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Negatively charged sites and calcium binding in the isolated rat heart sarcolemma*)

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With 4 figures and 2 tables

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Summary

Colloidal iron staining, calcium binding and enzyme activities were studied in the isolated rat heart sarcolemma. Colloidal iron staining of the sarcolemma revealed a high density of negatively charged sites associated with the cell surface. This membrane fraction was found to have calcium binding activity at both low (0.1 mM) and high (1.25 mM) concentrations of calcium. Pretreatment of the sarcolemma with either trypsin, phospholipase C or neuraminidase, was associated with a reduction in colloidal iron staining as well as decreased calcium-binding activity at high concentrations of calcium. Calcium binding at low concentrations was decreased by both trypsin and neuraminidase. Mg^{2+} ATPase, Ca^{2+} ATPase, and Na^{+}-K^{+} ATPase activities were altered by neuraminidase and trypsin treatments, whereas phospholipase C treatment altered Na^{+}-K^{+} ATPase only. It is concluded that both surface negative charge and calcium-binding sites associated with the isolated rat heart sarcolemma are contributed by a mosaic of biomolecules including proteins, phospholipids and glycoproteins, and alterations in the surface charge may influence the activities of membrane-bound enzymes.

Many studies have employed the colloidal iron (CI) stain of Mowry (23) to demonstrate the presence of anionic sites on the cell surface of whole tissue, isolated cells and cell membranes (11, 14, 15, 24, 37), and the stain is believed to bind to negatively charged membrane sites. Such sites could include acid mucopolysaccharides, acidic amino acids and phospholipids (4). Almost total loss of CI stain in studies on rat liver membranes (3) and myocardium (15) following treatment with neuraminidase indicated that

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CI was binding to N-acetyl neuraminic acid (sialic acid). Biochemical analysis of the same preparations, however, indicated only 60–70 % of the total membrane sialic acid was sensitive to neuraminidase, suggesting that some membrane sialoglycoproteins were insensitive to or inaccessable to both the colloidal iron stain and neuraminidase action.

The cell membrane and the surface material of the myocardial cell are known to bind calcium and are intimately involved in the regulation of the intracellular supply of this cation (6, 14, 15). The ability of other cations, such as lanthanum, to uncouple excitation from contraction in the myocardium, presumably by replacing calcium at the cell surface (26), indicates that calcium is bound to negatively charged membrane sites. Recently, it has been shown that in a sarcolemmal fraction, isolated from rat hearts perfused with lanthanum, binding of the stain is mainly to the surface coat (31). Treatment of the isolated sarcolemma with lanthanum resulted in a significant decrease in both calcium binding and calcium ATPase activities of the membrane (31). The present study was undertaken to investigate the distribution of negatively charged sites on the isolated rat heart sarcolemma by means of the colloidal iron technique. Preincubation of membranes with either neuraminidase, trypsin or phospholipase C was performed to assess the contribution of sialic acid, proteins and/or glycoproteins and phospholipids respectively to the negatively charged sites stained by colloidal iron. In addition, membranes were examined for their calcium-binding activity and for the activities of Mg^{2+} ATPase, Ca^{2+} ATPase, and Na^{+}-K^{+} ATPase.

Materials and methods

Neuraminidase (Cl. perfringens), phospholipase C (Cl. welchii) and trypsin (bovine pancreas) were obtained from Sigma. Trypsin inhibitor (egg white) was purchased from Calbiochem. $^{45}CaCl_2$ was supplied by New England Nuclear.

Enzyme treatments of sarcolemma

Sarcolemmal membranes were incubated for 20 min at 37 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 20 mM KCl to which neuraminidase (0.31 U/mg protein) or trypsin (100 μg/mg protein) had been added. Incubation with phospholipase C (1.0 U/mg protein) was carried out in the same buffer in the presence of 2 mM $CaCl_2$. Trypsin treatment was terminated by the addition of 2–3× trypsin inhibitor. Phospholipase C treatment was terminated by the addition of 2 mM EDTA, and neuraminidase treatment was terminated by cooling in ice. The specifications for these enzyme treatments of the sarcolemma were adopted from studies reported elsewhere (28, 30).

Calcium binding

Treated and control membranes were used to study Ca^{2+} binding. Approximately 0.15 mg/ml membrane protein was suspended in a medium containing 50 mM Tris-HCl, pH 7.4. Membranes were preincubated for 3 min at 37 °C, and the reaction was started by the addition of 100 μl of $^{45}CaCl_2$ at a final concentration of either 10^{-4} M or 1.25×10^{-3} M. The reaction was terminated after 5 min by the millipore filtration technique (29).

