The Plasma Membrane of Leading Pacemaker Cells in the Rabbit Sinus Node
A Qualitative and Quantitative Ultrastructural Analysis

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SUMMARY We studied the fine structure of the plasma membrane of electrophysiologically identified leading pacemaker cells from the rabbit sinus node, using both ultrathin sections of fixed tissue and replicas of freeze-cleaved material. We found that differences exist between sinus node and working myocardial membranes, but these are only quantitative. The caveolae or sarcolemmal invaginations are present in very large numbers; they increase the surface area of the plasma membrane by about 100%. The small macular nexuses that are present represent 0.2% of the membrane surface area. Nexuses are therefore about 10 times less numerous in leading sinus node cells than in working myocardium cells. A simple equivalent electrical representation of the sinus node shows, nevertheless, that an appreciable electrical coupling may be expected.

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SINUS NODE cells have special electrophysiological properties that make them different from other heart cells. The most typical of these properties are: (1) a low resting potential, (2) the presence of a diastolic depolarization, and (3) the absence or permanent inactivation of a rapid sodium channel (see for review Brooks and Lu, 1972). This physiological behavior seems to indicate that the pacemaking process is related to special properties of the sarcolemma. We thought it interesting to know whether these special physiological properties could be related to a special ultrastructural organization of the sarcolemma. Therefore, we studied the fine structure of the plasma membrane of electrophysiologically localized leading pacemaker cells from the rabbit sinus node. Both ultrathin sections of fixed material and replicas of freeze-cleaved tissue were observed. Special attention has been paid to the membrane invaginations or caveolae and to the nexuses or gap junctions which have been quantified. The results have been compared to those found in other myocardial cell types described in literature (e.g., McNutt and Weinstein, 1970; McNutt, 1975; Ishikawa and Yamada, 1975; Mazet and Cartaud, 1976; Kensler et al., 1977; Gros et al., 1978; Gabriella, 1978).

Methods

Preparation

Rabbits of both sexes weighing 2.5-3 kg were anesthetized with Hypnorm (10 mg fluanison + 0.2 mg fentanyl base/kg, im). A small part of the right atrium, including the sinus node and the crista terminalis, was isolated and pinned to a perforated silicon rubber block in a tissue bath so as to expose the endocardial surface. The upper part of the crista terminalis was cleaved to obtain a good exposure of the sinus node. The bath was perfused continuously with a solution containing, in mmol/liter NaCl, 130.6; NaHCO3, 24.2; KCl, 5.6; CaCl2, 2.2; MgCl2, 0.6; glucose, 11.1; saccharose, 13.2; saturated with a mixture of 95% O2 and 5% CO2, pH 7.4. Temperature was kept constant within 0.1° at 38°C.

Electrophysiological Localization of the Leading Pacemaker Site

The method is described in detail elsewhere (Bleeker et al., unpublished observations). Briefly, action potentials were recorded by means of the conventional glass microelectrode technique. The exploring microelectrode was mounted in a micro-manipulator on a mechanical stage, the lateral movements of which were read with vernier scales accurate to 0.01 mm. Two coordinates were recorded for each impaled cell.

The area of the early-discharging cells was located after mapping the propagation of excitation in the nodal area, by recording the electrical activity from a great number of cells. As the activation time of a cell, we chose the moment at which the transmembrane potential reached half the amplitude of the action potential. The activation moment of the impaled cells was timed with respect to an atrial electrogram which served as reference.

Electron Microscopy

At the end of the electrophysiological mapping, which took 1-3 hours, the site of the leading pacemaker group was marked iontopheretically. For that purpose, the microelectrode was filled with a...
1% solution of Alcian blue (Lee et al., 1969), and two dots were made 200 μm apart, one on each side. Then the preparation, still mounted to the rubber block, was immersed in cold 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The fixation was performed during one night for the preparations intended for ultrathin sectioning, and for half an hour for the preparations destined for freeze fracturing. Afterward, the preparations were washed in 0.15 M cacodylate buffer, and the small selected piece was cut 50 to 80 by 200 μm, parallel to the crista terminalis, using two razor blades glued together.

