PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 148, 113–117 (1975)

Ultrastructural Effects of *in Vitro* Experimentation on Right Ventricular Papillary Muscle from Cats in Hypovolemic Shock[1] (38487)

R. D. GOLDNER, N. B. RATLIFF, R. I. KOPELMAN, AND D. B. HACKEL

Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710

Characteristic structural lesions develop in cardiac muscle during hypovolemic shock. One category of lesion consists of subendocardial hemorrhage and necrosis which has been related to hypoxia (1, 2). The other is the "zonal lesion" which may represent focal supercontraction adjacent to intercalated discs (3) and which probably is not related to hypoxia (2). There is evidence that ventricular function deteriorates in hypovolemic shock, but the etiology of this functional deterioration is poorly understood (4, 5).

Examination of the relationship between structural and functional changes induced in cardiac muscle by hypovolemic shock may require sustaining papillary muscles *in vitro* for several hours during which time they are stimulated and various pharmacologic agents are added to the muscle perfusate. Lefer *et al.* have demonstrated the isolated cat papillary muscle preparation to be useful for the *in vitro* study of the physiologic effects of hemorrhagic shock on cardiac muscle (6).

The purpose of the present study is to determine if repeated electrical stimulation and two inotropic agents alter the ultrastructure of right ventricular papillary muscle from cats subjected to hypovolemic shock or from controls.

Materials and Methods. Three healthy adult cats were anesthetized with an intraperitoneal injection (0.25 ml/kg) of a 1:1 mixture by volume of sodium pentobarbital (Nembutal) and allobarbital with urethane (Dial-urethane). The latter mixture contained 30 mg pentobarbital, 50 mg allobarbital, and 200 mg urethane per ml. Penicillin (50,000 units) and streptomycin (0.125 g) were administered intramuscularly. An endotracheal tube was inserted, each animal was placed on a heating blanket to maintain body temperature at 37°, and the electrocardiogram was recorded throughout the procedure.

Heparin (500 units/kg) was administered intravenously through a polyethylene cannula inserted in the right femoral vein. Cannulas were inserted into both femoral arteries. The cannula placed in the left femoral artery was connected to a saline manometer to record mean arterial pressure, and the one in the right femoral artery was connected to a closed, plastic reservoir bottle (containing 4000 units heparin) for bleeding. The cats were bled into the reservoir during a 30 min period so that the mean arterial pressure fell slowly to 50 mm Hg from the mean control level of 125 mm Hg. The pressure was maintained at this level for 1–2 hr by placing the reservoir at the appropriate level.

The heart was quickly removed through a midline thoracotomy and placed in modified Krebs–Henseleit solution (7) equilibrated with 95% O_2 and 5% CO_2, and the right ventricle was opened with scissors. While immersed in perfusate, silk sutures were tied around each end of two right ventricular papillary muscles (≤ 1 mm in diameter), and the muscles were excised. One isolated right ventricular papillary muscle was gently stretched and immediately fixed, as described below, for electron microscopy. The other isolated muscle was attached by the sutures to an RCA 5743 force transducer and to a stationary glass hook, and was placed between two coiled platinum electrodes in a 20 ml lucite chamber. The electrodes were connected to a Grass stimulator. The chamber was filled with modified Krebs-Henseleit solution gassed with 95% O_2 and 5% CO_2 which was kept at 37° with a water jacket connected to a temperature controlled water circulator.

The isometric twitch tension signal from

[1] This work was supported in part by a grant from the American Heart Association, with funds contributed in part by the N.C. Heart Association, and by a grant from the USPHS (HL-05875). Dr. Hackel is the recipient of a Career Research Award from the USPHS (HL-K6-14188).

the transducer was amplified on a Beckman recorder which was calibrated with a 1 g weight on the transducer. At the point at which passive tension on the muscle produced a visible signal on the recorder, threshold voltage was identified. At the peak of the length-tension curve produced at this voltage, threshold was determined again; the peak of a second length-tension curve was found, and the muscle was set at that length. The Grass stimulator was set to deliver repetitive square wave pulses 2 V greater than the threshold voltage, at a rate of 1 per sec and a duration of 20 msec.

