PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 161, 435–437 (1979)

Effects of Low Ca^{2+} on the External Lamina of Cardiac Cells (40568)

R. G. KNIGHT, T. H. ROSENQUIST, AND T. M. NOSEK

Departments of Physiology and Anatomy, Medical College of Georgia, Augusta, Georgia 30901

The membrane that separates the intracellular from the extracellular compartment of mammalian myocardial cells has two components, the lipid bilayer and an external protein–polysaccharide coat approximately 50-nm thick (1). This external coat has been referred to as the basal lamina, external lamina, basement membrane, or glycocalyx and corresponds to the basement membrane of epithelial cells. Howse *et al.* (2) propose that the external lamina, as well as the lipid bilayer, acts as a selective permeability barrier between intracellular and extracellular compartments. Their histochemical studies have shown that the external lamina is rich in acidic residues which impart a high density of negatively charged sites. The electrostatic field produced by the polyanionic glycosaminoglycans hinders diffusion of anions into the cell while strongly binding cations, most notably Ca^{2+} (3). Loss of membrane-bound Ca^{2+} by simple diffusion into a Ca^{2+}-free medium results in a decrease in the selective permeability characteristics of the membrane (4). Winegrad (5) has found that the divalent cation chelating agents ethylenediaminetetraacetic acid (EDTA) and [ethylenebis(oxyethylene-nitrile)]tetraacetic acid (EGTA) further unselectively increase membrane permeability to small ions to such an extent that they functionally "skin" the myocardial cells. Winegrad (5) has suggested that EDTA and EGTA produce this effect by chelation of membrane-bound calcium. Accordingly, the number of free cation binding sites should be larger in EDTA-treated muscles than in preparations bathed in Ca^{2+}-free media. The purpose of this study was to test this hypothesis.

A preliminary communication of a part of this work has already appeared (6).

Materials and methods. Male guinea pigs, weighing between 200 and 400 g were used in all experiments. The guinea pigs were stunned by a blow to the head, and their hearts were rapidly excised. The right ventricle from each heart was divided into two parts of approximately equal size, one-half serving as a control and the other as the test preparation. Both muscle halves were initially bathed in a modified Krebs solution of the following composition: 128 m*M* NaCl, 5.6 m*M* KCl, 1.2 m*M* $MgCl_2$, 2.5 m*M* $CaCl_2$, 10.0 m*M* Tris buffer, and 10 m*M* dextrose at pH 7.4. The control muscle remained in this solution, maintained at 30° and oxygenated with 100% O_2, for the duration of the experiment.

The test muscle, trimmed to a width of approximately 3 mm and length of 7 mm, was clamped at one end into a hollow aluminum tube and secured to a steel post in a 1.5-ml Plexiglas bathing chamber. The other end of the muscle was similarly clamped to an isometric strain gauge mounted on a micromanipulator. The force of the isometric contractions of the muscle strips was displayed on a strip chart recorder. These records were graphically analyzed for resting tension and maximum developed tension.

The test muscle strip was superfused with physiological solution at a rate of 3 ml/min by a gravity flow-through system. The temperature of the bathing solution was continuously monitored by a thermocouple and telethermometer. A thermoelectric module and feedback circuitry controlled the temperature of the inflowing solution and maintained the temperature of the solution bathing the muscle at 30°.

An initial resting tension sufficient to obtain maximal developed tension was applied to the muscle strip. The muscle was stimulated at 60 cpm through two platinum electrodes (one in contact with the muscle and the other juxtaposed) by an electrically isolated stimulator. Both active and resting tensions were allowed to reach steady-state values before pacing was stopped. The muscle was then superfused with a membrane-disrupting solution of the following composition: 3 m*M* EDTA, 10 m*M* dextrose, 10 m*M* Tris