For thin sectioning, the small fixed fragment subsequently was postfixed in 1% OsO₄ in 0.1 M cacodylate buffer for 1 hour at 4°C, washed again in cacodylate buffer, immersed in 0.5% magnesium uranyl acetate in 0.9% NaCl for 20 minutes, dehydrated in acetone, and embedded in Araldite. The sections were cut on a Reichert ultramicrotome with glass or diamond knife and stained with lead citrate.

Lanthanum (according to the method of Revel and Karnovsky, 1967) was used to mark the extracellular spaces in two preparations. The blocks were postfixed in 1% osmium tetroxyde-1% lanthanum nitrate in collidine buffer, dehydrated in acetone, and embedded in Araldite.

For freeze cleaving, the briefly fixed specimen was washed quickly in 0.15 M cacodylate buffer and infiltrated successively with 10, 20, and 30% glycerol in the same buffer. The piece then was mounted on a gold disc and frozen in Freon 22 cooled with liquid nitrogen. Freeze fracturing and platinum-carbon shadowing were carried out on a Balzer BAF 300 freeze-etch unit at −100°C and in a vacuum of 3×10⁻⁶ KPa. The freeze-cleaved leading pacemaker sites from three hearts have been used in this study. Replicas and ultrathin sections were examined with a Philips EM 301 electron microscope.

Measurements

Mean Surface Area of the Gap Junctions and Density of the Intramembranous Particles

The mean surface area of the gap junctions and the density of the intramembranous particles (IMP) on the protoplasmic fracture (PF) and external fracture (EF) faces were evaluated on micrographs of replicas at a final magnification of 188,000. Careful attention was paid to the choice of the sites used for these measurements; only those sites that appeared flat and parallel to the plane of fracture were used. Surface areas of gap junctions and of nonjunctional membranes used for counting the particles were measured by superimposing transparent graph paper, with a spacing of 1 mm. Then the outlines of the surface areas to be measured were traced and the surfaces were calculated.

Occurrence of Gap Junctions in Cell Borders

Three hearts were used for these measurements, two being cut longitudinally and the other perpen-

dicularly to the crista terminalis. Micrographs of ultrathin sections at a final magnification of 30,000 were used for measurements of cell borders and gap junctions. A map reader was rolled along the images of the borders, and the lengths were recorded. The circumferences of more than 40 adjacent cells were recorded for each heart. Gap junctions were identified, measured, and their length recorded in a similar fashion. Application of this method to circles, arcs, and polygons of known circumferences, lengths, and perimeters gave maximal errors of about 1% in the measurements.

Results

Aspect of the Membranes

The PF and EF faces of the plasma membranes of the leading pacemaker cells, as practically all plasma membranes examined so far, are studded with IMP about 9 nm in diameter and distributed randomly (Figs. 1 and 2).

The number of particles counted is 1339 ± 69 (SEM) per μm² on PF faces and 347 ± 33 (SEM) per μm² on EF faces; i.e., PF faces exhibit about 4 times more particles than do EF faces.

On the top of a rather large number of these IMP, small central depressions 1-3 nm in diameter are observed (Fig. 3). Such particles are present on PF faces as well as on EF faces.

Caveolar Invaginations

Numerous membrane vesicles or caveolae are present under the sarcolemma (Figs. 4 and 5). Some

![Figure 1](http://circres.ahajournals.org/)

**FIGURE 1** General view of fractured cells from the sinus node. The cleavage plane has passed through some myocardial cells (three of which can be clearly discerned: C1, C2, and C3) revealing the intracellular organelles and a part of the PF face of the plasma membrane of cells C2 and C3. es = extracellular space; m = mitochondria, mf = myofilaments.
of them show a connection with the sarcolemma; some do not (Fig. 4). However, all of the caveolae contain the extracellular marker lanthanum to a comparable degree (Fig. 6), even when no connection was visible between the caveolae and the sarcolemma. No lanthanum-free vesicles lying in the cytoplasm have been identified.

Measurements made on caveolar profiles, in thin sections and on replicas, give an average long dimension in nm of 104.8 ± 14.2 (mean ± sd; n = 170) and an average short dimension in nm of 80.1 ± 11.5 (mean ± sd; n = 170).

Two or more caveolae sometimes join one another (Fig. 4) and have a common opening to the extracellular space. The number of caveolae per caveolar neck has not yet been determined very precisely, but is approximately 2.