The muscle was stimulated for 2–3 hr at the peak of its length-tension curve. Adrenalin chloride (1.0 μg/ml) and calcium carbonate (1.0 mg/ml) were intermittently added to the Krebs–Henseleit perfusate bathing the muscle. The muscle was washed twice with fresh Krebs–Henseleit perfusate before the addition of each different solution. All solutions added to the chamber were prewarmed to 37°; and after the addition of a new solution, at least 10 min were allowed for equilibration.

The isometric twitch tension produced at the beginning of the experiment with the muscle bathed in Krebs–Henseleit perfusate was compared to that produced at the completion of the experiment with the muscle in the same perfusate.

At the completion of the experiment, the chamber was lowered from around the muscle; and within 2 sec the muscle, still stretched to the peak of its length-tension curve, was in Karnovsky's fixative. It was fixed for 1 hr at room temperature and then transferred to fresh fixative at room temperature for an additional hour. It was then postfixed in 2% *s*-collidine buffered osmium tetroxide for 1.5 hr, dehydrated in a series of graded ethanols followed by propylene oxide, and embedded in Epon 812. Thick sections (0.5 μm) were cut on a Porter-Blum microtome with a glass knife and were stained with toluidine blue. Adjacent thin sections (25–50 nm) of each block were then cut with a diamond knife, picked up on collodion coated grids and sequentially stained with 2% uranyl magnesium acetate and lead citrate and photographed with Hitachi HS-8 or HU-11E microscopes.

Seven control cats were anesthetized either with Nembutal and Dial-urethane as described above or with only Nembutal (35 mg/kg). Two were immediately sacrificed and their papillary muscles were excised and fixed as described above. Five were maintained under anesthesia for the same length of time as the shock cats (1–2 hr) but were not bled. One of their right ventricular papillary muscles was excised and immediately fixed as described above. The other was placed in the muscle chamber and was stimulated for 2–3 hr at the peak of its length-tension curve and subsequently fixed in a fashion identical to that described above for the shock cats.

Results. The papillary muscles that were excised and fixed immediately after anesthetization of the two cats were judged normal by light and electron microscopic examination (conforming to previous description (8) of normal cat right ventricular papillary muscle) as were each of the muscles excised from the five other control cats and fixed without further manipulation.

The light and electron microscopic appearance of muscles stimulated for 2–3 hr in oxygenated, modified Krebs–Henseleit solution at the height of their length-tension curves, was also normal in the five control animals (Figs. 1 and 4). The isometric twitch tension of each muscle was the same at the beginning and the end of the stimulation period. The ultrastructural appearance of muscles from control cats anesthetized with Nembutal and Dial-urethane was the same as that from animals anesthetized with only Nembutal.

There were no light microscopic or ultrastructural differences between those shock muscles stimulated for 2–3 hr in oxygenated, modified Krebs-Henseleit perfusate and those shock muscles fixed without further manipulation (Fig. 2). Muscles from the three cats put into hemorrhagic shock all demonstrated ultrastructurally characteristic zonal lesions (Figs. 3 and 5). The zonal lesions consisted of marked cellular contraction and distortion of myofilaments adjacent to intercalated discs, fragmentation of Z bands, displacement of mitochondria from the center to the periphery of the lesion and convolution of the sarcolemma in this area. The zonal lesions

