Distribution of Sialic Acid in Frog Skeletal Muscle and Effect of Neuraminidase on Ca Uptake and ATPase Activity of Sarcoplasmic Reticulum

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Abstract The contents of sialic acid in the sarcolemma, sarcoplasmic reticulum (SR) and myofibrils obtained from frog skeletal muscle homogenate were determined. The total sialic acid contents of the sarcolemma and fragmented SR were 2.95 and 3.34 nmols per mg of protein, respectively, while that of myofibrils was 1.47 nmols per mg of protein. Treatment of the fragmented SR with neuraminidase (EC 3.2.1.18; NAase) resulted in the release of sialic acid. Ca uptake and ATPase activities were measured in the NAase-treated fragmented SR. When the fragmented SR stood for 1 hr after treatment and washing, the Ca uptake was decreased slightly and neither basic nor extra ATPase activities were affected. In contrast, when the fragmented SR was allowed to stand for 24 hr after similar treatment, Ca uptake and extra ATPase activity were markedly inhibited, while the duration of extra ATP splitting was markedly prolonged and final ATP hydrolysis was increased without noticeable change in basic ATPase activity. The results obtained suggest that in frog skeletal muscle, sialic acid locates mainly at the surface and SR membranes and that sialic acid is not directly involved in active Ca transport of the SR membrane.

It is well known that phospholipids and proteins are the main components of cell membranes and play an important role in the functions of the membrane. Polysaccharide, which is usually present as a glycoprotein, has also been found histochemically and biochemically in the membranes of many tissues (RAMBOURG et al., 1966; LUFT, 1971; WARREN, 1959). It has been reported that sialic acid in the glycoprotein plays an important role in Ca binding of the soluble Ca-binding protein from ox liver (SOTTOCASA et al., 1972) and rat liver mitochondria (LEHNINGER, 1971). Glycoprotein is also essential for Ca uptake of the isolated SR in rat uterus muscle (VACCARI et al., 1971) and of the mitochondria in rat liver (VASINGTON et al., 1972). In skeletal muscle, there have been a few reports describing the existence of sialic acid in the surface and SR membranes (ANDREW...
and APPEL, 1973; SEVERSON et al., 1972), but the physiological role of sialic acid has not yet been clarified.

In the present paper, we determined the content of sialic acid in various centrifugal fractions of frog skeletal muscle homogenate, and examined the effect of the NAase on the Ca uptake and ATPase activities of the fragmented SR, in order to elucidate the role of sialic acid in SR Ca transport.

MATERIALS AND METHODS

Materials. Leg skeletal muscles of the frog (Rana japonica) were used.

Preparation of muscle fractions. Various centrifugal fractions (fractions I, II, III and IV) were prepared according to the procedure described by TANIGUCHI (1969): The muscles were minced with scissors and homogenated with a Waring Blender for 30 sec at maximal speed with 4 volumes of extraction solution (80 mM KCl, 20 mM histidine-HCl buffer, pH 7.0). The homogenate was centrifuged for 15 min at 1,000×g. The supernatant was filtrated through gauze. The filtrate was then centrifuged for 15 min at 2,000×g. The sediment (fraction I) was suspended in 0.1 M KCl. The supernatant was centrifuged for 15 min at 12,400×g, and the sediment (fraction II) was washed twice in 0.1 M KCl and resuspended in the same solution. The supernatant was centrifuged for 40 min at 67,500×g. The supernatant (fraction IV) was separated and the sediment (fraction III) was washed twice in 0.1 M KCl and resuspended in the same solution. TANIGUCHI (1969) reported that fractions II and III can be regarded as the fragmented SR, on the basis of his findings that the amount of Ca uptake by fractions I and IV were less than one tenth of the uptake values by fractions II and III. Fractions II and III consisted of large amounts of vesicles with diameters of 150–300 nm. Contamination of mitochondria and the tubular membrane structures was slight. In the present study, fraction II was used as the fragmented SR, according to the report by TANIGUCHI (1969). The myofibrils used in the present study were prepared following the procedure of NAGAI et al. (1960). The sarcolemmal fraction was prepared according to the procedure reported by SULAKHE et al. (1973). All procedures for the preparation of these muscle fractions were performed at 0–4°C and the suspensions were stored at 4°C.

Measurements of sialic acid content. Each fraction was hydrolyzed in 0.05 N H₂SO₄ for 1 hr at 80°C and the sialic acid contents were measured by WARREN'S method (1959).