In replicas, the openings of the caveolae are found as circular depressions 20–30 nm in diameter on PF faces (Fig. 7), and as small walled craters on EF faces (Fig. 8). No regular array of the caveolae has been observed. They seem to be distributed at random, sometimes densely packed over large areas, sometimes sparsely packed.

Preliminary measurements made on replicas give a mean value of 20 caveolar necks per μm² of apparent external sarcolemmal surface area. This corresponds to about 40 caveolae per surface area of 1 μm². If we assume that a caveola is an ellipsoid, the calculated membrane surface area of a caveola is 0.027 μm². Therefore, the caveolae would increase the surface area of the external sarcolemma by about 100%.

Gap Junctions or Nexuses

The three types of specialized junctions found between myocardial cells, fasciae adhaerentes, des-
mosomes, or maculae adhaerentes, and gap junctions or nexuses, also are present between leading sinus node cells, but they are smaller in size and less numerous than in working myocardium.

Gap junctions have been found in thin-sectioned (Fig. 9) as well as in freeze-cleaved (Fig. 10) nodal tissue. In replicas, their presence is indicated on PF faces as arrays of particles with a center-to-center distance of about 9 nm (Figs. 10 and 11) which are partially hexagonally organized, and on EF faces as similar arrays of depressions (Figs. 10 and 13).

Central depressions are present on the top of the particles on PF faces (Fig. 11).

A picture like that shown in Figure 10, where four or five gap junctions are found side by side, is exceptional. Gap junctions are often found isolated or in pairs, with virtually no relation with the other types of specialized junctions; but they are also observed occasionally in primitive, nonfolded intercalated discs (Fig. 12), where they are generally associated with one or two fasciae adhaerentes (Fig. 12) and/or one or two desmosomes (Fig. 12).

In ultrathin sections, punctate junctions are often observed (Fig. 14). In replicas, no membranous structure which could correspond to punctate junctions has been observed so far.

The gap junctions present between sinus node cells are generally small in size. Their surface area measured on replicas ranges from 0.01 to 5 × 10⁻² μm², with a mean of 0.87 × 10⁻² ± 0.12 × 10⁻² μm² (mean ± SEM; n = 15). The density of particles present in nexuses has been found to be: 9711 particles/μm² ± 461 (mean ± SEM; n = 9, on PF faces only).

The average length of cell borders measured is indicated in column 2 of Table 1. Caveolae borders have not been followed by the wheel of the map reader during the measurements of the length of cell borders. We have calculated that caveolae would increase the membrane surface area by about 100%.

In column 3 of Table 1, the length of the cell borders corrected for caveolae is given, and the length of these borders specialized as nexuses is
shown in column 4. Because (1) the nexuses are not found in specific areas of the cell membrane, as is the case in working myocardium, but seem to be randomly distributed over the cell surface, and because (2) the cells in the sinus node are much more poorly organized than in working myocardium and therefore are cut more or less at random, we can assume that the measurements given in columns 3 and 4 are representative samples of the whole surface area. In this case, as shown in column 5, the percentage of cell border occupied by nexuses is 0.2% ± 0.03.

**Discussion**

It is generally accepted that intramembranous particles seen on replicas of freeze-cleaved membranes represent membrane proteins (McNutt, 1977). A quantitative estimate of the IMP has been performed to compare their density in spontaneous impulse-generating heart cells and working myocardial cells. Preliminary results for the crista terminals of the rabbit do not indicate a significant difference between atrium and leading pacemaker cell plasma membranes with respect to IMP density. Gros et al. (unpublished observation) recently have obtained data about particle density in mouse ventricular myocardial cells (1320 particles/µm² membrane on PF faces, and 225 particles/µm² membrane on EF faces) that are in the same range as our findings. It seems thus that, as far as the intramembranous particle density is concerned, leading pacemaker cells and working myocardium heart cells are comparable.

The depressions present on the top of some in-
tramembranous particles (Fig. 3) have been observed in other cell types (Orci et al., 1977). They are identical to those observed on the nexal particles on PF faces (Fig. 6). These central depressions might be hydrophilic channels as they have been interpreted for the nexal particles by McNutt and Weinstein (1970) and McNutt (1977). It can be imagined that in nonjunctional zones, the particles having such a depression traverse the membrane completely, and that their hydrophilic channel connects extra- and intracellular spaces. These channels could be considered as the morphological equivalent of the pores postulated in biological membranes, as already suggested for other tissues by Orci et al. (1977), and/or for the ionic channels.

