Electrophysiological and Electron Microscopic Correlations Concerning the Effects of Neuraminidase on Canine Heart Cells

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SUMMARY To assess the importance of the glycocalyx in cardiac cells, isolated preparations of sinus node (SN), atrial working muscle (AM), false tendon (FT), and ventricular working muscle (VM) were studied electrophysiologically with intracellular electrodes and then structurally with the electron microscope. Twenty isolated canine right atria with attached ventricular tissue were arterially perfused (SN artery, FT’s were superfused) and at the close of each experiment, ruthenium (Ru) red in a glutaraldehyde fixative solution was substituted for the normal perfusate; this step arrested all cardiac cell activity. The Ru red was found aggregated in a thick (>500 Å) layer outside the external leaflet of the cell membrane in SN, AM, FT, and VM cells; this layer corresponded to the location of the glycocalyx. Similar deposits of Ru red were found inside caveolae, transverse tubules, and intercellular junctions. In the SN, the glycocalyx demarcated by Ru red was different, in that it surrounded the peripheries of P cell clusters rather than individual P cells. Neither the junction between two P cells nor that between P and transitional cells was invaded. Fifteen complete sets of cardiac tissues were treated with neuraminidase (1.0 U/ml) for 1 hour or more before the addition of Ru red. In nine different preparations, the Ru red-positive layer became virtually absent after this treatment in SN, AM, and VM cells, but the glycocalyx in FT cells remained normal in appearance. Intracellular electrodes in each tissue sample recorded the electrophysiological changes during neuraminidase treatment. Functional importance of the glycocalyx in AM and VM cells was demonstrated by their inability to conduct impulses after neuraminidase treatment. The same treatment in SN cells ultimately abolished their automaticity, whereas, in quiescent FT cells, it evoked spontaneous firing. Thus, the glycocalyx (or sialic acid removed by neuraminidase) may play a different role in each of the two types of automatic cells. These electrophysiological and ultrastructural results support an important role for the glycocalyx in the canine heart. Removal of part or all of it by neuraminidase promotes aberrant electrical activity in each different type of canine cardiac cell studied. Circ Res 50: 228-239, 1982

CARDIAC cells have a coat known as the basement membrane or glycocalyx (Martinez-Palomo, 1970; Parsons and Subjeck, 1972; McNutt and Fawcett, 1974). Importance of the glycocalyx has recently been investigated by use of the enzyme neuraminidase (EC 3.2.1.18) which selectively removes it by cleaving one of its major components (sialic acid). For example, Langer and associates observed that when the glycocalyx was removed from neonatal, adult, and cultured mammalian heart cells (Frank et al., 1977; Langer et al., 1979; see Langer, 1980, for review), Ca\(^{2+}\) permeability became enhanced. A concurring interpretation has been made in the guinea pig heart by Bailey and Pawzi (1980). However, Isenberg and Klockner (1980) reported that slow inward (Ca\(^{2+}\)) current in adult rat heart cells was not altered by neuraminidase treatment. Other work in the guinea pig heart (Grupp et al., 1980; Harding and Halliday, 1980) has demonstrated no functional effect of neuraminidase.

One reason for the controversy over function of the glycocalyx is that it may have a different chemical composition or accessibility in heart cell preparations from different species, or even from different parts of the same heart. The functions of glycocalyx in different preparations might also vary. Furthermore, the particular type of cell under study and its physiological state could dictate the tissue’s apparent response to glycocalyx removal, irrespective of the composition or independent function of the glycocalyx.

In previous studies we examined the normal anatomy (James, 1974), cellular fine structure (James et al., 1966; James and Sherf, 1968; Kawamura and James, 1971), and electrophysiological behavior (Woods et al., 1976, 1979) of special regions in the canine heart. Our aim in the present study was to determine how arterial perfusion of the canine heart with neuraminidase might affect the electrical prop-
properties of the sinus node, atrial and ventricular working muscle, and ventricular false tendon cells. Physiological observations have been correlated with electron microscopic studies of the same tissues. We found that neuraminidase treatment produced important physiological changes in canine heart cells, but the nature of the effect depended upon which type of heart cell was being studied.

