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EFFECTS OF ELECTRON-MICROSCOPIC FIXATIVES ON CELL MEMBRANES OF THE PERFUSED RAT HEART

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SUMMARY

Left ventricles from rat hearts were pre-perfused through the coronary blood vessels with physiological saline containing variable concentrations of the fixatives formaldehyde or osmic acid, then perfused for 30 min with physiological saline. The distribution of K^+, Na^+, Ca^{2+}, Mg^{2+}, water, and anions in fixative-treated ventricles was then compared with that of ventricles perfused in fixative-free physiological saline. After exposure to fixatives there is an exchange of cellular K^+ for extracellular Na^+ similar to that which occurs when active transport of Na^+ is inhibited. The selective permeability of the cell membrane to ions was partially preserved at all but the highest fixative concentrations. The osmolality of the fixative therefore affects volumes of cells and subcellular structures in fixed tissues. Osmium-treated ventricles accumulate very large amounts of Ca^{2+} (average: 46 times the normal ventricular Ca^{2+} content) from a medium containing 1.4 mM Ca^{2+}. They also take up large amounts of osmium; after perfusion with 32.7 mM OsO_4, the accumulated osmium compound accounts for 46 % of the tissue dry weight.

INTRODUCTION

Recent physiological observations on the nature of tissue compartments in heart muscle have suggested a need for correlating the tissue compartments defined by physiological methods with structures seen in electron micrographs of cardiac cells[1–3]; indeed, several reports have appeared containing conclusions about physiological properties of cardiac membrane systems based on electron micrographs of tissue fixed in aldehyde and osmium tetroxide[3,4]. These conclusions rest on an important assumption: it is assumed that the fixative completely destroys the selective permeability of the cell membrane. As a result, fixation is considered to proceed without distortion of cell volume. If the selective permeability of cellular membranes were not abolished, cellular volumes would be distorted by the osmotic effects of the fixative, or by specific actions of the fixative on passive ion permeability or on active ion transport.

We shall present evidence that this assumption does not hold in mammalian heart muscle. In this tissue the selective permeability of the cell membrane is at least partially preserved after exposure to osmic acid or formaldehyde. Our experiments have yielded two additional measurements: first, we have estimated the amount of

osmic acid taken up by heart muscle when the tissue is fixed uniformly with this agent; secondly, we have shown by chemical analyses that fixation with osmic acid leads to a remarkable uptake of calcium by the tissue, a calcium uptake probably localized at least in part to the plasma membrane.

Portions of this work have previously appeared in abstract form[5].

METHODS

Experimental design

The experiments were designed to measure the effects of electron-microscopic fixatives on the permeability of the cardiac cell membrane to ions. To this end, isolated rat hearts were pre-perfused through the coronary vessels for 30 min with physiological salt solution to establish a steady state with respect to cellular ionic composition. Next, the steady state was perturbed by changing the perfusing solution to one containing the fixative. After 10 min of perfusion with physiological salt solution containing fixative, the fixative was washed out by re-perfusing the heart with fixative-free physiological salt solution for an additional 30 min. The period of re-perfusion with physiological salt solution served as a test of how well the membranes of fixative-treated cells were able to maintain the normal gradients of ionic concentration between the cellular and extracellular solutions. At the end of this period, left ventricles were analyzed for their contents of water, K^+, Na^+, Ca^{2+}, and Mg^{2+}. The ionic distributions so obtained were compared with those of hearts perfused with salt solution without exposure to a fixative.

Procedure

Rats weighing 200–250 g, were weighed, injected with heparin, and anesthetized with intraperitoneal pentobarbital. The hearts were isolated and perfused on the Langendorff cannula as described elsewhere[6]. At the end of the re-perfusion with physiological salt solution, the heart was removed from the perfusion apparatus and the left ventricle was quickly dissected out by the method of KRAMES AND VAN LIERE[7]. The left ventricle was cut into 2 approximately equal portions, each of which was weighed in a separate Vycor crucible. One portion was dried overnight in an oven at 100°, reweighed to obtain the dry weight, and ashed at 500° in a muffle furnace. The other half was extracted for 48 h without drying. The extraction was done in tightly closed polyethylene bottles containing 10 ml of redistilled 0.1 M HNO_3, or 10 ml of the $LaCl_2$–HCl–NaCl mixture used for determining Ca and Mg. All perfusions were carried out at 22–24°.