Treatment of fragmented SR with NAase. The fragmented SR (fraction II) was incubated in a tube containing NAase and 0.05 M Na phosphate buffer (pH 6.5), at 25°C. The reaction was stopped by placing the tube in ice water, and then the reaction mixture was centrifuged at 12,400×g for 15 min at 0°C. The sediment was washed twice and resuspended in cold 0.1 M KCl. This suspension was stored at 4°C for 1 and 24 hr for the measurement of Ca uptake and ATPase activities,
respectively. During storage, no sialic acid from the fragmented SR was released. The amount of sialic acid released by NAase treatment was determined by measuring the free sialic acid in the supernatant by the thiobarbituric acid method (WARREN, 1959).

Ca uptake. The reaction mixture contained 40 mM Tris-HCl buffer (pH 7.2), 50 mM KCl, 1 mM MgCl₂, 1 mM ATP, 10 μM CaCl₂, a trace amount of ⁴⁵Ca (1 μCi per ml) and 0.2 mg of protein in a final volume of 1 ml. The reaction was started by the addition of ATP at 20°C and terminated by passing the mixture through a Millipore filter after 3 min. The radioactivity of the filtrate was counted in a Bray's scintillator with a liquid scintillation spectrometer (Beckman, LS-250).

ATPase activities. The ATPase activity of the fragmented SR was determined in a mixture containing 40 mM Tris-HCl buffer (pH 7.2), 50 mM KCl, 1 mM MgCl₂, 5 mM oxalate, 0.4 mg of protein with and without 50 μM CaCl₂ in a final volume of 2 ml. The reaction was started by the addition of ATP at 20°C and stopped by adding 2 ml of 10% TCA. The liberated Pi was measured by the FISKE-SUBBAROW method (1925). The Na⁺-K⁺-stimulated ATPase activity of the sarcolemmal fraction was measured in a medium containing 50 mM Tris-HCl buffer (pH 7.4), 100 mM NaCl, 20 mM KCl, 5 mM MgCl₂, 5 mM ATP and 0.2 mg of protein per ml, for 10 min at 37°C.

Protein concentration. The concentration of protein was determined by the biuret reaction with bovine serum albumin as a standard.

Reagents. All reagents used were of analytical grade. Neuraminidase (EC 3.2.1.18) from Clostridium perfringens was obtained from Boeringer Mannheim GmbH and had an activity of 0.6 units per mg. One unit of activity equals 1 μmols of N-acetyl neuraminic acid released per min at 37°C at pH 5. This enzyme preparation has been demonstrated to be free from N-acetyl neuraminic acid aldolase, protease, RNase, DNase, NADH-oxidase, β-galactosidase and phospholipase C (a report from Boeringer Mannheim GmbH, Kontroll-Labor Biochemie). ATP and ⁴⁵Ca were obtained from Sigma Chemical Co. and from International Nuclear Corp., respectively.

RESULTS

Sialic acid contents

Table 1 shows the sialic acid contents in various fractions of muscle homogenate. Fractions II and III contained 3.22±0.54 and 3.45±0.43 nmols of sialic acid per mg of protein, respectively. For reference, the sialic acid content of fraction I, which consisted of a large amount of myofibrils and a small amount of mitochondria, and of fraction IV, which was the 67,500×g supernatant fraction, were also measured: Fractions I and IV contained 1.48±0.17 and 1.72±0.40 nmols of sialic acid per mg of protein, respectively. Thus, contents of sialic acid were higher in fractions II and III than in fractions I and IV. The sarcolemma also
Table 1. Sialic acid contents in various fractions of frog muscle homogenate.

<table>
<thead>
<tr>
<th>Muscle fractions</th>
<th>× g</th>
<th>Sialic acid (nmols per mg of protein)</th>
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<tbody>
<tr>
<td>Fraction I</td>
<td>1,000-2,000</td>
<td>1.48±0.17 (n=12)</td>
</tr>
<tr>
<td>II</td>
<td>2,000-12,400</td>
<td>3.22±0.54 (n=11)</td>
</tr>
<tr>
<td>III</td>
<td>12,400-67,500</td>
<td>3.45±0.43 (n=13)</td>
</tr>
<tr>
<td>IV</td>
<td>67,500&lt;</td>
<td>1.72±0.40 (n=13)</td>
</tr>
<tr>
<td>Myofibrils</td>
<td></td>
<td>1.47±0.25 (n=4)</td>
</tr>
<tr>
<td>Sarcolemmal fraction</td>
<td></td>
<td>2.95 (n= 2)</td>
</tr>
</tbody>
</table>

The method of preparation of muscle fractions and the procedure for determining sialic acid contents are described in the MATERIALS AND METHODS section. Values are given as means± S.E.

contained about 2.95 nmols of sialic acid per mg of protein, while myofibrils contained 1.47±0.25 nmols of sialic acid per mg of protein. Moreover, with respect to the purity of this sarcolemma preparation, it was observed that its Na⁺-K⁺-stimulated ATPase activity was 7.8–10.8 μmols P₁ per mg of protein per hr (mean value 9, n=4), at 37°C. These values are similar to those obtained by SULAKHE et al. (1973) using rabbit skeletal muscle.