Methods

Cardiac Tissue Preparation

Excision of whole hearts from 20 mongrel dogs (less than 6 months old) was performed under anesthesia with intravenous sodium pentobarbital (40 mg/kg). Details of the procedure have been previously reported (Woods et al., 1976). The excised hearts were transected 3 mm below and parallel to the atrioventricular sulcus. A catheter was advanced through the right coronary artery up to the sinus node branch where it was tied in place. Perfusion of the sinus node artery (5 ml/min) with a solution containing (mmol/liter) Na\(^+\) (145), K\(^+\) (4.19), Ca\(^{2+}\) (1.27), Mg\(^{2+}\) (0.85), Cl\(^-\) (124), HCO\(_3^-\) (25.0), H\(_2\)PO\(_4^-\) (2.39), SO\(_4^{2-}\) (0.85), and glucose (5.60) was maintained throughout each experiment, until the tissue was fixed for sectioning. Temperature was 36°C, pH was 7.40, and all arterial side branches that could divert the flow away from the sinus node region were ligated. A cut then was made beginning just proximal to the catheter insertion and extending along the underside of the right atrial appendage, along the ventral (anterior) surface of the interatrial septum, and finally up the dorsal (posterior) surface of the superior vena cava. All structures associated with the left atrium and left ventricle were trimmed away; a 3-mm strip of right ventricular wall was left attached. The perfused right atrium was pinned, epicardium up, to the wax bottom of 25-ml chamber and again, newly exposed arteria e side branches were ligated. Regular sinus rate and contractions were always maintained for at least 10 hours unless an experimental step interfered.

After the perfused atrium was in place, one false tendon from the right and two from the left ventricle of the same heart were pinned (at their diastolic lengths) to the wax bottom of the same chamber within 2 cm of the sinus node artery outflow. The perfusate passed through the sinus node artery for less than 3 seconds before bathing (by superfusion) the three false tendons. The solution was returned to a 100-ml perfusate reservoir for gas dispersion (95% O\(_2\), 5% CO\(_2\)) and recirculation to the atrium.

From three hearts, samples of right ventricular free wall were obtained for neuraminidase treatment followed by electron microscopic study. These areas were perfused by small unligated arterial branches from the major perfused arteries. Details of the electron microscopic studies are presented below.

Neuraminidase Treatment

Atria and false tendons were left to stabilize in the experimental environment for a minimum of 1 hour. After action potentials and other control data were recorded in 15 different preparations, sustained perfusions of neuraminidase began. The derivation of this enzyme is described in the catalog of Sigma Chemical Company (Saint Louis, Missouri, January, 1980, p. 423). Neuraminidase type V (1.0 U/ml) or the purer type IX (0.1 U/ml) was dissolved in the normal perfusate and delivered through the cannulated arteries. There was no significant difference between the electrophysiological responses to the two types of neuraminidase. Type IX, being more costly because of several more purification steps, was utilized in more dilute concentrations and required 1 or 2 extra hours of perfusion to produce the same effects as those of type V (1.0 U/ml), which had its maximum effect (electrical quiescence) within 2 ± 0.5 hours. Protease activity (casein substrate) and N-acetylneuraminic acid aldolase activity of type V were reported to be only one-hundredth (0.011 U/ml) and two-thousandths (0.002 U/ml), respectively, of the neuraminidase activity (1.0 U/ml).* The much purer form (type IX) was reported to have negligible levels of these impurities.

To verify the manufacturer's analysis, each sample of neuraminidase was preserved frozen and later assayed for protease activity by the Lowry et al. (1951) method. Lots numbered 77C-8590-3 and 118C-8020 (type V) and 89C-9005-1 (type IX) were so assayed and found to contain no more protease activity (albumin substrate) than claimed by the manufacturer. This was true for fresh and frozen samples.

To test whether the contaminating protease activity could be a significant factor, trypsin was added to the perfusate at a relatively high concentration (1 µg or 1.5 U/ml) and perfused for up to 2 hours in four atria. The amplitude of atrial contractions diminished slightly but there was no change in sinus rate. In working muscle cells, action potential upstroke velocity and amplitude became reduced in parallel with a declining diastolic transmembrane potential, but no electromechanical arrest occurred.

This brand of neuraminidase has been shown to contain less phospholipase activity than others that are commercially available (Kraemer, 1968). However, to test for possible contaminant effects relatively high concentrations of phospholipase A\(_2\) (1 µg or 1 U/ml) and phospholipase C (10 µg or 0.2 U/ml) were perfused separately for up to 2 hours in four atria. Afterwards, electrophysiological and mechanical changes were the same as observed with trypsin; sinus rate was essentially unchanged. As

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* Manufacturer's definitions: Protease activity unit = 1.0 µmol tyrosine per min; pH = 7.5, 37°C. N-acetylneuraminic acid aldolase activity unit = 1.0 µmol pyruvate per min; pH = 7.2, 37°C.
described in Results, the responses to neuraminidase were qualitatively different from these and more pronounced.