A very high number of irregularly distributed caveolae is observed under the sarcolemma. The presence of these structures is well known, but they have received different names in the literature such as: membrane vesicles (Lorber and Bertaud, 1971), pinocytotic vesicles (e.g., Forssman and Girardier, 1970), and caveolar invaginations or caveolae (e.g., Masson-Pevet et al., 1978, Gabella, 1978). Caveolae are much more numerous in sinus node cells (where they increase the surface area of the plasma membrane by about 100% in the rabbit) than in working heart myocardium. They increase surface area by 27% in rat papillary muscle (Gabella, 1978) and by 17-26% in rabbit papillary muscle (Levin and Page, 1977).

The physiological role of these caveolae is still unknown, but some observations deny them a pinocytotic function in the sinus node. (1) In the cells exposed to lanthanum, all of the caveolae were marked, whether or not they appeared to be attached to the sarcolemma. No vesicle free of electron-dense material has been observed in the cytoplasm. Since lanthanum is an extracellular marker that is administrated after death of the cells, it can be concluded that all the caveolae communicate with the extracellular space, although the neck is not always visualized in ultrathin sections. (2) Another argument is that the cell coat has been shown to follow the sarcolemmal invaginations into the vesicles (Masson-Pévet and Gros, in press). The hypothesis of Pollack (1977) that catecholamines are stored within the caveolae of sinus node cells and discharged from the cell into the extracellular space by exocytosis therefore lacks morphological support.

It is possible that the caveolae are an integral part of the T system, since T tubules appear first as caveolae and are formed by caveolar proliferation in developing myocardium (Ishikawa and Yamada, 1975). However, in sinus node, where caveolae are especially numerous, the cells do not contain any T tubules. This is in agreement with the observation of Forssmann and Girardier (1970) that the apparent density of caveolae was inversely proportional to the development of the T system. Contrary to what is found in the large transversal invaginations of the T system, the basal lamina does not follow the plasma membrane into the caveolae.

Whatever their function may be, the presence of caveolae greatly increases the membrane area and, thus, the surface-to-volume ratio of the cells. This point is of importance for the calculation of the linear electrical membrane properties. A detailed study of the caveolae is being pursued at present to gain insight into their function.

Sinus node cells have been called "primitive fibers" by Keith and Flack (1907). They can be compared to some extent to embryonic cells on the basis of a high nucleocytoplasmic ratio, scarcity of myofibrils, and a high glycogen content (Masson-Pevet et al., 1979). Moreover, the mean area of gap junctions is much smaller in leading sinus node cells than in working myocardium: their maximal area (5 × 10⁻² μm²) corresponds to that found in 10 days postcoitum mouse myocardium, and is much smaller than that measured in mammalian adult myocardium (54 × 10⁻² μm²) (Gros et al., 1978). As in mammalian myocardium (Gros et al. 1978), however, only macular gap junctions have been found. Gap junctions in formation: linear arrays of particles on PF faces and nexuses with arms and/or central particle-free zones never have been observed, as is the case during ontogenesis in myocardium (Gros et al., 1978). The size of the gap junctions thus would argue in favor of the sinus node being an embryonic remnant, but their form, which is comparable to that found in adult myocardium, suggests that sinus node cells differentiate in a way other than that in working myocardium fibers.

Punctate junctions observed in thin sections (Fig. 9) have the same appearance as those present between frog atrial cells (Mazet, 1977). Nevertheless, we never have observed on replicas the typical nexuses found in frog myocardium (Mazet and Car- taud, 1976; Kensler et al., 1977) or in other amphibian heart cells (Mazet, 1977). The possibility that punctate junctions are artefacts cannot be excluded.

The gap junctions with their hydrophilic channels interconnect contiguous cells and are widely held to represent the sites of current flow between cells. It has been stated in the literature that these junctions would be absent or exceedingly rare (Tranum-Jensen, 1976; James, 1977) between sinus node cells. This is one of the arguments used by Pollack (1977) to suggest that intercellular synchronization in the sinus node is not due to electrical interaction but to mechanical interaction. We have found that nexuses represent 0.2% of the cell surface area in leading sinus node cells from the rabbit, which is about 10 times less than in working myocardial cells (Table 2).