Membrane enzyme assays

ATP hydrolysis of both treated and control membranes was studied by suspending 40–50 μg/mg protein in a medium containing 50 mM Tris-HCl, pH 7.4. Na^+-K^+ ATPase was studied in the presence of 0.1 M NaCl, 10 mM KCl, 4 mM $MgCl_2$, 1 mM EDTA. The activity of Mg^{2+} ATPase was studied in the presence of 4 mM $MgCl_2$, 1 mM EDTA and the activity of Ca^{2+} ATPase was studied in the presence of 4 mM $CaCl_2$. Membranes were preincubated for 3 min at 37 °C, and the reaction was started by the addition of 4 mM Tris-ATP, pH 7.4. The reaction was terminated by the addition of 12 % ice-cold trichloroacetic acid. Phosphate determination was made according to the procedure of Taussky and Shorr (32) and protein concentration was estimated by the method of Lowry et al. (18).

Sialic acid determination

Sialic acid released by either neuraminidase treatment or acid hydrolysis of sarcolemmal membranes was measured by the method of Warren (35). For determination of the neuraminidase-sensitive component of the sialic acid contained in sarcolemma, membranes were incubated with the enzyme as described above, and the supernatant was assayed for sialic acid. The pellet was washed and resuspended in 0.1 N H_2SO_4 for 1 hr at 80 °C to hydrolyze the remaining sialic acid. Some membranes were hydrolyzed without prior enzyme treatment under the conditions described above to determine total sialic acid content.

Cytochemical procedures

Colloidal iron staining and electron microscopy

Two cytochemical protocols were followed each yielding a different topological view of the distribution of the colloidal iron stain. In both procedures membranes were treated with trypsin, phospholipase C or neuraminidase prior to fixation and staining in order to determine the effect of removing or modifying the different membrane components on the distribution of negatively charged sites. In the first procedure both treated and control membranes were suspended in 2 % glutaraldehyde in phosphate buffer, pH 7.4, for 45 min at 4 °C. Following centrifugation, the pellet was washed in the same buffer, followed by two changes (5 min each) of 12 % acetic acid and resuspended in freshly prepared colloidal iron solution, pH 1.2–1.3, for a period of 1 hr at room temperature (11). Following staining, the membranes were centrifuged, washed in four changes (3 min each) of 12 % acetic acid and postfixed in 1 % osmium tetroxide for 1 hr at 4 °C. The membranes were dehydrated in a graded ethanol series and embedded in Epon 812 (19). Thin sections were cut on a Porter-Blum ultramicrotome, placed on Formvar-coated grids, stained with uranyl acetate and lead citrate and examined in a Zeiss EM-9 electron microscope.

In the second procedure sarcolemmal membranes, fixed and subsequently stained with colloidal iron in the manner described above, were applied directly to Formvar-coated grids and examined in the electron microscope (3, 24). This procedure yields a surface view of the colloidal iron distribution on the sarcolemma.

Results

Colloidal iron staining

It has been reported earlier that the membrane fraction obtained by hypotonic shock-LiBr method (22) originates from the myocardial cell membrane (31), and electron microscopic examination has shown it to be

free of contaminants such as myofialments, mitochondria, and nuclei. Marker enzyme studies of this membrane preparation also revealed minimal contamination (3–5 %) with other cytoplasmic organelles (1). The fraction used in the present study showed a high Na^+-K^+ ATPase activity (14.27 ± 1.06 μmoles Pi/mg protein per hr) which was 80–90 % inhibited by 2 mM ouabain. The preparation was found to consist of vesicles of variable shape and size. The outer surface of most vesicles was not smooth due to the presence of surface material.

Distribution of the colloidal iron stain in untreated sarcolemmal membranes is shown in figure 1a and b. Electron dense particles of colloidal iron (CI) in the sectioned membranes (fig. 1a) were present largely on the