Solutions

The physiological salt solution used for pre-perfusion and re-perfusion had the following composition (in mM): K^+ 5.93, Na^+ 153.4, Cl^- 138.7, HCO_3^- 22.0, HPO_4^{2-} 0.59, $H_2PO_4^-$ 1.45, Ca^{2+} 1.4, and Mg^{2+} 0.56. The solution was brought to pH 7.3 and maintained at this pH by passing a stream of water-saturated gas (5 % CO_2–95 % O_2) through it. Buffered fixatives were prepared by adding osmic acid or paraformaldehyde to the physiological salt solution. The three concentrations (mM) of osmic acid tested were, respectively, 0.327, 3.27, and 32.7 (calculated as OsO_4); the corresponding three concentrations of formaldehyde were 25 and 250 mM (calculated

on the assumption that paraformaldehyde dissociates into formaldehyde monomers in dilute aqueous solution) and 4 %. In some experiments an extracellular tracer (^{35}S-labeled sulfate) was incorporated in the solution used for re-perfusion.

Analyses

K, Na, Cl, and ^{35}S were determined as described elsewhere[6]. Ca and Mg were measured on an atomic absorption spectrophotometer. For this purpose the undried halves of the left ventricle were extracted in the same solution of $LaCl_2$, HCl, and NaCl subsequently employed as the blank for the spectrophotometry. This solution was made as follows: 1 l of 10 times concentrated stock solution was prepared by dissolving 11.73 g lanthanum oxide in 50 ml conc. HCl and diluting to 1 l with deionized, redistilled water. 1 l of the solution used for extraction was then made by adding 100 ml of the concentrated stock to 100 ml of 1.0 M NaCl and diluting to a final vol. of 1 l. The extract was centrifuged to remove suspended matter. It was used directly for determination of Ca, and diluted 10-fold with the same blank for determination of Mg. The 100 mM NaCl was added to the blank to render negligible effects of Na on the signals due to Ca and Mg. The concentrations of K and of phosphate present in the extracts were found to be without significant effect on the Ca and Mg signals. Dissolved osmic acid at a concentration more than 100 times greater than that which could have been present in any of the tissue extracts used gave a small (approx. 10 %) increase in the signal due to Ca; interferences at the much lower osmic acid concentrations actually prevailing in the extracts were therefore negligible. All glassware used for Ca and Mg determinations was washed with HCl and rinsed in doubly distilled, deionized water.

Reagents

The salts used for preparation of the perfusing solutions were of analytical reagent grade. The sources of other chemicals were as follows: paraformaldehyde, Fisher Scientific; OsO_4, General Chemical Division, Allied Chemical; ^{35}S-labeled Na_2SO_4, New England Nuclear.

RESULTS

Although the contractions of the heart were arrested by all the osmic acid concentrations used (0.327–32.7 mM), the coronary perfusion remained adequate for uniform fixation and for washing out of the excess fixative. A useful criterion of adequate fixation was a uniform blackening of the tissue upon inspection under a dissecting microscope. Adequate rates of coronary perfusion (>1 ml/min) were also maintained during perfusion with formaldehyde-containing solution at formaldehyde concentrations of 2.5–250 mM; by contrast, in salt solution containing 4 % formaldehyde, the rate of perfusion fell quickly to very low values (<0.02 ml/min). Spontaneous contractions ceased during perfusion with a medium containing 25 mM formaldehyde, but began again after 5–10 min of perfusion in formaldehyde-free solution. Although spontaneous contractions were not usually abolished during perfusion with 2.5 mM formaldehyde, atrio-ventricular dissociation and other disturbances of cardiac rhythm were observed at this low formaldehyde concentration. After exposure to formaldehyde at high concentrations (250 mM and 4 %), the left ventricles

had an inelastic, leathery consistency even after washing out the fixative. Fixation in formaldehyde at concentrations of 25 mM or lower did not apparently change the normal consistency of the muscles.