Treatment of fragmented SR with NAase

Table 2 shows the amount of sialic acid released from the fragmented SR (fraction II) by treatment with NAase. When 30 mg of fragmented SR was incubated in Na phosphate buffer solution (pH 6.5) with 1.2 units of NAase at 25°C, the amount of sialic acid released increased with the lengthening period of incubation and reached 2.59 nmols per mg of protein after 20 min of incubation. The total sialic acid content of the fragmented SR was determined to be 3.26 nmols per mg of protein. Thus by treatment with 1.2 units of NAase at 25°C for 20 min,

Table 2. The amount of sialic acid released from the fragmented SR by NAase treatment.

<table>
<thead>
<tr>
<th>Period of treatment</th>
<th>Amount of sialic acid released (nmols per mg of protein)</th>
</tr>
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<tbody>
<tr>
<td>With NAase</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>0.71</td>
</tr>
<tr>
<td>10 min</td>
<td>1.05</td>
</tr>
<tr>
<td>20 min</td>
<td>2.59</td>
</tr>
<tr>
<td>Without NAase</td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>0.51</td>
</tr>
<tr>
<td>10 min</td>
<td>0.51</td>
</tr>
<tr>
<td>20 min</td>
<td>0.51</td>
</tr>
</tbody>
</table>

Thirty mg of fraction II was incubated in 0.05 M Na phosphate buffer (pH 6.5) solution with or without 1.2 units of NAase at 25°C. The amount of sialic acid was measured by WARREN'S method (1959).
about 80% of the total sialic acid was released. On the other hand, when the fragmented SR was incubated in Na phosphate buffer (pH 6.5) without NAase, the amount of sialic acid released was independent of the period of incubation and was 0.51 nmols per mg of protein. Similar tendencies were observed in the other three preparations.

**Effect of NAase treatment on Ca uptake**

Figure 1 shows the relation between the amount of sialic acid released by NAase treatment and the amount of Ca uptake. The amounts of sialic acid given on the abscissa were taken from Table 2.

In the case of the fragmented SR that stood for 1 hr at 4°C in 0.1 M KCl solution following washing after NAase treatment, the amount of Ca uptake decreased as the amount of sialic acid released increased. The extent of the decrease (inhibition) was only 20% at the release of 40% of the total sialic acid from the fragmented SR and the extent of inhibition (20%) was not further increased even at more than an additional 40% release. In contrast, in the case of the fragmented SR that stood for 24 hr at 4°C in 0.1 M KCl solution following washing after NAase

![Figure 1](image-url)

Fig. 1. Amount of sialic acid released from the fragmented SR by treatment with NAase and the Ca uptake. The conditions and procedure of NAase treatment and washing of the fragmented SR were the same as in Table 2. Ca uptake activity by the fragmented SR that stood for 1 and 24 hr after NAase treatment was determined in reaction mixture containing 40 mM Tris-HCl buffer (pH 7.2), 50 mM KCl, 1 mM MgCl₂, 1 mM ATP, 10 μM CaCl₂, a trace amount of ⁴⁵Ca and 0.2 mg of protein in a final volume of 1 ml. Reaction time was 3 min at 20°C. The ordinate represents the percentage of the amount of Ca uptake by the fragmented SR which was not treated with NAase. One hundred per cent of the amount of Ca uptake corresponds to 42 nmols of Ca per mg of protein. The abscissa represents the percentage of the total sialic acid released from the fragmented SR, 3.26 nmols of sialic acid per mg of protein. △, 1 hr after NAase treatment (20 min) and washing; ▲, 24 hr after NAase treatment (20 min) and washing.

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treatment, the amount of Ca uptake markedly decreased as the amount of sialic acid released increased and inhibition was 80% at the release of 80% of the total sialic acid. In addition, sialic acid was not detected in the supernatant which was centrifugally separated from the suspension of fragmented SR, which had stood either for 1 or 24 hr at 4°C after NAase treatment and washing. This indicates that no more sialic acid was released from the fragmented SR during storage under these conditions. It was also observed that Ca uptake activity in the untreated fragmented SR was not affected by standing for only 24 hr at 4°C.