0037-9727/79/090435-03$01.00/0

buffer, and 140 m*M* KCl, pH 7.0. After 15 min of exposure to this solution, three different contracture solutions were introduced to the muscle strip to determine the completeness of membrane disruption. These solutions contained 3 m*M* EGTA, 10 m*M* dextrose, 10 m*M* Tris buffer, 140 m*M* KCl, and 1 m*M* $MgCl_2$ at pH 7.0. An apparent stability constant for Ca-EGTA of $10^{6.69}$ M^{-1} at pH 7.0 (7) was used to determine the appropriate amount of $CaCl_2$ to be added to 3 m*M* EGTA to produce Ca^{2+} ion concentrations of 1×10^{-7}, 1×10^{-6}, and 1×10^{-5} *M*. Resting tension was followed for three consecutive 10-min periods during which the muscle was exposed to each of the contracture solutions.

Following the third contracture solution, a relaxation solution of the same composition but containing no $CaCl_2$ superfused the preparation for 10 min. The test muscle and its control were then quickly frozen in liquid nitrogen. Serial sections of the muscles 20-μm thick were cut in a cryostat at −20° and mounted on microscope slides. The slides were immersed overnight in a Ca^{2+}-free 0.05% Alcian blue 8GX medium containing 0.1 *M* $MgCl_2$, 0.2 *M* sodium acetate, and 0.2 *M* acetic acid. Alcian blue has from two to four cationic groups per molecule which confer high electrostatic attraction of the dye for the polyanions of the external lamina and nuclear material (8–10). Copper is present in a 1:1 molar ratio in the Alcian blue molecule. Therefore, the amount of Alcian blue bound to the muscles was determined by measuring the copper content with atomic absorption spectrophotometry (11). Nuclear staining was assumed to be uniform throughout each ventricle. Therefore, any effect of EDTA on Alcian blue binding to the entire myocardial cell was attributed to a change in the amount of dye bound to the external lamina which is in turn an index of the cation binding capacity of this structure.

Results. To substantiate that the EDTA-disrupting media increased the permeability of the myocardial membrane, we measured the effect of various extracellular Ca^{2+} ion concentrations on the resting tension of all disrupted muscle strips. Resting tension increased 10-fold as the external calcium concentration was raised from 1×10^{-7} to 1×10^{-5} *M*. These results are similar to those reported by Winegrad (5). Because changes in the Ca^{2+} concentration over this range have no effect on the resting tension of untreated muscle preparations (5), these results suggest that EDTA significantly reduced the membrane permeability barrier of our test muscles, particularly to Ca^{2+}.

Light microscopy demonstrated that Alcian blue stained both the nuclei and external lamina of the cardiac cells. Atomic absorption spectrophotometry revealed that EDTA disruption significantly reduced the amount of dye bound to the muscle by an average of 21 ± 3% as indicated by the amount of copper found in the muscles (see Table I). This result indicates that EDTA reduced the cation binding capacity of the external lamina.

Discussion. An increase in the number of cation binding sites on the external lamina in EDTA-treated muscles would be consistent with the hypothesis that EDTA skins myocardial cells by chelating membrane-bound Ca^{2+}. That the number of cation binding sites was reduced by EDTA does not disprove this hypothesis. However, it suggests that the chelation of Ca^{2+} by EDTA does more to the membrane than merely decrease the amount of Ca^{2+} bound to the external lamina. The decreased number of cation binding sites can be explained in at least three ways: (i) The number of cation binding sites available for binding the dye has been reduced due to competition between EDTA and the dye for the sites; (ii) the number of cation binding sites has been reduced by destruction of the external lamina; (iii) chelation of divalent cations by EDTA has caused conformational changes in the external lamina such that a smaller number of the cation binding sites are available for binding the dye.