The ion contents of muscles perfused for 10 min with progressively increasing concentrations of osmic acid and formaldehyde are shown in Tables I and II, respectively. These values were obtained after re-perfusing the fixed muscles with fixative-free control solution. The tables show that exposure to both fixatives causes

TABLE I

ION AND WATER CONTENTS OF VENTRICLES EXPOSED TO OSMIC ACID

Data in this and the following table are given as mean ± S.E. Figures in parentheses give the number of experiments.

Concn. of osmic acid (mM)	*Tissue ion content (mmoles/kg dry wt.)*				*Tissue water content (kg water/kg dry wt.) × 100*
	K^+	Na^+	Ca^{2+}	Mg^{2+}	
0 (17)	382 ± 5*	320 ± 5*	5.1 ± 0.2	43.2 ± 0.8	81.9 ± 0.2
0.327 (7)	202 ± 6	542 ± 34	15 ± 2	44 ± 1	80.4 ± 0.8
3.27 (6)	56 ± 12	430 ± 26	68 ± 10	35 ± 2	82.1 ± 0.3
32.7 (5)	36 ± 5	689 ± 75	127 ± 10	35 ± 1	80.4 ± 0.8

* From PAGE AND PAGE[6].

TABLE II

ION AND WATER CONTENTS OF VENTRICLES EXPOSED TO FORMALDEHYDE

See legend of Table I.

Concn. of formaldehyde (mM)	*Tissue ion content (mmoles/kg dry wt.)*				*Tissue water content (kg water/kg dry wt.) × 100*	$\frac{SO_4^{2-}\ space \times 100}{total\ water}$
	K^+	Na^+	Ca^{2+}	Mg^{2+}		
25 (6)	339 ± 21	361 ± 55	—	—	82.5 ± 0.5	42 ± 2
250 (7)	221 ± 9	534 ± 17	—	—	82.0 ± 0.4	41 ± 5
4% (7)	91 ± 13	555 ± 34	7–14*	41–47*	79.2 ± 0.5	—

* Range of 3 determinations.

a redistribution of ions when muscles are subsequently perfused with a physiological salt solution without fixative. The magnitude of the changes increases with the concentration of the fixative. The changes occur at concentrations of osmic acid much lower than those of formaldehyde. The redistribution of ions consists of a marked net loss of K^+ and an uptake of Na^+ by the tissues. In addition, fixation with osmic acid leads to a striking net uptake of Ca^{2+}. For example, at a fixative concentration of 32.6 mM, ventricles exposed to OsO_4 accumulate an average of 25 times the normal Ca^{2+} content from a solution having the physiological (1.4 mM) Ca^{2+} concentration (Table I). At osmic acid concentrations of 3.27 and 32.7 mM such ventricles also undergo a small loss of Mg^{2+}; the loss is statistically highly significant ($P>0.01$),

and is remarkable because the ventricular Mg^{2+} content is usually stable even under rather extreme variations in external ionic composition or osmolality (E. PAGE, unpublished observations). In this connection, it is of interest that exposure to 4% formaldehyde brings about only a small net uptake of Ca^{2+} and no significant loss of Mg^{2+} (Table II).