From the amount of gap junctions, we conclude that there will be an appreciable electrical coupling between the nodal cells (see Appendix), which agrees with the measurements of the passive electrical properties of the rabbit sinus node (Bonke, 1973; Bukauskas et al., 1977). The fact that there
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Table 2  Occurrence of Nexuses in Different Myocardial Cell Types

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue</th>
<th>% nexus length intercalated disc length</th>
<th>% nexus length cell border length</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>R atrium</td>
<td>5.7</td>
<td>(1.03)*</td>
<td>Spira (1971)</td>
</tr>
<tr>
<td>Rat</td>
<td>Papillary RV</td>
<td>7.5</td>
<td>(1.26)†</td>
<td>Matter (1973)</td>
</tr>
<tr>
<td>Rat</td>
<td>Papillary LV</td>
<td>12</td>
<td>3.7</td>
<td>Page and McCallister (1973)</td>
</tr>
<tr>
<td>Rat</td>
<td>Papillary LV</td>
<td>0.84</td>
<td></td>
<td>Page (1978)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Papillary RV</td>
<td>3.04</td>
<td></td>
<td>Page (1978)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Papillary LV</td>
<td>0.75</td>
<td></td>
<td>Nakata cited in Page (1978)</td>
</tr>
<tr>
<td>Calf</td>
<td>Bundle RV</td>
<td>3.3</td>
<td></td>
<td>Arluk and Rhodin (1974)</td>
</tr>
<tr>
<td>Sheep</td>
<td>Purkinje LV</td>
<td>17</td>
<td></td>
<td>Mobley and Page (1972)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Leading sinus node</td>
<td>0.20</td>
<td></td>
<td>This study</td>
</tr>
</tbody>
</table>

* This value is not given by the author. The cell has been considered to be a smooth cylinder 92.2 μm long and 10.2 μm in diameter (values given by the author) with a ratio of disc surface to cross-section surface of 2. The nexuses present along the longitudinal membranes of the cylinder have not been taken into consideration.

† This value is not given by the author. The cell has been considered to be a cylinder 100 μm long and 15 μm in diameter, with a folding factor of 3.56 for the intercalated discs. T system has been considered to enlarge the curved surface by 33% (as stated by Page and McCallister, 1973).

are about 10 times fewer gap junctions between leading sinus node cells than between working myocardial cells does not exclude the mechanism of electrical communication, because for synchronization weak electrical coupling is sufficient (Torre, 1976).

Appendix

by Wim K. Bleeker

To gain some insight into the extent of the electrotonic interaction between nodal cells, we represented the node by a simple equivalent electrical circuit.

1. A three-dimensional model was made, in which the node is regarded to be built in layers of cells. From electron microscopic observations, we estimated that each nodal cell is connected to six other cells, so the central cell of our model is connected by its low resistance junctions to six cells. This layer again is connected by low resistance junctions to a next layer, which is assumed to consist of 18 cells. Four layers are considered in the model; the third and fourth are composed of 38 and 66 cells, respectively (see Fig. a).

2. The nodal cells are roughly spindle shaped, having a diameter of about 6 μm in the thickest part and a length of about 20 μm. Taking into account a doubling of the surface area by folding of the membrane and another doubling by the presence of the caveolae, we estimated the surface area of one nodal cell to be about 1000 μm² and, consequently, the total area of gap junctions per cell, 2 μm².

3. For the specific membrane resistance (Rm) a value of 10,000 Ω·cm² was chosen, as was calculated for sinus nodal cells by Bukauskas et al. (1977). This value compares closely with those calculated for trabecular muscle by Weidmann (1970) and for the atrioventricular node by De Mello (1977). The input resistance of an isolated cell is therefore 1000 MΩ.

4. For the specific resistance of gap junctions (Rg), we used the data of Jongsma and Van Rijn (1972), who calculated a value between 0.25 and 1.25 Ω·cm². The resistance of the cytoplasm and the extracellular spaces were considered low enough, with respect to the resistance of the gap junctions, to be neglected.

