^+ (Na_S).

Table I summarizes all the diffusion experiments and lists both the diffusion rate *a* and the binding index *b*. The higher the value of *b* the greater the restriction imposed on the mobility of the particles by the barrier. The table shows that the diffusion of Na^+ through the resting muscle is significantly reduced by 2,4-dinitrophenol, iodoacetate, ouabain and procaine and increased by insulin and PCMB. Diffusion of K^+ is enhanced by 2,4-dinitrophenol and PCMB. Through the stimulated muscle, Na^+ diffusion is reduced by eserine and anoxia, accelerated by 2,4-dinitrophenol, iodoacetate, PCMB and insulin. K^+ diffusion is decreased by iodoacetate, fluoroacetate, ouabain, adrenaline and eserine.

According to the manner in which they modify the ion movements associated with excitation, the drugs can be divided into the following groups. (a) 2,4-Dinitrophenol and iodoacetate reverse the tendency of Na^+ to slow down during excitation and prevent the acceleration of K^+ movement associated with the process. The latter effect is also exerted by PCMB. (b) Fluoroacetate, eserine, anoxia and perhaps ouabain actually slow down K^+ movement in the stimulated muscle. Ouabain and anoxia also prevent the decrease in Na^+ diffusion. (c) Insulin and procaine abolish the changes associated with excitation. It is to be noted that both substances decrease excitability of the muscle.

TABLE I

DIFFUSION OF Na^+ AND K^+ ACROSS RESTING AND STIMULATED RAT DIAPHRAGM; ACTION OF METABOLIC INHIBITORS AND OTHER DRUGS

Each value represents the mean of at least 6 experiments; S.D. = standard deviation. Stimulation of the phrenic nerve was done at double of the threshold voltage, 6 cycles for 1 h.

Drug	*Resting*				*Stimulated*			
	Na^+		K^+		Na^+		K^+	
	a ± S.D.	*b* S.D.	*a* ± S.D.	*b* ± S.D.	*a* ± S.D.	*b* ± S.D.	*a* ± S.D.	*b* ± S.D.
Control	0.299 ± 0.068	0.758 ± 0.073	0.141 ± 0.053	0.847 ± 0.078	0.188 ± 0.060	0.914 ± 0.084	0.243 ± 0.071	0.679 ± 0.059
2,4-Dinitrophenol	0.190 ± 0.040	0.906 ± 0.122	0.266 ± 0.075	0.639 ± 0.062	0.249 ± 0.025	0.827 ± 0.079	0.259 ± 0.060	0.674 ± 0.064
Iodoacetate	0.146 ± 0.034	1.027 ± 0.135	0.141 ± 0.032	0.877 ± 0.092	0.267 ± 0.075	0.795 ± 0.081	0.157 ± 0.034	0.853 ± 0.081
Fluoroacetate	0.224 ± 0.032	0.839 ± 0.091	0.173 ± 0.044	0.750 ± 0.081	0.140 ± 0.065	1.055 ± 0.101	0.120 ± 0.025	0.891 ± 0.088
PCMB	0.444 ± 0.126	0.571 ± 0.062	0.292 ± 0.048	0.591 ± 0.052	0.303 ± 0.106	0.677 ± 0.064	0.263 ± 0.082	0.671 ± 0.065
Insulin	0.348 ± 0.072	0.697 ± 0.065	0.153 ± 0.050	0.831 ± 0.080	0.325 ± 0.082	0.720 ± 0.069	0.204 ± 0.053	0.745 ± 0.079
Ouabain	0.213 ± 0.056	0.898 ± 0.079	0.182 ± 0.038	0.773 ± 0.068	0.250 ± 0.036	0.847 ± 0.085	0.136 ± 0.030	0.903 ± 0.085
Adrenaline	0.272 ± 0.063	0.756 ± 0.076	0.163 ± 0.040	0.795 ± 0.075	0.145 ± 0.057	1.082 ± 0.098	0.127 ± 0.042	0.930 ± 0.093
Eserine	0.240 ± 0.048	0.802 ± 0.079	0.174 ± 0.046	0.805 ± 0.080	0.121 ± 0.020	1.095 ± 0.099	0.101 ± 0.048	0.993 ± 0.102
Procaine	0.174 ± 0.050	0.913 ± 0.106	0.197 ± 0.052	0.716 ± 0.074	0.221 ± 0.038	0.899 ± 0.090	0.192 ± 0.038	0.716 ± 0.073
Anoxia	0.240 ± 0.082	0.806 ± 0.077	0.179 ± 0.050	0.826 ± 0.079	0.153 ± 0.065	0.983 ± 0.094	0.115 ± 0.058	0.974 ± 0.099

To correlate the diffusion of ions under some of the conditions just enumerated with possible modifications of protein structure, the ionizable sulfhydryl content of diaphragm was estimated. Table II shows that stimulation of the muscle, as previously described in the brain, causes a significant increase in SH groups. A similar unmasking was observed when resting muscle was treated with 2,4-dinitrophenol or ouabain.