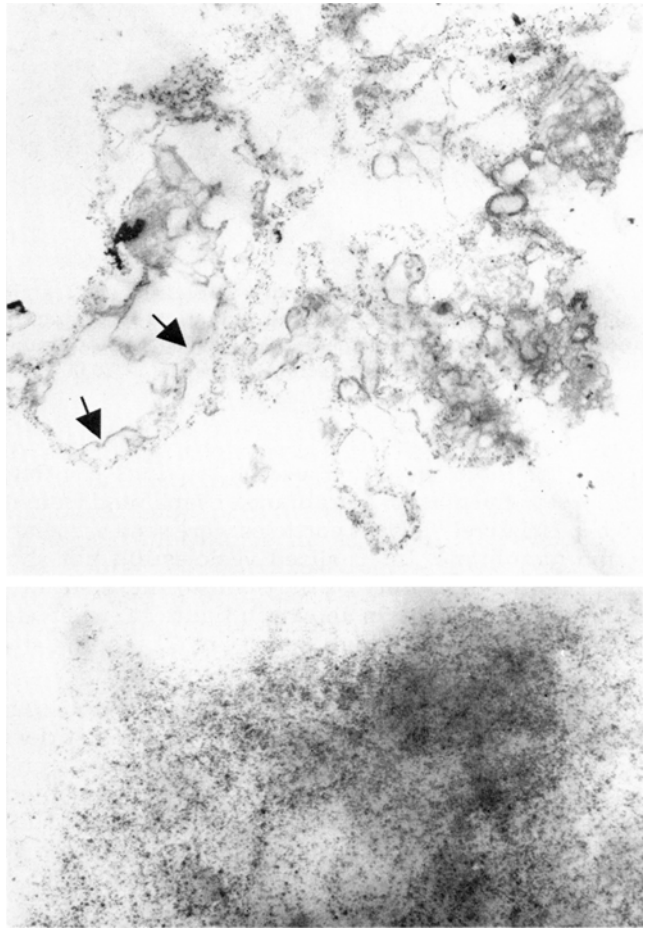

Fig. 1. Colloidal iron staining of the isolated rat heart sarcolemma. a) Sectioned membranes: CI particles are seen mostly in the fuzzy layer. This layer at places is separated from the unit membrane (arrows): × 22,500. b) Membranes in surface view: CI forms a dense uniform pattern over the membrane surface, × 78,000.

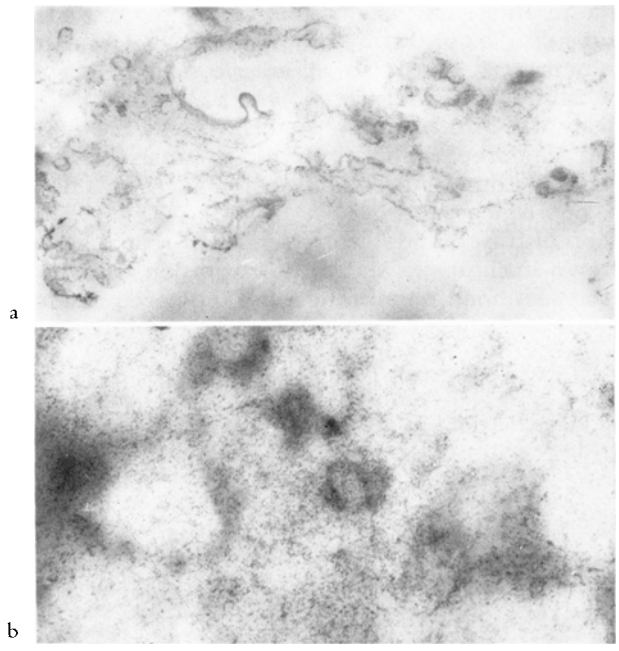

Fig. 2. Effects of trypsin treatment (100 μg/mg protein) on CI staining of the sarcolemma. a) Sectioned membranes: marked reduction of CI staining as well as poor membrane definition due to this treatment, × 23,000. b) Membranes in surface view: uniform reduction in the CI staining, × 70,000.

surface material. The latter varied between 0.1 and 0.3 μ in thickness and was either apposed to the plasma membrane or separated from it by a clear gap of up to 0.5 μ. Relatively few CI particles were seen associated directly with the plasma membrane. Internalized vesicles did not show any CI, probably due to non-accessibility of the stain to these structures. Membranes from the same preparation shown in figure 1a, when examined in surface view (fig. 1b), revealed a high density of CI binding sites, and the distribution of CI in this view was fairly uniform (fig. 1b).

Trypsin treatment of membranes altered their morphological appearance as well as subsequent binding of the CI to the cell surface material. The results of a typical experiment are shown in figure 2a and b. In the sectioned material sarcolemmal membranes were represented by membranous sheets (fig. 2a), rather than by vesicles seen in the untreated preparation. CI staining of the cell surface material, seen both in sectioned and surface views, was considerably reduced after trypsin treatment, however, distribution of the stain remained uniform.

The effects of phospholipase C treatment on the density and distribution of CI are shown in figure 3a and b. In sectioned material, a morphological alteration in sarcolemmal membranes, manifested chiefly by a substantial reduction in the surface material as well as vesicle size, was