Effect of NAase treatment on ATPase activity

Figure 2 shows the effect of the removal of sialic acid from the fragmented SR on basic and extra ATPase activities. NAase treatment was performed by incubating 25 mg of the fragmented SR with 1.0 unit of NAase at 25°C for 20 min. With this treatment, 2.07 nmols of sialic acid per mg of protein was released and this value corresponded to 77% of the total sialic acid of the fragmented SR. Basic and extra ATPase activities of the NAase-treated fragmented SR were not affected by standing for 1 hr at 4°C after treatment and washing. On the other hand, when allowed to stand for 24 hr at 4°C after the above-described NAase treatment, ATP hydrolysis in the initial early phase of its time course (extra splitting,
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Fig. 3. ATPase activity of the fragmented SR that stood for 24 hr after NAase treatment and washing. The conditions and the procedure of NAase treatment of the fragmented SR were the same as in Fig. 2. The fragmented SR was allowed to stand for 24 hr at 4°C. Other conditions and symbols are the same as in Fig. 2.

HASSELBACH and MAKINOSE, 1962) was strongly inhibited in the presence of Ca and the duration of extra splitting was markedly prolonged, while ATP hydrolysis in the secondary late phase (final ATP hydrolysis) was significantly increased, without changing basic ATPase activity (Fig. 3).

DISCUSSION

As mentioned in the MATERIALS AND METHODS section, the NAase used in the present study was stated to be free from N-acyl neuraminic acid aldolase, protease, RNase, DNase, NADH-oxidase, β-galactosidase and phospholipase C (information from Boeringer Mannheim GmbH, Kontroll-Labor Biochemie). Among these enzymes, we particularly noted phospholipase C, because this enzyme is known to be able to release sialic acid from guinea pig taenia coli (ISHIYAMA et al., 1975). The following confirms that the NAase used in the present experiments did not contain phospholipase C. Previously, MARTONOSI et al. (1968) have reported that the Ca uptake and ATPase activities of the fragmented SR were markedly inhibited by treating the SR with phospholipase C for 20 min. If the NAase used in the present study was contaminated with phospholipase C, the Ca uptake and ATPase activities of the SR should have been markedly inhibited. However, the present results (see the case after standing for 1 hr in Figs. 1 and 2) indicate that after treating the SR with NAase for 20 min, Ca uptake by the fragmented SR was slightly inhibited and the ATPase activity was not affected. Therefore, it is evident that the NAase used was free from phospholipase C.

It has been reported that the plasma membrane and the intracellular membrane
VASINGTON et al. (1972) reported that the glycoprotein containing sialic acid in the mitochondrial membrane of rat liver is related to energy-linked Ca translocation. HASSELBACH and MAKINOSE (1962) reported that the Ca uptake activity by the fragmented SR of skeletal muscle is parallel with extra ATPase activity. In the present work, whether or not the sialic acid contained in the SR membrane of frog skeletal muscle is related to the energy-linked Ca uptake by the SR was examined. The results obtained in this experiment showed that considerable sialic acid was released from the fragmented SR by NAase treatment (Table 2) as mentioned above, and that in spite of this release the amount of Ca uptake and both basic and extra ATPase activities were only slightly affected in the case of the fragmented SR that stood for 1 hr after NAase treatment and washing (Figs. 1 and 2). In contrast, in the case of the fragmented SR that stood for 24 hr after treatment, Ca uptake and extra ATPase activities were greatly inhibited, while the duration of extra splitting was markedly prolonged and final ATP hydrolysis was significantly increased without noticeable change in basic ATPase activity (Figs. 1 and 3). These results clearly indicate that the efficiency of the energy-linked Ca uptake was reduced by allowing the SR to stand for 24 hr after NAase treatment and washing. A similar decrease in the efficiency of Ca uptake has been observed in the case of treatment of the SR with aging (MARTONOSI and FERETOS, 1964) or with low concentrations of Triton X-100 (YAMAMOTO and TONOMURA, 1967). This suggests that the decrease in the efficiency of active Ca uptake by the SR stored for 24 hr after NAase treatment may be attributed to the leaky character of SR membrane. It is therefore concluded that the loss of sialic acid is not directly related to the active Ca transport of the SR membrane.

DÖRRSCHEIDT-KÄFER (1977) briefly reported that in frog sartorius the shift in the contraction threshold after treatment with NAase can be attributed to the release of sialic acids producing a decrease in surface charges on the muscle membrane. In this connection, it is necessary to examine in detail the effect of NAase on the electrical and mechanical responses in frog skeletal muscle. This problem will be dealt with in a future paper.
REFERENCES


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