TABLE II

CHANGES IN THE SULFHYDRYL CONTENT OF RAT DIAPHRAGM UNDER THE INFLUENCE OF STIMULATION, ANOXIA AND DRUGS

	SH (μmoles/g)	*±S.D.*	*Number of experiments*
Resting	5.32	1.08	30
Stimulated*	8.55	2.05	18
Anoxia	6.40	1.56	8
Insulin, resting	4.14	0.84	22
Insulin, stimulated*	4.60	1.02	12
DNP	8.49	2.42	12
Ouabain	8.15	1.80	10

* Stimulated through the phrenic nerve at double of the threshold voltage, 6 cycles for 30 min.

Addition of insulin to the bathing fluid produced the opposite effect[15] and stimulation of insulin-treated diaphragm caused only a slight elevation of SH groups.

Fig. 4 shows the correlation between the binding index (b) of the ions and the ionizable SH of the muscle. It is seen that, as the SH groups are unmasked, the freedom of Na^+ to move across the diaphragm decreases while that of K^+ increases. The only exception is the insulin treated muscle in which K^+ diffusion increases on stimulation without a corresponding rise in SH. The middle points correspond to anoxia and their position suggests that the muscle deprived of oxygen loses its ability to discriminate between K^+ and Na^+.

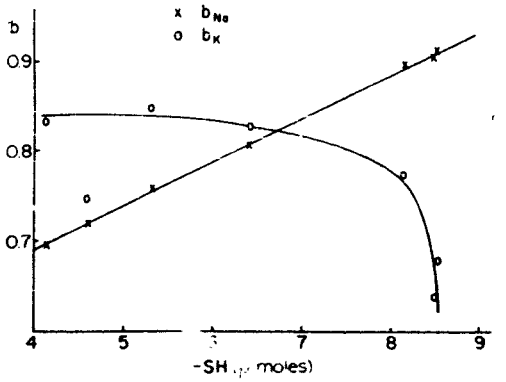

Fig. 4. Correlation between ion "binding" and protein structure. Abscissa: ionizable sulfhydryl groups in μmoles/g wet weight of diaphragm; ordinate: binding index. The points represent from left to right: insulin-treated diaphragm at rest, the same during stimulation, normal resting diaphragm, anoxia, ouabain-treated, 2,4-dinitrophenol-treated, and normal stimulated muscle. [illegible] the unmasking of SH groups (indicating a loosening of protein structure) while K^+ fixation shows the opposite trend.

DISCUSSION

Interpretation of the results just summarized is made difficult by the fact that the diaphragm is a comparatively complex structure. McLENNAN[16] used it for measuring diffusion across muscle tissue with the basic assumption that diffusion takes place exclusively in the extracellular spaces. This is, however, difficult to reconcile with the changes observed under the influence of stimulation and other conditions studied in the present work. Moreover, large molecules such as inulin or serum proteins which can probably diffuse through extracellular spaces, are almost completely stopped by the diaphragm.

If the diaphragm is considered too composite a structure for the study of transport across cells, the same objection could be raised against frog skin, toad bladder and other widely used preparations. The necessity of establishing a steep gradient between the two compartments which places the muscle in somewhat unphysiological conditions could be avoided by using radioactive Na^+ and K^+ and this will be attempted in the future.

If one tries to interpret the results of these experiments in terms of membrane permeability, one reaches the paradoxical conclusion that stimulation decreases permeability to Na^+ and increases it to K^+. The fact that excitation slows down the diffusion of Na^+ and accelerates the passage of K^+ may, however, mean that stimulation results in an increase in binding sites for Na^+ and a decrease in these sites for K^+. The nature of the binding, as well as its compatibility with the osmotic and electrical behavior of the cell is still a matter of conjecture and the problem cannot be settled until more information is available, particularly on the state of water in the cell.

The cell proteins, as a large potential source of anionic sites, are assumed to be involved in the cation-binding process whether it occurs in the membrane, as suggested by DANIELLI[17] or in the matrix of the cell. The magnitude of the changes taking place in the protein side groups is in favor of the latter hypothesis. The present trend in our understanding of cell structure minimizes the distinction between plasma membrane and endoplasmic reticulum. The ubiquitous presence of membrane-like lamellar structures has all but abolished the representation of the cell as a sac containing a solution of enzymes, substrates and ions and tends to substitute to it the idea of a macromolecular matrix[18,19] or cytoskeleton[20]. The matrix may function as an ion exchange system[5,21] holding preferentially K^+ at rest and exchanging it for Na^+ when activated. The configuration of the proteins which make up the system could therefore be the key factor in ion transport.

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