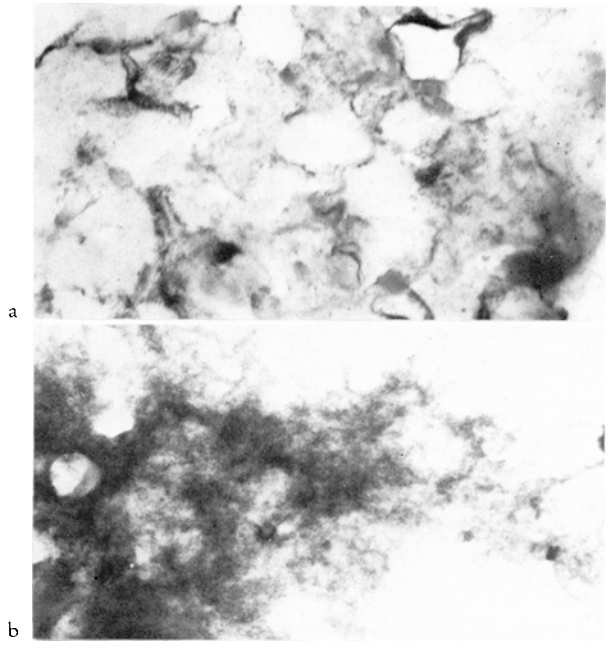

Fig. 3. Effects of phospholipase C treatment (1.0 U/mg protein) on CI staining of the sarcolemma. a) Sectioned membranes: marked reduction in the CI staining as well as size of the vesicles is apparent, × 58,000. b) Membranes in surface view: marked decrease in CI staining, × 35,000.

apparent. In addition, CI staining of the surface material, in both sectioned and surface views, was reduced from control (fig. 3a and b).

Neuraminidase treatment of the membranes had no apparent effect on their morphological appearance (fig. 4a and b). However, CI staining of the surface material after neuraminidase treatment was consistently less than in controls (fig. 4a and b). In surface view, the loss of CI staining was found to be patchy in appearance, and remaining CI particles were seen to aggregate in a random fashion.

Sialic acid content

In this series of experiments, sarcolemmal membranes were incubated with neuraminidase (0.31 U/mg protein) as described in Methods. Neuraminidase treatment released 25.4 ± 2.70 nmoles sialic acid/mg protein. Subsequent acid hydrolysis of the neuraminidase-treated membranes released a further 14.99 ± 0.08 nmoles of sialic acid/mg protein, yielding a total sialic acid content of 40.3 ± 3.47 nmoles/mg protein. Acid hydrolysis of control membranes which had not been treated with neuraminidase was performed and yielded a sialic acid content of 40.4 ± 3.14 nmoles/mg protein; a result virtually identical to that obtained in the above experiment. Neuraminidase treatment therefore released approximately 63 % of the total sarcolemmal bound sialic acid.

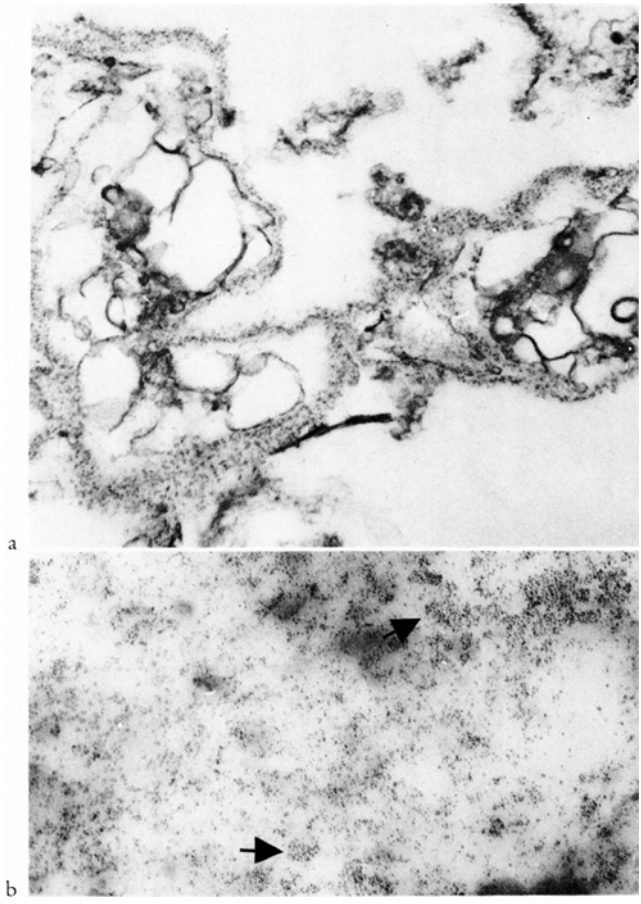

Fig. 4. Effects of neuraminidase treatment (0.31 U/mg protein) on CI staining of the sarcolemma. a) Sectioned membranes: In this view only marginal reduction in the CI staining is noticed, × 26,500. b) Membranes in surface view: A definite reduction in the CI staining as well as a random aggregation (arrows) of the CI particles is noticeable, × 72,500.

Calcium binding

Rat heart sarcolemma is reported to have both high and low affinity calcium binding sites with affinity constant values of 2×10^{-5} M and 5.6×10^{-2} M respectively (17). In this study the binding characteristics of the sarcolemmal fraction both before and after treatment with trypsin, phospholipase C and neuraminidase. The results are shown in table 1. Control membranes bound 24.5 ± 1.96 nmoles Ca^{2+}/mg protein per 5 min in the presence of 0.1 mM Ca^{2+}. Increasing the Ca^{2+} concentration to 1.25 mM resulted in a tenfold increase in calcium binding to 248.5 ± 14.34 nmoles Ca^{2+}/mg protein per 5 min. Calcium binding in the presence of 0.1 mM calcium was reduced by both trypsin and neuraminidase treat-

Table 1. Effects of trypsin, phospholipase C and neuraminidase on calcium binding by rat heart sarcolemma.