The comparatively less pronounced redistribution of cations after perfusion with salt solution containing 25 mM and 250 mM formaldehyde suggested that the membranes of aldehyde-treated cells might at least partially retain their selective permeability to ions. We therefore examined the permeability of the membrane to sulfate, an anion which can be used to characterize tissue compartments in the rat's heart[6]. The values for the $^{35}SO_4^{2-}$ space after 30 min of perfusion with $^{35}SO_4^{2-}$ (Table II) indicate that SO_4^{2-} does not distribute itself uniformly throughout tissue water, a finding consistent with the interpretation that the cellular membrane systems retain their selectivity toward this anionic species. Preliminary observations on the distribution of Cl^- after aldehyde fixation lead to the same conclusion. Comparable data for 4% formaldehyde could not be obtained because the slow rate of coronary perfusion after exposure to this solution precluded the establishment of an equilibrium with respect to the tissue distribution of $^{35}SO_4^{2-}$.

The values of the dry weight in Table I have not been corrected for the osmium content of the tissue. This correction becomes appreciable after treatment with 32.7 mM osmic acid. The values for the Ca^{2+} content in Table I therefore underestimate the Ca^{2+} content per kg (osmium-free) dry wt.: the corrected value, obtained as described below, would be nearly twice that given in the table (see next paragraph), that is approx. 231 mmoles/kg dry wt., or approx. 46 times the normal tissue content of Ca^{2+}. Similarly corrected values for other ions are (in mmoles/kg dry wt.) K^+ 66, Na^+ 1253, and Mg^{2+} 64.

The data of Table I permit an estimation of the amount of osmium compound taken up by the ventricles. The highest osmic acid concentration used (32.7 mM) is of the same order of magnitude as that commonly used by electron microscopists for fixation. At this concentration we observed a striking increase in the wet and dry weights of the ventricles after perfusion with osmic acid. KRAMES AND VAN LIERE[7] have published a statistical analysis which allows prediction of the left ventricular weights from the weights of the rats. From a graphical plot of their data, we have obtained the normal wet and dry weights of the left ventricles from rats of known weight. The difference between these predicted weights and those observed after perfusion with osmic acid was attributed to the net uptake of osmium compound. This method yields a minimal estimate of the osmium compound accumulated, since the method assumes that the fixative does not leach out any gravimetrically significant component of the dry weight. For hearts perfused with 32.7 mM osmic acid (Table I), the gravimetric technique gives a minimal estimate of 0.45 ± 0.03 for the ratio (weight of osmium compound)/(total dry wt.).

DISCUSSION

The importance of uniform fixation

Two findings which bear on the nature of the interaction of osmium-containing fixatives with tissues are the striking net uptake of Ca^{2+} and the unexpectedly large

accumulation of osmium compound. These two findings have not previously been observed because studies directed at chemical changes produced in tissues by fixatives have had to rely on fixation of the specimen by immersion in the fixative. This technique produces non-uniform fixation: the inward diffusion of osmic acid from the surface of the tissue in contact with the fixative produces a gradient of fixative concentration, the highest concentration being found near the surface, the lowest near the core of the tissue. Moreover, the leaching out of tissue constituents by the fixative will also tend to be non-uniform—it will be greatest near the surface of the tissue. With the perfusion technique employed by us, it became possible to achieve very nearly uniform fixation throughout the left ventricle, and therefore to examine the chemical effects of osmic acid by analyses of whole ventricles.

Changes in the volume of cells and organelles as the result of fixation

In kidney tissue MAUNSBACH[8] has achieved excellent fixation for electron microscopy by perfusion with aldehyde concentrations as low as 25 mM. Since 25 mM aldehyde causes only very small disturbances in the cardiac contents of ions and water (Table II), fixation with such low concentrations would appear to merit more serious consideration than heretofore.

Conclusions about the relative volumes of cells, extracellular space, and cellular organelles have been based on electron micrographs of cardiac cells after physiological experiments. In such experiments cell volume has been changed by altering the extracellular tonicity and the cells have then been fixed[3]. Inferences about the interrelation of function and ultrastructure have been based on this evidence[4].