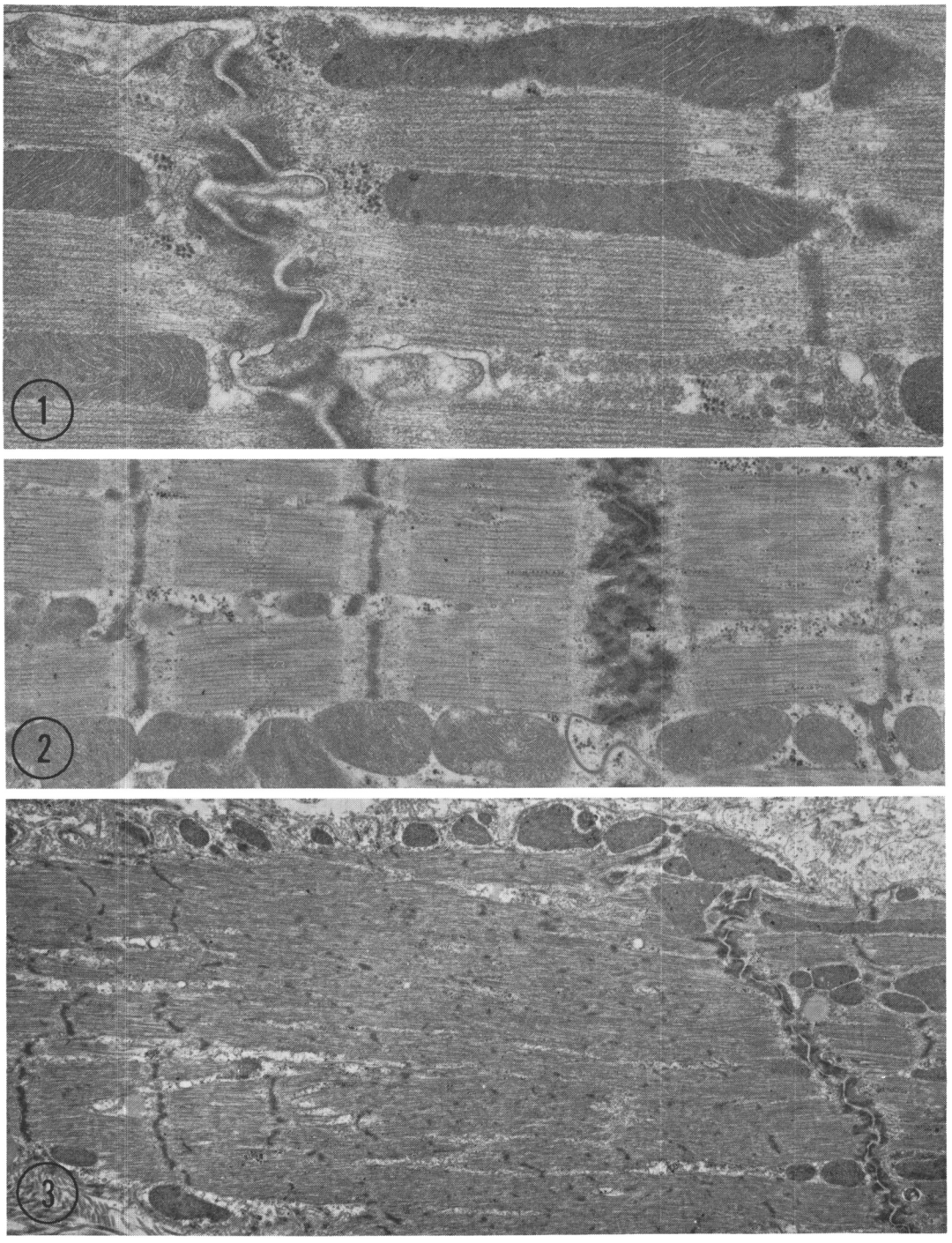

FIG. 1. Portion of normal, unstimulated right ventricular papillary muscle (RVPM) from a control cat. Elongated mitochondria extend to both sides of intercalated disc (left). A, I, and Z bands are distinct and structurally normal. 30,000 X.

FIG. 2. Normal portion of unstimulated RVPM from a shock cat. Mitochondria are between sarcomeres and extend to the intercalated disc. Note the regular arrangement of A, I, and Z bands. 15,400 X.

FIG. 3. Zonal lesion in stimulated RVPM from a shock cat. Mitochondria are present under the folded sarcolemma at the periphery of the lesion (top) but are absent between sarcomeres and adjacent to the left side of the intercalated disc. Myofilaments are distorted, and Z bands are fragmented. A and I bands are not discernable. 11,600 X.