Ca^{2+} concn. (mM)	Calcium binding (nmoles Ca^{2+}/mg protein per 5 min) Control	Trypsin	Phospholipase C	Neuraminidase
0.1	24.5 ± 1.96	7.2 ± 0.41*	22.1 ± 1.52	14.4 ± 0.77*
1.25	248.5 ± 14.34	126.4 ± 8.58*	169.1 ± 2.75*	142.4 ± 20.12*

Assay medium as described in Materials and methods. Each value is the mean ± S.E. of 4–6 experiments.
* Significantly different from the control value ($P < 0.05$).

Table 2. Effects of trypsin, phospholipase C and neuraminidase on the activities of Mg^{2+} ATPase, Ca^{2+} ATPase and Na^+-K^+ ATPase of rat heart sarcolemma.

Enzyme activity (μmoles Pi/mg protein per hr)	Treatment			
	Control	Trypsin	Phospholipase C	Neuraminidase
Mg^{2+} ATPase	32.3 ± 1.15	39.3 ± 3.57*	30.4 ± 2.03	21.6 ± 1.94*
Ca^{2+} ATPase	35.5 ± 2.13	44.9 ± 2.80*	32.6 ± 1.40	23.0 ± 1.62*
Na^+-K^+ ATPase	14.37 ± 1.06	1.62 ± 0.88*	2.18 ± 0.29*	8.01 ± 0.90*

Na^+-K^+ ATPase was measured in the presence of 100 mM NaCl, 10 mM KCl, 4 mM $MgCl_2$ and Mg^{2+} ATPase activity was determined in a medium containing 1 mM EDTA and 4 mM $MgCl_2$. Ca^{2+} ATPase activity was determined in the presence of 4 mM $CaCl_2$. All other conditions were as described in Methods. Each value is the mean ± S.E. of 4–8 experiments.

* Significantly different from the control value ($P < 0.05$).

ments but was unaffected by treatment with phospholipase C. Furthermore, trypsin was found to be more effective than neuraminidase in reducing calcium binding at this concentration. Calcium binding activity of the membranes in the presence of 1.25 mM Ca^{2+} was reduced by treatment with any of the membrane perturbing agents employed in this study with the order of potency, starting with the most effective agent, being trypsin > neuraminidase > phospholipase C (table 1).

Sarcolemmal enzyme activities

The effects of trypsin, phospholipase C and neuraminidase treatments on the activities of three sarcolemmal enzymes are shown in table 2. Treatment of membranes with trypsin resulted in an increase in the specific activities of both Mg^{2+} ATPase and Ca^{2+} ATPase by 22 % and 26 % respectively over control values. In contrast, Na^+-K^+ ATPase activity was reduced 90 % by this treatment. Phospholipase C caused a slight depression in the activities of Mg^{2+} ATPase and Ca^{2+} ATPase, but these changes were not significant. However, a marked reduction (85 %) in the activity of Na^+-K^+ ATPase was apparent following phospholipase C treatment. Treatment of sarcolemma with neuraminidase caused a comparable reduction of approximately 30–40 % in the activities of all three enzymes, and these changes were statistically significant.

Discussion

It is well documented that CI at pH 1.2–1.4 employed in the present study binds to the cell surface (3, 10, 11, 12, 15, 21, 24, 37). Although there is still no agreement with respect to the chemical nature of the sites binding CI, it is generally agreed that such sites are negatively charged. The present study demonstrates the presence of negatively charged sites in the isolated rat heart sarcolemmal fraction by two different cytochemical procedures using CI stain as a common probe. A comprehensive picture regarding the distribution of negative charge could be constructed by combining the observations from both the sectioned and the surface views of the CI-stained membranes. The high density of CI particles observed in the surface view was most likely due to superimposition of particles scattered in the surface coat and on the plasma membrane. The distribution of CI in the sectioned material is comparable to that reported in rat and rabbit myocardial tissue preparations (10, 14). Furthermore, the reported separation of the basement membrane from the unit membrane seen in rat hearts perfused with Ca^{2+} free medium for a prolonged time (10, 33) may in fact correspond to the occurrence of a wide gap between the two layers observed in the isolated sectioned membranes. In this regard it should be noted that isolation of sarcolemma in the present study involved homogenization and washing in the presence of 1 mM EDTA, a known chelator of calcium. The results of this cytochemical study are qualitatively similar to CI staining reported for cultured heart cells and myocardial tissue (10).