The results of the present experiments with formaldehyde suggest, however, that estimates of the volumes of tissue components from aldehyde-fixed material are subject to two types of artifacts. The first originates from the fact that tissues fixed at the lower concentrations of aldehyde do not become indiscriminately permeable to small solutes. The cellular and sub-cellular membranes therefore retain at least partially their ability to respond to an osmotic gradient with a net movement of water into or out of the membrane-limited compartment. A second type of artifact results when fixed tissues are washed free of fixative with physiological salt solution, a procedure commonly used in preparing heart muscle for electron microscopy. Tables I and II show that treatment with fixatives gives rise to a marked loss of cellular K^+ and a net uptake of Na^+. These ion movements resemble those which occur when the active transport of Na^+ out of cardiac cells is inhibited[9]. Qualitatively similar effects of fixatives on ion distributions have been reported to occur in the frog's urinary bladder[10], and in the eggs of *Linnea stagnalis* L. (see ref. 11). However, both osmic acid[12] and formaldehyde (ref. 13; H. A. FOZZARD, personal communication) produce a change in resting potential and other electrophysiological evidence for an altered passive permeability to ions. It cannot be decided on the basis of the present experiments whether the loss of K^+, the uptake of Na^+, and the change in membrane potential which follow treatment of cardiac cells with osmic acid or formaldehyde are due to an inhibition of active transport, to a change in passive permeability to ions, or to both factors. Whatever the cause, it seems probable that these changes are accompanied by net movements of water across cellular and sub-cellular membranes. The distortions thus produced in the physiological volumes do not require that the fixative be hypertonic, but should occur even in isotonic fixatives.

The uptake of calcium and osmium by osmium-fixed tissues

REVEL reported that the plasma membranes of skeletal muscles fixed in osmic acid in the presence of 10 mM Ca^{2+} appeared to 'stain' darkly in electron micrographs, even in the absence of the usual electron microscopic 'staining' with salts of uranium or lead. He did not investigate this phenomenon further, since the reaction could not be reproducibly obtained. With the uniform fixation achieved in the present study, we have been able to reproduce at will a large net uptake of Ca^{2+} during fixation in 3.27 and 32.7 mM osmic acid in the presence of 1.4 mM $CaCl_2$. REVEL's observations make it seem probable that a substantial fraction of the Ca^{2+} uptake found by us is localized to the plasma membrane. Recent experiments on embryonic tissue, including heart muscle, have disclosed the presence of an electron-opaque layer, about 50 Å thick, at the outward-facing surface of the plasma membrane[14]. This layer has an affinity for the divalent lanthanum ion, an ion whose physiological effects resemble those of Ca^{2+} in important respects[15]. If Ca^{2+} is being taken up selectively by the plasma membrane, the fact that the uptake renders the membrane opaque to an electron beam implies that the amount of Ca^{2+} per unit volume of membrane must be large. Ca, an element of low atomic number, scatters electrons less well than heavier elements; it follows that relatively large amounts of Ca^{2+} must be taken up per unit volume of membrane to achieve a density of scattering sites sufficient to opacify the plasma membrane in electron micrographs. The fact that osmium-fixed plasma membranes bind Ca^{2+} also suggests the possibility that they may similarly take up heavier alkaline earth metals which scatter electrons more effectively.

The present results raise the question of why osmium-treated tissues take up such large amounts of Ca^{2+}. KORN[16–18] has recently shown that OsO_4 reacts with olefinic groups in lipids to form the stable osmic acid esters of glycols. The osmium appears to be covalently bound to the hydrocarbon portions of lipids in membranes fixed with OsO_4. The results of this paper would suggest that the amount of osmium compound so fixed is unexpectedly large. Since we have not investigated the degree to which our method of fixation saturates the potential sites of combination with OsO_4, it is possible that even greater amounts of osmium could be bound. The black osmium compounds present in fixed tissues are difficult to define precisely[19]; for this reason we have not calculated either the amount (in moles) of osmium bound per unit dry wt. or the value of the ratio (moles Ca^{2+} taken up)/(moles osmium bound). The stoichiometry of the reaction between Ca^{2+}, osmic acid, and cardiac membranes, as well as the identification of the reacting groups in the membrane, remain as problems for future experiments.

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