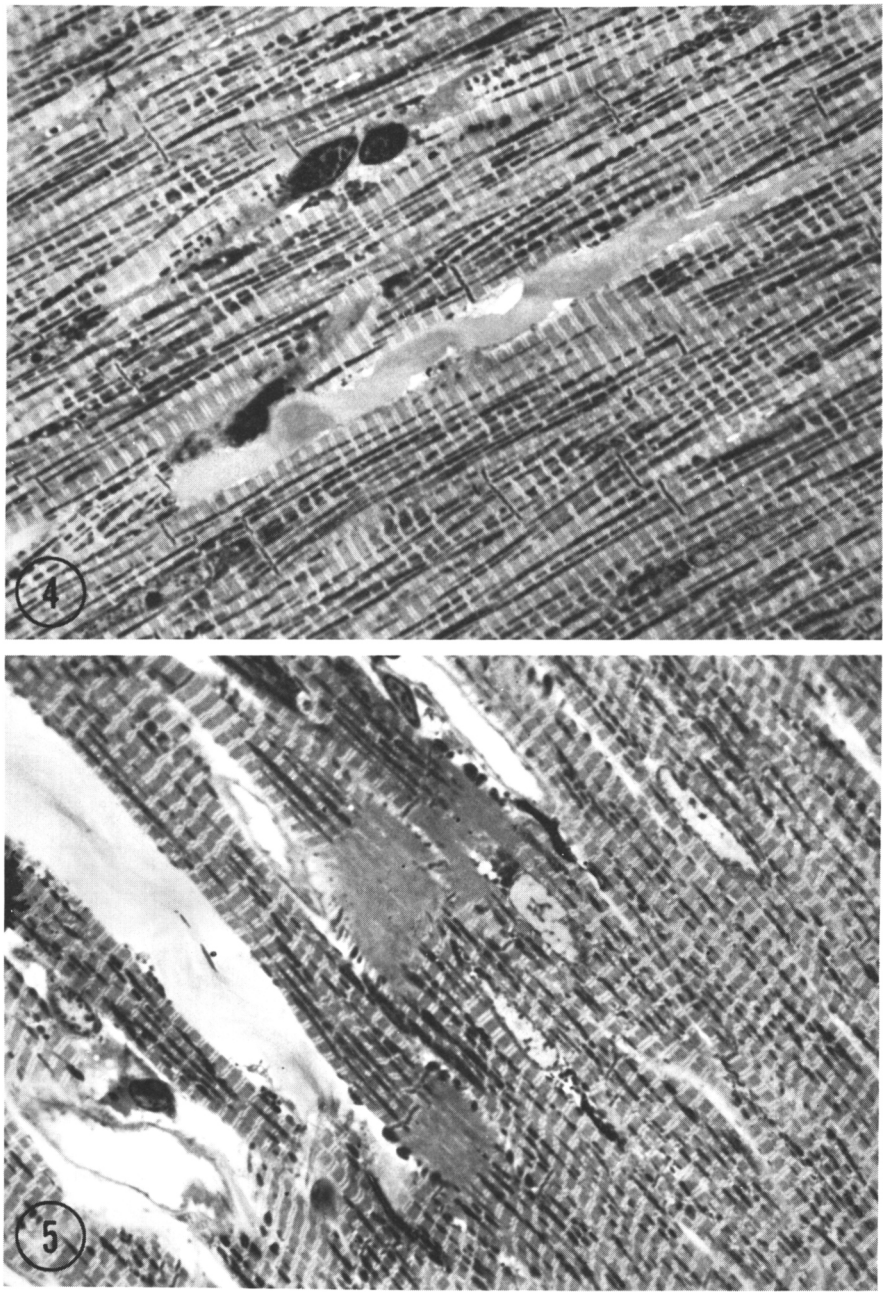

FIG. 4. Portion of normal, unstimulated RVPM from a control cat. A bands, I bands, and intercalated discs are distinct, structurally normal and are in register. Light micrograph, Epon-embedded, 0.5 μm section stained with toluidine blue. 1000 X.