Mowry (23) considered CI to be specific for acidic polysaccharides at pH 1.1–1.3 but warned of non-specific staining at pH 1.4 or higher. The CI

stain has been used for the cytochemical demonstration of membrane-bound sialic acid (3, 10, 11, 15, 21, 24) as well as hyaluronic acid and sulfated mucopolysaccharides (21, 37). It has been noted that the positively charged colloid can bind not only to carboxyl and sulfate groups of mucopolysaccharides but also to carboxyl groups of acidic amino acids and phosphate groups of phospholipids (4). The results of this study support this contention inasmuch as pretreatment of sarcolemmal membranes with either trypsin, phospholipase C or neuraminidase reduced CI staining. It appears the negative charge on the heart sarcolemma is due to several different chemical groups, since no one treatment completely prevented CI staining. Reduction in CI staining following proteolytic digestion has been demonstrated in red cell membranes (24), and this treatment has also been reported to release membrane-bound sialic acid as a sialoglycopeptide in red cells (20, 24, 34), rat liver membranes (3), and rat hepatoma cultures (2). In the present study, removal of over 60 % of sialic acid by neuraminidase was not as effective in reducing CI staining as was trypsin treatment. This result could be anticipated if CI is bound not only to sialic acid but also to membrane proteins. In that event release of peptides and sialoglycopeptides by trypsin should have an additive effect in reducing negatively charged sites. Furthermore, lanthanum, which is believed to displace calcium at the cell surface (26), has been found associated with surface layer which was sensitive to trypsin and pronase (25). Others, however, have shown lanthanum bound to membrane phospholipids (7, 16). Our results on the isolated rat heart sarcolemma strengthen the view that the surface negative charge is due to a mosaic of chemical groups.

The data described here on Ca^{2+} binding properties of trypsin and neuraminidase treated sarcolemma indicate that proteins and glycoproteins may also play a major role in both the high affinity and low affinity Ca^{2+} binding pools (9, 17, 27). Limas (27) reported calcium binding to rat heart sarcolemma which was dramatically reduced by trypsin treatment but relatively unaffected by treatment of the membranes with phospholipases or neuraminidase. In contrast, we observed a significant reduction in membrane Ca^{2+} binding at both high (1.25 mM) and low concentrations of calcium (0.1 mM) with neuraminidase, and at high concentrations of calcium following phospholipase C treatment. The reason for these differences between the two studies is not clear at present. However, reduction in the number of available cation binding sites, indicated by the fall in Ca^{2+} binding following phospholipase C and neuraminidase treatments in the present study, is supported by a corresponding decrease in CI staining of the treated membranes. Sialic acid has been reported to bind calcium in a selective manner (13), thus the lack of any significant effect of neuraminidase treatment on subsequent Ca^{2+} binding to the sarcolemma in Limas' (17) study is intriguing. Our results indicate that proteins, phospholipids and sialic acid are involved in membrane calcium binding. Furthermore, the failure of phospholipase C to significantly reduce Ca^{2+} binding at low concentrations (0.1 mM) of calcium may suggest that phospholipids contribute more to low affinity Ca^{2+} binding pool.

Neuraminidase has been reported by a number of workers to substantially prevent subsequent binding of CI in a variety of tissues (3, 10, 15, 24).

While neuraminidase always reduced staining in our preparation, it never eliminated it. Benedetti and Emmelot (3), Langer et al. (15), and Frank et al. (19) all reported 60–70 % of membrane sialic acid was released by neuraminidase. At the same time almost total abolition of CI staining was noted in these studies, indicating the remaining sialic acid was insensitive to or inaccessable to both the stain and neuraminidase. The study of Nicolson (24), on the other hand, reported hydrolysis of 90 % of membrane-bound sialic acid by neuraminidase with concomitant decrease in CI staining to 85 % of control. In the present study removal of 63 % of membrane-bound sialic acid resulted in a definite reduction in staining, although the exact percentage decrease could not be ascertained from this essentially qualitative approach. It appears that binding sites remaining following neuraminidase treatment either are not sialic acid or if they are, they possess α-ketosidic linkages not cleaved by the enzyme under our experimental conditions. Such neuraminidase-resistant bonds have been reported by Drzeniek (8).

The effects of trypsin and phospholipase C, two membrane-perturbing agents, on three sarcolemmal enzymes are not unexpected as loss of proteins or lipids not only alters the microenvironment of the membrane but may also have direct effects on the enzyme molecules (5). However, a rather uniform depression of Mg^{2+} ATPase, Ca^{2+} ATPase, and Na^{+}-K^{+} ATPase activities following neuraminidase treatment would suggest either sialic acid forms a part of the enzyme molecules or the distribution of surface charge plays a role in the regulation of the activities of these membrane-bound enzymes. The latter view is consistent with a recent report suggesting that the activities of membrane enzymes with charged substrates may be regulated by the density of the surface charge, and alterations in that charge may change the concentration of a substrate in the immediate vicinity of the membrane (36).