Electron Microscopic Studies

Nine of the hearts (60% of all specimens) were selected at random for electron microscopic study. Staining of the glyocalyx with Ru red was performed according to the method of Luft (1966, 1971). Following the physiological observations, each sinus node was fixed by perfusing cold 3% glutaraldehyde (0.1 M phosphate buffer at pH 7.3) containing Ru red (1500 ppm in distilled water) through the cannula inserted into the sinus node artery. Free-running false tendons excised from both ventricles were treated with the same solution by superfusion rather than arterial perfusion, and fully satisfactory fixation was obtained for these fine strands. Cubes (2 mm³) dissected from the sinus nodes and false tendons were minced into smaller pieces, immersed in the same solution for 2 hours at 4°C, and rinsed overnight with 0.1 M phosphate buffer (pH 7.3) at 4°C. Then they were postfixed with 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.3 with ruthenium red for 3 hours at room temperature, dehydrated as usual through an ethanol series, and embedded in epon. Ultrathin sections were examined in a Philips EM-300 electron microscope at 60 kV, either with or without additional heavy metal staining.

Physiological Studies

Electrophysiological characteristics of cells in the cardiac tissues prepared as described above were examined with microelectrodes as reported previously (Woods et al., 1976, 1979). Action potential recordings (10 ± 5 seconds in sinus node cells and 60 ± 30 seconds in all others) were also used to establish the anatomical boundaries of specific regions such as the sinus node (see Woods et al., 1976). The electronically differentiated action potential upstroke velocity was displayed with some action potentials on an oscilloscope screen. A bipolar silver electrode from a Grass S4E stimulator (with stimulus isolation and constant current) provided external pacing in some experiments. The stimulus spike (threshold current, 3 msec duration) caused deflections in the transmembrane potential trace and was used for measuring conduction intervals between stimuli and action potentials. Bipolar electrograms (1 mm interpolar distance) were recorded from the atrial epicardial surface simultaneously with the action potentials in order to measure conduction intervals.

To monitor changes in contraction amplitude the free (left atrial) edge of the perfused atrium was attached to a U.F.I. model 1030 force transducer. This provided a signal proportional to the tension developed with each contraction. Changes from control contraction amplitude (mm displacement) were readily detected. The strain gauge output was displayed on a Hewlett-Packard 7754A recorder along with a tachogram triggered by the strain gauge signal for direct reading of heart rate.

All electrophysiological results were analyzed with Student's t-test for unpaired data. Results are expressed as mean ± 1 standard deviation from the mean.

Results

Fine Structure of the Normal Glyocalyx

Ru red was bound to outer layers of the cell surface that appeared to be made up of irregular aggregates of granules. Single grains were less than 20 Å in diameter. A strong reaction with Ru red was localized to the glycoprotein surface coat (glyocalyx). This was composed of external laminae with a network of fine filaments and thin cell coats attached to the underlying plasma membrane. Thickness of the glyocalyx was not always regular over the entire surface, and because of this, as well as the fact that some sections were unavoidably oblique rather than transverse, thickness is expressed only as an approximation of the average thickness around any particular cell type. Ru red stained not only the external cell surface but also intercellular junctions, vesicular invaginations (caveolae, Gabella, 1978), and T-tubules, as well as the endothelial cell surfaces of the capillaries and the membranes surrounding nerve axons adjacent to the cardiac muscles. Ru red was not present in the space within the unit membrane of the plasma membrane, the membrane systems of mitochondria and sarcoplasmic reticulum, or any other intracellular site.

Cell Coat of the Sinus Node (P cells and Transitional Cells)

Ru red-positive material enclosed clusters of P cells (James et al., 1966) and was precipitated deeply in the intercellular clefts between P cells at the periphery of each cluster. However, the intercellular junctional spaces between contiguous P cells were never stained by Ru red. Thickness of the entire deposit covering the P cell clusters averaged about 550 Å. In the regions of deep intercellular clefts, Ru red grains were seen uniformly in the surface coat adjacent to the plasma membrane and in the outer zone of the surface coat. The outer layer network of fine filaments was absent in the depths of the infoldings. At intercellular clefts, numerous caveolae associated with the cell surfaces possessed Ru red-positive membranes. No caveolar membranes near other intercellular spaces showed Ru red deposit (Fig. 1).