Figures b and c show the equivalent electrical circuit based on $R_g = 0.25 \ \Omega \cdot cm^2$ and $R_g = 1.25 \ \Omega \cdot cm^2$, respectively. The figures give the steady state voltage displacements in the four layers of cells, resulting from a 20-mV voltage step in the
central cell. Furthermore the total current (i), flowing from this cell to the neighboring cells, is given. This model is certainly an oversimplification of the complex structure of the nodal tissue, but, on the other hand, it can be seen that the current from the central cell (i) is dependent largely on the resistance of the gap junctions of this cell, which is the most reliable element in the equivalent circuit. Therefore, we think that at least the calculated voltage response in the cells of the first layer gives a realistic impression of the actual electrotonic interaction in the sinus node.

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Serotonin Metabolism in the Normal and Failing Hamster Heart

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SUMMARY Serotonin has been found in the heart. Because cardiac serotonin is a potential endogenous source of inotropic support, an understanding of its metabolism in normal and failing hearts may be important. Cardiac serotonin was assayed in cardiomyopathic hamsters and their controls. Hearts were flushed free of platelets. Cardiac serotonin stores were 30-40% of those of norepinephrine. They were not affected by repeated injections of a mast cell granule depletor (H48/80) or the neurotoxins, 6-hydroxydopamine and 5,7-dihydroxytryptamine. Thus, cardiac serotonin appeared to be extraneural and not secondary to mast cell or platelet contamination. Inhibition of serotonin synthesis resulted in a prompt decrease, and inhibition of serotonin degradation led to a rapid increase in cardiac serotonin stores, demonstrating actual serotonin synthesis within the heart. Serotonin content (0.45 ± 0.012 μg/g in controls vs. 0.24 ± 0.009 μg/g in failing myopathic hearts) and synthesis (0.71 ± 0.016 μg/g per hour in controls vs. 0.028 ± 0.011 μg/g per hour in failing myopathic hearts) were significantly reduced in heart failure. Serotonin stores of uterus (a "control" organ) were identical for both strains. There was no difference in cardiac serotonin between the two strains in young hamsters. Human papillary muscles, taken at cardiac surgery, had serotonin levels (0.388 ± 0.027 μg/g) comparable to that found in hamster hearts. Thus, there are significant stores of serotonin synthesized within the heart. Both the stores and synthesis of serotonin are reduced in the failing myopathic hamster heart.

Serotonin has been shown to exert a direct positive inotropic effect on mammalian myocardium (Buccino et al., 1967, Benfey et al., 1974). Recent studies have demonstrated the presence of this indolealkylamine in the heart and blood vessels of rats, cats, and dogs (Beauvallet et al., 1968; Berkowitz et al., 1974; Votavova et al., 1971; Madan et al., 1970). Though cardiac serotonin, like cardiac norepinephrine, appears to contribute little to basal myocardial contractility (Buccino et al., 1967), the presence of serotonin in the heart suggests it may be an endogenous source of inotropic support during physiological or pathological cardiac stress. Alterations in serotonin metabolism also have been associated with cardiac injury (Spatz, 1969; Crawford, 1963). Therefore, we decided to characterize the metabolism of cardiac serotonin and to determine whether cardiac stores of this compound were affected in a natural model of heart disease—the cardiomyopathic Syrian hamster (Gertz, 1972).

Methods

The dystrophic Syrian hamster is a useful, reproducible, spontaneous model for human myocardial disease (Gertz, 1972). Female cardiomyopathic (Bio 53.58) and matched control (Bio R.B.) Syrian hamsters, 90-110 and 240-270 days old, were used in these experiments. The former represented an early stage of the cardiomyopathy and the latter represented the stage of cardiac decompensation and failure. All hamsters were allowed at least 2 weeks to acclimatize to our laboratory animal facility after delivery from the breeder (TELACO). A 12-hour light, 12-hour dark cycle was maintained in the animal housing area. Hamsters were allowed water but deprived of food (Purina rat chow) for 24 hours preceding the experiment.

The hamsters were killed by decapitation between 10 a.m. and 2 p.m. The hearts, in situ, were flushed free of blood with 20 ml of heparinized saline, administered by retrograde perfusion under...
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