It can be concluded that the density and distribution of the surface charge is intimately related to both cation binding and enzymatic activities in rat myocardial sarcolemma and alterations in this parameter by removal or modification of membrane components contributing to the surface charge will necessarily result in changes in the functional properties of this membrane system.

Zusammenfassung

Beim isolierten Sarkolemm vom Rattenherzen wurde die Färbung mit kolloidalem Eisen, Ca-Bindung und Enzym-Aktivitäten untersucht. Die Färbung mit kolloidalem Eisen zeigte eine hohe Dichte negativ geladener Stellen an der Zelloberfläche. Es wurde festgestellt, daß diese Membranfraktion über Ca-Bindungsaktivität sowohl bei niedriger (0,1 mM) als auch bei hoher (1,25 mM) Ca-Konzentration verfügt.

Vorbehandlung des Sarkolemms mit Trypsin, Phospholipase C oder Neuraminidase führte sowohl zu einer Abnahme der Färbung mit kolloidalem Eisen als auch zu einer verminderten Bindungsaktivität für Calcium bei hohen Ca-Konzentrationen. Die Ca-Bindung bei niedrigen Konzentrationen wurde sowohl durch Trypsin als auch durch Neuraminidase vermindert. Die Aktivität der Mg^{2+}-ATPase, Ca^{2+}-ATPase und Na^{+}-K^{+}-ATPase wurde durch Neuraminidase und Trypsin-Behandlung verändert, während Behandlung mit Phospholipase C nur die Na^{+}-

K^+-ATPase beeinflußte. Es ergibt sich die Schlußfolgerung, daß sowohl den negativen Oberflächenladungen als auch den Ca-Bindungsstellen des isolierten Sarkolemms vom Rattenherzen ein Mosaik von Biomolekülen zugrunde liegt, welches Proteine, Phospholipide und Glycoproteine enthält, und daß Änderungen der Oberflächenladung die Aktivität membrangebundener Enzyme beeinflussen können.

Key words: heart sarcolemma, sarcolemmal calcium binding, surface charge, colloidal iron staining, membrane-bound enzymes

References

1. Anand, M. B., M. S. Chauhan, N. S. Dhalla: Ca^{2+}/Mg^{2+} ATPase activities of heart sarcolemma, microsomes and mitochondria. J. Biochem. **82,** 1731 (1977).
2. Baumann, H., D. Doyle: Effect of trypsin on the cell surface proteins of hepatoma tissue culture cells. Characterization of a carbohydrate-rich glycopeptide released from a calcium binding glycoprotein. J. Biol. Chem. **254,** 3935 (1979).
3. Benedetti, E. D., P. Emmelot: Studies in plasma membranes IV. The ultrastructural localization and content of sialic acid in plasma membranes isolated from rat liver and hepatoma. J. Cell. Sci. **2,** 499 (1967).
4. Blanquet, P. R., A. Loiez: Colloidal iron used at pH's lower than 1 as electron stain for surface proteins. J. Histochem. Cytochem. **22,** 368 (1974).
5. Coleman, R.: Membrane bound enzymes and membrane ultrastructure. Biochim. Biophys. Acta **300,** 1 (1973).
6. Dhalla, N. S., A. Ziegelhoffer, J. A. C. Harrow: Regulatory role of membrane systems in heart function. Can. J. Physiol. Pharmacol. **55,** 1211 (1977).
7. Doggenweiler, C. F., S. Frenk: Staining properties of lanthanum on cell membranes. Proc. Nat. Acad. Sci. USA **53,** 425 (1965).
8. Drzeniek, R.: Substrate specificity of the neuraminidases. Histochem. J. **5,** 271 (1973).
9. Duffy, M. H., V. Schwartz: Calcium binding by the erythrocyte membrane. Biochim. Biophys. Acta **330,** 294 (1973).
10. Frank, J. S., G. A. Langer, L. M. Nudd, K. Seraydarian: The myocardial cell surface, its histochemistry and the effect of sialic acid and calcium removal on its structure and cellular ionic exchange. Circulat. Res. **41,** 702 (1977).
11. Gasic, G., L. Berwick: Hale stain for sialic acid containing mucins: adaptation to electron microscopy. J. Cell. Biol. **19,** 223 (1963).
12. Howse, H. D., V. J. Ferrans, R. G. Hibbs: A comparative histochemical and electron microscopic study of the surface coatings of cardiac muscle cells. J. Mol. Cell. Cardiol. **1,** 157 (1970).
13. Jaques, L. W., E. B. Braun, W. S. Barrett, W. S. Brey, W. Weltner: Sialic acid. A calcium binding carbohydrate. J. Biol. Chem. **252,** 4533 (1977).
14. Langer, G. A.: The structure and function of the myocardial cell surface. Amer. J. Physiol. **235,** H461 (1978).
15. Langer, G. A., J. S. Frank, L. M. Nudd, K. Seraydarian: Sialic acid. Effect of removal on calcium exchangeability of cultured heart cells. Science **193,** 1013 (1976).
16. Lesseps, R. J.: The removal of phospholipase C of a layer of lanthanum staining material external to the cell membrane in embryonic chick cells. J. Cell. Biol. **34,** 173 (1967).
17. Limas, C. J.: Calcium binding sites in rat myocardial sarcolemma. Arch. Biochem. Biophys. **179,** 302 (1977).