Transitional cells of the sinus node (James et al., 1966) exhibited a glycoprotein coat approximately 650 to 800 Å in thickness. As in P cells, a more uniform dense layer approximately 100 Å thick
covered the outer aspect of the plasma membrane. Transitional cells made contact with working myocytes as well as other transitional cells, and in those places formed junctional specializations (intercalated discs) within which Ru red-positive material was observed. However, the regions of the intercellular contact between transitional cells and P cells did not show this positive reaction. In fact, no P cell junctions (which normally occur only with other P cells or with transitional cells) contained Ru red. The caveolar membranes were positive to Ru red underneath the sarcolemmal areas and along the intercalated discs present between all myocytes except P cells.

Cell Coat of Atrial and Ventricular Working Myocardial Cells

In the atrium, the glycoprotein surface coat stained by Ru red varied in thickness from approximately 600 to 1100 Å. Again, the 100 Å layer adhering to the outer leaflet of the plasma membrane bilayer was much more dense. This dense deposit could be enhanced by using uranyl acetate and lead citrate stains, and then it appeared to have an intermittent anchoring to its outer leaflet (Fig. 2). The membranes at all specialized regions of the intercellular junction between two working myocytes, and the junction between working myocytes and a transitional cell all were stainable with Ru red (Fig. 2), as were the membranes of caveolae in subsarcolemmal areas of the atrial cells and in the intercellular junctional areas. At these membranes, Ru red grains seemed to be bound just superficially to the lipid bilayer of the plasma membrane.

Cells from the three right ventricular (free wall) muscle samples reacted with Ru red similarly to the atrial cells. The cell coats and other structures revealed by this procedure appeared to be the same as those of the atrial working muscle cells.

Cell Coat of False Tendon Cells

Right and left ventricular false tendon cells reacted in the same way to treatment with Ru red. Accumulations of Ru red seen in the surface coat were approximately 800 Å thick. However, in comparison with the cells in the sinus node, Ru red-positive materials appeared much denser and more homogeneous throughout the layer (Fig. 3). All membranes of the false tendon intercalated disc appeared surrounded by Ru red-positive materials that were closely attached to the outer leaflet of the unit plasma membrane (Fig. 3). The caveolae beneath the sarcolemma and at the intercellular junction, which seemed to be fewer than those of the cells in the sinus node, were well labeled with Ru red (Fig. 3).

Fine Structural Effects of Neuraminidase in the Sinus Node

Neuraminidase (0.1 U/ml, 30 minutes) had several effects on external coat of P cell clusters in the sinus node. At the P cell cluster periphery, the surface coat formed by the filamentous web was decreased to approximately 350 Å in thickness and showed a marked reduction in density (Fig. 1). The external leaflet of the unit plasma membrane was covered only discontinuously by a more dense line positive to Ru red. As was observed before neuraminidase treatment, there was no Ru red present.
FIGURE 2 A: Intercellular junctions (ij) between atrial working cells showing the distribution of Ru red deposits. Glycoprotein coat (GC) positive to Ru red encloses each cell periphery. Ru red-positive materials are seen inside almost all caveolae associated with the intercellular junctions and the sarcolemma (open arrows). Counterstained. B: Electron micrograph from atrial myocardium treated with neuraminidase (0.1 U/ml, 30 minutes) and stained with Ru red. The thickness of glycoprotein coat (GC) at the periphery of working cells (W) is much decreased. The thin coat closely related to the outer layer of plasma membrane remains positive to Ru red. Several working cells and a transitional cell make contact by intercellular junctions between adjacent cells, forming side to side or end to end contact. Intercellular junctions are well-labeled with Ru red. A large number of caveolae shows staining with Ru red (open arrows). Counterstained.

in the intercellular space between contiguous P cells or between P cells and transitional cells. Several caveolar membranes associated with the sarcolemma and the intercellular clefts were labeled with Ru red (Fig. 1).

Increasing neuraminidase activity to 1.0 U/ml for 2 hours removed more of the cell coat and also may have slightly disrupted the sarcolemma. Only a very thin dense layer was seen on the cell surfaces. The dense deposit never occurred in the intercellular spaces. A few caveolar membranes of the subsarcolemmal portions were lightly stained by Ru red. Several caveolae were found in the extracellular spaces, presumably having crossed through the sarcolemma.