FIG. 5. Multiple severe zonal lesions, stimulated RVPM from a shock cat. The zonal lesions appear as relatively homogeneous areas adjacent to the intercalated discs with loss of recognizable A and I bands. In the remainder of the myocytes the A and I bands are less in register than in Fig. 4. Fully developed lesions such as these are not difficult to recognize by light microscopy, but the absence of such changes does not insure that less fully developed zonal lesions are not present in the muscle. Light micrograph, Epon-embedded, 0.5 μm section stained with toluidine blue. 1000 X.

were frequently not demonstrated by light microscopy of thick sections stained with toluidine blue, even when adjacent thin sections examined by electron microscopy showed numerous, unequivocal zonal lesions.

The isometric twitch tension of muscles from cats subjected to hypovolemic shock was the same at the beginning and at the end of the stimulation period.

Discussion. The present study confirms that normal cat papillary muscle can be stimulated for 2–3 hr in an oxygenated solution of modified Krebs-Henseleit buffer without developing either ultrastructural changes (8) or changes in the recorded isometric twitch tension between the beginning and the end of the experimental period (6). It demonstrates for the first time that such *in vitro* experimentation can be performed on muscles injured by hypovolemic shock (lesions documented by electron microscopy) without altering the ultrastructure of the myocyte or of the zonal lesions and without producing changes in function as determined by comparing the isometric twitch tension at the beginning and the end of the experimental period.

The data indicate that electron microscopy is important to the evaluation of papillary muscles from animals subjected to hypovolemic shock, because zonal lesions and early necrotic changes may not be demonstrated by routine light microscopy (1). By light microscopy of frozen sections incubated for succinic dehydrogenase activity, severe zonal lesions do appear as a clear zone adjacent to the intercalated disc (1–3). A similar zone is visible in Epon-embedded, 0.5 μm sections stained with toluidine blue (Fig. 5). This clear zone results from translocation of the mitochondria from the involved area, but it is not visible in lesions with less pronounced mitochondrial translocation, i.e., zonal lesions in the formative stages (9).

The procedure described is particularly applicable to electron microscopic study because muscles can be placed in fixative within 2 sec after removal from oxygenated perfusate, and entire papillary muscles can be fixed at the peak of their length-tension curves. In addition their small size facilitates economic examination of the entire muscle by electron microscopy, thereby minimizing sampling errors.

Summary. The present data demonstrate for the first time that repeated stimulation (2–3 hr in an oxygenated perfusate) of right ventricular papillary muscles from cats subjected to hypovolemic shock does not alter the ultrastructure of the shock lesions. Neither does the *in vitro* experimentation cause functional impairment as determined by the measured isometric twitch tension. The data also indicate that electron microscopy is important to the evaluation of papillary muscles from cats subjected to shock.

1. Martin, A. M., Jr., and Hackel, D. B., Lab. Invest. **12,** 77 (1963).
2. Ratliff, N. B., Hackel, D. B., and Mikat, E., Amer. J. Pathol. **51,** 341 (1967).
3. Martin, A. M., Jr., Hackel, D. B., and Kurtz, S. M., Amer. J. Pathol. **44,** 127 (1964).
4. Crowell, J. W., and Guyton, A. C., Amer. J. Physiol. **203,** 248 (1962).
5. Siegel, H. W., and Downing, S. E., Amer. J. Physiol. **218,** 772 (1970).
6. Lefer, A. M., Craddock, G. B., Cowgill, R., and Brand, E. D., Amer. J. Physiol. **211,** 687 (1966).
7. Lefer, A. M., J. Pharmacol. Exp. Ther. **151,** 294 (1966).
8. Fawcett, D. W., and McNutt, N. S., J. Cell Biol. **42,** 1 (1969).
9. Ratliff, N. B., Kopelman, R. I., Goldner, R. D., and Hackel, D. B., Amer. J. Pathol. **74,** 77a (1974).

Received May 21, 1974, P.S.E.B.M. 1975, Vol. 148.