It is unlikely that EDTA is capable of competing with Alcian blue for the negatively

TABLE I. EFFECTS OF EDTA ON COPPER CONTENT OF MUSCLES STAINED WITH ALCIAN BLUE 8GX[a]

Treatment	Myocardial weight (mg)	Alcian blue concentration[b]
Control	0.323 ± 0.020	1.688 ± 0.065
EDTA	0.277 ± 0.054	1.329 ± 0.056*

[a] Values are means ± SE of six paired experiments.
[b] Micrograms of Cu per cc per mg tissue.
* Significantly smaller than control, $P < 0.01$ by a Student's *t* test.

charged binding sites because EDTA itself is a multidentate ligand and carries a strong negative charge at pH 7.0 (12). Eastwood *et al.* (13) have correlated an irreversible loss of ion-selective permeability of the sarcolemma of chemically skinned human and rabbit skeletal muscles with severe disruption of the sarcolemma; large gaps in the membrane were observed in electronmicrographs. In contrast, Winegrad (5) and McClellan and Winegrad (14) have demonstrated that chemically skinned myocardial preparations display no gross disruption of the sarcolemma. Therefore, it is unlikely that EDTA treatment reduces the number of cation binding sites by destroying the external lamina. On the other hand, studies on various solutions of glycosaminoglycans have demonstrated that they undergo changes in conformation in response to small changes in the ionic composition of their microenvironment (15–17). Electronmicrographs of chemically skinned rat myofibers show effects of skinning on the external lamina; it becomes less electron dense and more fibrous in appearance (14). Our results suggest that those changes in appearance of the external lamina reflect conformational changes induced by a decrease in the Ca^{2+} concentration of the microenvironment of the external lamina below a critical level. These changes, which are not always completely reversible (5), decrease the number of cation binding sites available for the Alcian blue dye and increase the permeability of the membrane unselectively to small ions. Our results are supportive of the concept that membrane-bound Ca^{2+} provides a stabilizing influence on membrane permeability by affecting the conformation of molecules that constitute the membrane.

Summary. EDTA decreases the binding of Alcian blue to the external lamina of the myocardium and increases membrane permeability to small ions unselectively. The combination of these events support the notion that chelation of calcium by EDTA causes conformational changes in the external lamina to which the changes in membrane permeability may be attributed.

1. Fawcett, D. W., and McNutt, N. S., J. Cell Biol. **42,** 1 (1969).
2. Howse, H. D., Ferrans, V. J., and Hibbs, R. G., J. Mol. Cell. Cardiol. **1,** 157 (1970).
3. Scott, J. E., *in* "The Chemical Physiology of Mucopolysaccharides" (G. Quintarelli, ed.), p. 171. Little–Brown, Boston (1968).
4. Peach, M. J., *in* "The Pharmacological Basis of Therapeutics" (L. S. Goodman and A. Gilman, eds.), p. 892. Macmillan, New York (1975).
5. Winegrad, S., J. Gen. Physiol. **58,** 71 (1971).
6. Knight, R. G., Nosek, T. M., and Rosenquist, T. H., The Physiologist **21,** 66 (1978).
7. Hellam, D. C., and Podolsky, R. J., J. Physiol. London **200,** 807 (1969).
8. Scott, J. E., Quintarelli, G., and Dellovo, M. C., Histochemie **4,** 73 (1964).
9. Salthouse, T. N., Stain Tech. **36,** 63 (1961).
10. Scott, J. E., and Dorling, J., Histochemie **5,** 221 (1965).
11. Rosenquist, T. H., Histochem. J. **9,** 127 (1977).
12. Fritz, J. S., and Schenk, G. H., "Quantitative Analytical Chemistry," p. 244. Allyn and Bacon, Boston (1974).
13. Eastwood, A. B., Wood, S., and Bock, K. L., Biophy. Soc. Annu. Meet. Abstr. **21,** 185a (1978).
14. McClellan, G. B., and Winegrad, S., J. Gen. Physiol. **72,** 737 (1978).
15. Cleland, R. L., and Wang, J. L., Biopolymers **9,** 799 (1970).
16. Mathews, M. B., Biochim. Biophys. Acta **35,** 9 (1959).
17. Obrink, B., Pertoft, H., Inverius, P. H., and Laurent, T. C., Connective Tissue Res. **3,** 187 (1975).

Received November 3, 1978. P.S.E.B.M. 1979, Vol. 161.