Sinus node transitional cells showed a similar response to neuraminidase. With the weaker neuraminidase treatment (0.1 U/ml, 30 minutes), the width of the glycoprotein coating at the cell periphery layer measured only 450 Å. A thin 100 Å cell coat closely related to the plasma membrane showed heavy deposits of Ru red. The junctional intercellular spaces were still labeled with Ru red. But after the stronger neuraminidase treatment (1.0 U/ml, 2 hours), the filamentous cell coat was almost completely removed, and Ru red-positive materials

FIGURE 3 False tendon cells from canine right ventricle. A: At the cell periphery, heavy accumulations of Ru red are seen throughout the layer of glycoprotein coat (GC), as well as inside caveolae (open arrows), which seem to be fewer than found in the atrial cells. Counterstained. B: Electron micrograph of false tendon cells from right ventricle treated with neuraminidase (1 U/ml, 2 hours) and stained with Ru red. Thick dense layer of glycoprotein coat (GC) persists at the cell periphery. Caveolae with Ru red-positive materials are scattered (open arrows).
were restricted to the thin layer less than 100 Å thick next to the plasma membrane bilayer. Ru red staining in the intercalated discs was visible as a faint dense line. Caveolae were sometimes observed in the extracellular space as also observed for some P cells after prolonged exposure to neuraminidase.

**Fine Structural Effects of Neuraminidase in Atrial and Ventricular Working Muscle**

The structural responses of atrial and ventricular working muscle cells to neuraminidase were virtually identical. The weaker neuraminidase treatment (0.1 U/ml, 30 minutes) in atrial cells resulted in a reduction of surface coat thickness to approximately 400 ± 100 Å. In some places the staining was almost eliminated. The thin layer directly apposed to the outer leaflet of the plasma membrane bilayer preserved its normal staining density very well. There was no separation of junctional membranes in any of the cells studied. The intercellular junctions exhibited almost the same intense staining with Ru red as in untreated (control) tissue. The membranes of the caveolae were not ruptured after exposure to the enzyme. Ruthenium red-positive materials covered a considerable portion of the caveolar membranes (Fig. 2).

After stronger neuraminidase treatment (1.0 U/ml, 2 hours), atrial and ventricular muscles showed fine structural changes similar to those of P cells and transitional cells. At the cell periphery, the dense thick zone of the glycoprotein coat was no longer present, and in certain places only a thin 100 Å coat covering the outer leaflet of the plasma membrane showed a weak positive reaction. At the intercalated discs, the deposits of Ru red grains were seen in a very thin layer adhering to the outer plasma membrane bilayer. Caveolae were seen in the extracellular spaces close to the sarcolemma. Sometimes caveolae were perforated. The caveolae, either associated with Ru red-positive materials or not, were also scattered in subsarcolemmal areas and adjacent to the intercalated discs.

**Fine Structural Effects of Neuraminidase in the False Tendons**

In contrast to its effect upon cells in the sinus node, neuraminidase did not appear to affect the cell coat or any other structure in false tendon cells despite treatment with the higher concentration of the enzyme and a longer (2-hour) exposure time. Neither was a significant change observed in the thickness of the Ru red-positive layer covering the cell periphery, nor in the density of staining (Fig. 3). The region of the intercellular junction was structurally intact and remained strongly positive to Ru red. No rupture of caveolar membranes occurred and no free caveolae were found in the extracellular spaces. Almost all caveolar membranes were heavily labeled with Ru red.

**Physiological Studies of the Sinus Node**

After the first 10 minutes of neuraminidase (1.0 U/ml) perfusion through the sinus node artery, sinus rate (control = 118 ± 24 beats/min) began to increase. The maximum rate (150 ± 31 beats/min or 127% control, P < 0.05) was attained 36 ± 24 minutes later. Then as neuraminidase perfusion continued for another 60 to 90 minutes, sinus rate fell progressively until atrial impulses ceased at 2 hours (±30 min) after neuraminidase perfusion began (Fig. 4). Canine atria perfused with normal physiological solution beat regularly for at least 10 hours (Woods et al., 1976).

For 60 ± 30 minutes after the atrial arrest, action potentials of normal amplitude were still recorded in cells of the sinus node, but these were not transmitted to the surrounding atrium. These action potentials slowed in frequency and became fragmented in shape prior to becoming irregular in rate (Figs. 5 and 6). Action potential amplitude decreased progressively until, finally, only low amplitude oscillations were recorded in the sinus node cells. The last recorded sinus node action potentials with amplitudes of 20 mV or greater showed that the APD 90 (time required to repolarize by 50%) had increased (P < 0.05) from 91 ± 13 to 154 ± 46 msec. This was due to the fall in amplitude that occurred while total action potential duration remained relatively unchanged. Transmembrane potential at the point of sinus arrest had changed (P < 0.05) from −55 ± 4 to −37 ± 13 mV.