18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, R. J. Randall: Protein measurement with the folin phenol reagent. J. Biol. Chem. **193,** 265 (1951).
19. Luft, J. H.: Improvements in epoxy resin embedding methods. J. Biochim. Biophys. Cytol. **9,** 409 (1961).
20. Makela, O., T. Mieltinen, P. Pesola: Release of sialic acid and carbohydrates from human red cells by trypsin treatment. Vox Sanguinis **5,** 492 (1960).
21. Mareel, M., C. Dragonetti, M. C. Van Peteghem: Cytochemistry of colloidal iron binding to the surface of Hela cells and human erythrocytes. Histochemistry **48,** 71 (1976).
22. McNamara, D. B., P. V. Sulakhe, J. N. Singh, N. S. Dhalla: Properties of the heart sarcolemmal Na^+-K^+ ATPase. J. Biochem. **75,** 795 (1974).
23. Mowry, R. W.: Improved procedure for the staining of acidic polysaccharides by Mullers colloidal (hydrous) ferric oxide and its combination with the Feulgen and periodic-acid-Schiff reactions. Lab. Invest. **7,** 566 (1958).
24. Nicolson, G. L.: Anionic sites of human erythrocyte membranes. I. Effects of trypsin, phospholipase C and pH on the topography of bound positively charged colloid particles. J. Cell. Biol. **57,** 373 (1973).
25. Overton, J., Localized lanthanum staining of the intestinal brush border. J. Cell. Biol. **38,** 447 (1968).
26. Sanborn, W. G., G. A. Langer: Specific uncoupling of excitation and contraction in mammalian cardiac tissue. J. Gen. Physiol. **56,** 191 (1970).
27. Shlatz, L., G. V. Marinetti: Calcium binding to the rat liver plasma membrane. Biochim. Biophys. Acta **290,** 70 (1972).
28. Singal, P. K., M. S. Nijjar, K. S. Dhalla, M. B. Anand-Srivastava, N. S. Dhalla: Structure-function relationship in the heart sarcolemma. In: Advances in Myocardiology, Vol. 1, Eds. M. Tajuddin, P. K. Das, M. Tariq and N. S. Dhalla. University Park Press, Baltimore, 33 (1980).
29. Sulakhe, P. V., N. S. Dhalla: Excitation-contraction coupling in heart. VII. Calcium accumulation in subcellular particles in congestive heart failure. J. Clin. Invest. **50,** 1019 (1971).
30. Takeo, S., M. J. Daly, M. B. Anand-Srivastava, N. S. Dhalla: Influence of neuraminidase treatment on rat heart sarcolemma. J. Mol. Cell. Cardiol. **12,** 211 (1980).
31. Takeo, S., P. Duke, G. M. L. Taam, P. K. Singal, N. S. Dhalla: Effects of lanthanum on the heart sarcolemmal ATPase and calcium binding activities. Can. J. Physiol. Pharmacol. **57,** 496 (1979).
32. Taussky, H. H., E. Shorr: A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. **202,** 675 (1953).
33. Tomlinson, C. W., J. C. Yates, N. S. Dhalla: Relationship among changes in intracellular calcium stores, ultrastructure and contractility of myocardium. In: Recent Advances in Studies on Cardiac Structure and Metabolism, Vol. 4, Myocardial Biology, Ed. N. S. Dhalla, 331 (University Park Press, Baltimore 1974).
34. Triplett, R. B., K. L. Carraway: Proteolytic digestion of erythrocytes resealed ghosts and isolated membranes. Biochemistry **11,** 2897 (1972).
35. Warren, L.: The thiobarbituric acid assay of sialic acids. J. Biol. Chem. **234,** 1971 (1959).
36. Wojtezak, L., M. J. Nalecz: Surface charge of biological membranes as a possible regulator of membrane bound enzymes. Eur. J. Biochem. **94,** 99 (1979).
37. Yardley, J. H., G. D. Brown: Fibroblasts in tissue culture. Use of colloidal iron for ultrastructure localization of acid mucopolysaccharides. Lab. Invest. **14,** 501 (1965).

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