After the atria had been treated with neuraminidase for approximately 30 minutes, conduction from sinus node to atrium was markedly slower. More than 150 msec elapsed between the firing of the central sinus node and the activation of nearby atrial working muscle cells. Because of the normal anatomical distribution of the sinus node artery, our administered neuraminidase perfused juxtaglominal atrium as well as the sinus node itself.

Continued perfusion of neuraminidase resulted in further conduction disturbances within and near the sinus node. After only 15 minutes, some sinus node cells actually fired after the atrial working muscle became activated (see Fig. 6). After 45 minutes, these late-firing cells underwent a reduction in the amplitudes of their action potentials. After 75 minutes, the sinus node cells began firing irregularly, presumably because of further deterioration in intranodal conduction. However, sinoatrial conduction from the primary sinus node pacemaker region was still intact at this time.

In the late stage of the neuraminidase treatment, sinus node cell firing became chaotic (Fig. 6), first at the side nearest the superior vena cava and later at the side nearest the crista terminalis. Sinoatrial conduction to the adjacent atrial muscle at first persisted with some variability in atrial firing cycle lengths (Fig. 6). Partial conduction block toward the superior vena cava always eventually occurred,
FIGURE 4  This record shows changes observed in sinus rate and atrial contractions during perfusion of neuraminidase (N'ase) into the sinus node artery. Neuraminidase was added at the arrow. Irregular deflections in the tachogram are caused by hysteresis in the tachograph sensing circuit. Atrial arrest occurred after 3.5 hours of neuraminidase perfusion. The last panel shows that 1 hour later some atrial cells were still responsive to pacing stimuli (small contractions).

but at that point in time conduction was normally preserved toward the crista terminalis. Within 3 hours, the crista terminalis route always became blocked and, therefore, the sinoatrial block became complete. During the final period of atrial quiescence, no action potentials (see Fig. 7) could be evoked by external stimuli applied to either the atrial surface or the sinus node itself.

Physiological Studies of Atrial Working Muscle

Atrial cells closest to the sinus node artery were the first affected (Fig. 8) during neuraminidase perfusion and those farthest from it were the last affected. Thus, the action potentials recorded at any given moment ranged from depressed (Fig. 8) to normal. This produced large standard deviations in measurements (see Table 1) so that the changes were not statistically significant. However, the true
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reductions in diastolic potential, conduction velocity, upstroke velocity, and amplitude can be appreciated in Figure 8. These changes were accompanied by decreasing contraction amplitude (Fig. 4). The resting transmembrane potential recorded in right atrial appendage cells at the time of atrial arrest was $-57 \pm 23$ mV. They could no longer be stimulated with the pacing electrodes. Atrial working myocytes never became spontaneously automatic.

Physiological Studies of Ventricular False Tendon

All false tendon cells of the right and left ventricle were quiescent (transmembrane potential was $-69 \pm 6$ mV, $-85 \pm 4$ mV during pacing) when impaled with microelectrodes, and they remained quiescent until exposed to neuraminidase (1.0 U/ml, 2 hours). By $110 \pm 46$ minutes after starting neuraminidase, spontaneous action potentials began to appear in these cells (Fig. 9). Maximum diastolic potential was then $-82 \pm 8$ mV. Action potentials had the same maximum upstroke velocities, durations, and amplitudes as externally paced action potentials (Fig. 10; Table 1), before or after neuraminidase. Conduction velocity that was not significantly changed in the false tendons was estimated by pacing them at 60 impulses/min and measuring the interval between the stimulus spike and the action potential over a distance of $2 \pm 1$ mm.

Discussion

General Cardiac Effects of Neuraminidase

Even though it is a normal constituent of brain cells (Schengrund and Rosenberg, 1970), components of Clostridium (or Vibrio) toxin such as neuraminidase (Hipp et al., 1980) would be expected to have some deleterious effects. However, the functional consequences of removing the glycoalyx from certain types of heart cells has not been determined. In the present study, neuraminidase apparently removed the glycoalyx from all except the false tendon cells, but the major remaining membranous features (plasma membranes, intercellular junctions, reticular membranes) appeared normal, as reported by others (Frank et al., 1977). Hence,
TABLE 1 Electrophysiological Effects of Neuraminidase (N'ase) on Canine Heart Cells

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Maximum diastolic potential (mV)</th>
<th>Conduction velocity (mm/sec)</th>
<th>Maximum upstroke velocity (V/sec)</th>
<th>Amplitude (mV)</th>
<th>APD₉₀ (msec)</th>
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<tr>
<td></td>
<td>Control</td>
<td>N'ase</td>
<td>Control</td>
<td>N'ase</td>
<td>Control</td>
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<tr>
<td>Sinus node (sinus rhythm)</td>
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<tr>
<td></td>
<td>−55 ±4</td>
<td>−37*</td>
<td>[15]</td>
<td>[1]*</td>
<td>3 ±2</td>
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<td>(n = 7)</td>
<td></td>
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<tr>
<td>Atrial working muscle (sinus rhythm)</td>
<td>−75 ±1</td>
<td>−65</td>
<td>589 ±14</td>
<td>291*</td>
<td>50† ±16</td>
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<td>(n = 8)</td>
<td></td>
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<td>False tendon (stimulated action potentials)</td>
<td>−85 ±4</td>
<td>−82</td>
<td>918 ±14</td>
<td>668</td>
<td>231† ±26</td>
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<td>(n = 12)</td>
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* Significant difference (P < 0.05) between control and neuraminidase. [ ] = best estimates of sinus node conduction velocity between action potentials.

Diastolic depolarization obscured the exact instant at which the impaled pacemaker cells became activated.

Values reported were also reported in similar studies (Woods et al., 1979; Kraft et al., 1980).

n = Number of canine cardiac tissue preparations in which action potentials selected for this table were recorded. Of all hearts studied (20 in all), seven provided typical action potential data for each type of tissue studied (7 sinus nodes, 8 atrial muscle samples, and 12 false tendons, at least one of each from each heart). These examples were selected for this table so that responses to neuraminidase would be more comparable and standardized than a consolidation of action potentials recorded at different times from 20 different hearts. Five of the 20 hearts were used for "control" electron microscope experiments, and so they were never treated with neuraminidase.

some of the electrophysiological effects of neuraminidase may be caused by the absence of a glycolyx. If further structural changes were produced by contaminating substances, they would differ in functional effects (judging by the effects of protease and phospholipases).

The ultrastructural studies of sinus node, atrial working, and ventricular working cells each confirmed that treatment with neuraminidase for 30-60 minutes removes most of the Ru red binding components. This includes the surface coat found normally along the cell membrane, inside transverse

FIGURE 9 Panels A and C show the transmembrane resting potentials of cells in a right ventricular (RV) and a left ventricular (LV) false tendon (FT). These cells were always quiescent until neuraminidase was added. B and D show the spontaneous action potentials evoked by neuraminidase after 60 minutes. Differentiator calibration is not the same in B and D; maximum upstroke velocity was not significantly different. The two small deflections in transmembrane potential trace A were coincident with mechanical disturbance of the microelectrode; they should be considered artifactual.
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Figure 10  Pacing stimuli at 60/min produced the action potentials in cells of false tendon shown in these panels. There was no significant change in these action potentials after neuraminidase for 60 minutes. Both upstroke velocity and conduction velocity [based on the distance and time between stimulus (s) and the action potential upstroke] remained fast as shown in Table 1.

In each atrium we observed a decline in amplitude of contraction after the neuraminidase treatment. Ca²⁺ inward current was unaltered by cell coat removal in other studies (Isenberg and Klocker, 1980; Harding and Halliday, 1980). Our results are in agreement, since the decline in atrial contraction amplitude was probably not caused by a fall in contractility. Rather, when impulse conduction began to fail, fewer cells contracted with each beat. At the end point (inexcitability) there was no conducted beats and no cell could be stimulated to respond. Contractions could not be elicited, although fine structure of the contractile apparatus appeared normal. Our interpretation concurs with that of Harding and Halliday (1980) except that our dog atria became inexcitable; their guinea pig atria remained responsive. Other than the species difference (guinea pig vs. dog), the main differences between that study and the present one were the arterial perfusion and point stimulation used in the canine tissues. As to the first difference, the lack of effect of neuraminidase on the glycocalyx of these false tendon cells indicates that superfusion may not be the best way to deliver the enzyme. And to the second difference, single point electrical stimulation demonstrates conduction impairment more readily than does the field-type electrode stimulation used in the guinea pig atria.

Effects of Neuraminidase upon Sinus Node

The electrophysiological changes in the sinus node during neuraminidase treatment were always the same with each preparation of neuraminidase. After the initial acceleration conduction within and near the sinus node slowed, then sinus rhythm became less regular. Both the rate and shape of sinus node action potentials became irregular as the pacemaker became functionally fragmented and propagated impulses collided with each other. Sinoatrial conduction ceased suddenly in one beat, although sinus node action potential generation continued. Later, only low amplitude asynchronous oscillations could be recorded in the sinus node. This pattern is distinctly different from the one observed in sinus node cells with verapamil, the slow channel blocker (Zipes and Fischer, 1974; Woods et al., 1979). Instead, with verapamil, the rate of firing slows and action potential amplitude simply falls; irregularity in rate and fragmentation of action
potentials never occur. With neuraminidase, the pacemaker cells instead seemed to lose their synchrony. This indicates that electrical coupling between them became diminished. DeMello (1977) proposed that when intracellular \([\text{Ca}^{2+}]\) rises, electrical coupling between cells fails. This would support the \(\text{Ca}^{2+}\) overload hypothesis, since removal of the glycocalyx promotes \(\text{Ca}^{2+}\) influx in certain cells (Langer, 1980; Nathan et al., 1980).

**Effects of Neuraminidase upon Atrial Working Muscle Cells**

After a 2-hour treatment with neuraminidase, the atrial working cells could no longer transmit impulses. According to the hypothesis of DeMello (1977), \(\text{Ca}^{2+}\) influx may have overloaded the cell interior with \(\text{Ca}^{2+}\) and decreased intercellular electrical coupling to the point where no impulses could be transmitted from cell to cell.

**Effects of Neuraminidase upon False Tendon Cells**

We know of no barrier to diffusion of neuraminidase that would prevent it from reaching the cell coat in false tendon cells. The only significant effect of neuraminidase on these cells, however, was the induction of automatic activity. This contrasts with the elimination of automaticity observed in the sinus node with prolonged neuraminidase treatment, although it might correspond to the transient increase in sinus rate seen shortly after neuraminidase perfusion was started. The resting transmembrane potential in false tendon cells remained normal during the ensuing several hours of spontaneous activity, so the automaticity was not induced by a less negative resting potential.

Removal of \(\text{Ca}^{2+}\)-binding sites might produce a greater functional effect in the sinus node cells. Their depolarizing inward current is largely dependent upon \(\text{Ca}^{2+}\), while that of normal false tendon cells is more dependent upon \(\text{Na}^{+}\). Decreasing extracellular \([\text{Ca}^{2+}]\) below normal depresses canine (Woods et al., 1979) and lapine (Seifen et al., 1964) sinus node automaticity, but it enhances automaticity in canine false tendon cells (Hoffman and Suckling, 1956; Woods et al., 1979). The effects of neuraminidase in our study, thus, resembled the effects of lowering extracellular calcium. How could neuraminidase lower the \(\text{Ca}^{2+}\) in false tendon cells when it failed to remove their cell coats? Neuraminidase removes sialic acid residues, and these may be only a minor component of the cell coat of false tendon cells. However, these acids are thought to be the sites of some \(\text{Ca}^{2+}\) binding in other tissues (Dorrscheidt-Kafer, 1979a, 1979b; Suzuki et al., 1980; Taeko et al., 1980). If removal of the sialic acid residues reduces the availability of \(\text{Ca}^{2+}\) (at the outer leaflet of the false tendon cell membrane), this alone could account for the contrasting changes in automaticity we found for \(P\) cells of the sinus node compared to cells of false tendon. Modification of \(\text{Ca}^{2+}\) availability by the cell coat (Langer, 1980) is supported by the physiological results in these false tendons. However, most \(\text{Ca}^{2+}\) is bound to phospholipid in the lipid bilayer (Philipson et al., 1980). If neuraminidase reached the false tendon cells, then their glycocalyx contains many structural elements that attract Ru red besides sialic acid. The fact that superfused Ru red reached these cell surfaces supports the assumption that neuraminidase did also.

In summary, our results support an electrophysiologically important role for the glycocalyx. Continued presence of the glycocalyx may be essential to the preservation of automaticity in the sinus node. In all cells except those of the false tendon, it may provide protection from excess \(\text{Ca}^{2+}\) entry as previously suggested (Langer, 1980). In false tendon cells there was a seeming paradox in that neuraminidase did not at all influence fine structure of these cells, but it did cause them to become automatic. The presence of sialic acid may suppress some local automaticity-promoting factor. Thus, two kinds of action upon the glycocalyx by neuraminidase are suggested. One action typified in \(P\) cells of the sinus node includes removal of a Ru red-sustainable component which has a regulatory influence on ions in its environment. The other, observed in false tendon cells, has some action upon local ionic movements but does not remove the material that attracts Ru red. Aside from the fact that \(\text{Ca}^{2+}\) availability may have a different functional significance in each of these two kinds of cells, there may be fundamental biochemical differences between the glycocalyces of \(P\) cells in the sinus node and of false tendon cells in the same heart.

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