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Protein Analysis of Cardiac Sarcolemma: Effects of Membrane-Perturbing Agents on Membrane Proteins and Calcium Transport†

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ABSTRACT: Protein composition of cardiac sarcolemmal membranes was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were observed to contain about 20 polypeptide bands ranging from 18 000 to 200 000 dalton mass. Out of these, six bands were prominent and together comprised 57% of the membrane protein. When sarcolemmal membranes, phosphorylated by [γ-^{32}P]ATP in the presence of Ca^{2+} or Na^+ with and without K^+, were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis at pH 2.4, the band III region (M_r 105 000) of gels was found to contain active sites of monomeric Ca-ATPase and (Na,K)ATPase. Bands I (M_r > 200 000), II (M_r 150 000), III (M_r 105 000), and VI (M_r 47 000) were accessible to trypsin; the extent of proteolysis was dependent on the time of exposure to, and the concentration of, trypsin (i.e., ratio of sarcolemmal protein/trypsin). Addition of molar sucrose protected sarcolemmal proteins from the tryptic proteolysis. Calcium transport was reduced by the action of trypsin; the degree of reduction was influenced by the time of exposure of membranes to trypsin as well as the concentration of trypsin. (Mg,Ca)ATPase activity, on the other hand, was elevated moderately at lower concentration and reduced at higher concentration of trypsin. Treatment with phospholipase C caused a time- and concentration-dependent decrease in calcium transport and (Mg,Ca)ATPase activity; electrophoretic patterns were unaffected by this treatment. Addition of lecithin to phospholipase C treated membranes produced a moderate increase in calcium transport. Exposure to Triton X-100 (1%) specifically solubilized three protein bands (M_r 90 000, 67 000, and 57 000), whereas exposure to deoxycholate (1%) preferentially solubilized high-molecular-weight proteins, including band III (M_r 105 000); Lubrol-PX (1%) caused nonspecific solubilization of proteins, although the extent of solubilization with Lubrol-PX was considerably less than with either Triton or deoxycholate.

It is recognized that cardiac contractility is regulated by several hormones and affected by drugs, which are thought to interact with specific receptors located on the sarcolemmal membranes (plasma membranes), as well as by transsarcolemmal ion fluxes. In our laboratory, we have initiated a series of biochemical investigations of cardiac sarcolemma with the ultimate aim of understanding initial molecular events occurring in these membranes due to cholinergic-adrenergic hormone interactions with the cardiac muscle. We have reported a procedure for the isolation of cardiac sarcolemma in a high degree of purity (Sulakhe et al., 1976a) and have characterized these membranes both biochemically and morphologically (St. Louis and Sulakhe, 1976a). Heart sarcolemmal membranes were observed to contain a calcium-transport system (Sulakhe et al., 1976a), whose properties were studied in some detail (St. Louis and Sulakhe, 1976b) and which was regulated by cAMP[1]-dependent phosphorylation of sarcolemmal proteins (Sulakhe et al., 1976a; Sulakhe and St. Louis, 1978; St. Louis and Sulakhe, in preparation). Considerable amounts of adenylate and guanylate cyclase were present in these membranes (Sulakhe et al., 1976b), and the activities of these enzymes were increased moderately by β-adrenergic and cholinergic agents, respectively (St. Louis and Sulakhe, 1976c; Narayanan and Sulakhe, manuscript in preparation); specific β-adrenergic receptors (Narayanan and Sulakhe, unpublished work) and cholinergic receptor sites were also present (Ma et al., 1978; Wei and Sulakhe, in preparation).

Analysis of proteins of sarcolemma and their biochemical functions is of potential significance in understanding molecular aspects of ion-transport systems and of receptor(s)-cyclase

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[1] Abbreviations used: cAMP, cyclic adenosine 3′,5′-monophosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; PPO, 2,5-diphenyloxazole; Cl_3AcOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-Cl, 2-amino-2-hydroxymethyl-1,3-propanediol chloride.

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systems of these membranes. In this paper, we describe the protein composition of guinea pig heart sarcolemma. To our knowledge, this is the first time that proteins of a highly purified, well-characterized cardiac sarcolemmal preparation have been analyzed. Numerous investigators have effectively used membrane-perturbing agents to describe the composition and organization of proteins in membranes such as red blood cell membranes (Guidotti, 1972; Marchesi et al., 1976) and fragmented sarcoplasmic reticulum from skeletal muscle (MacLennan and Holland, 1976). We have therefore employed several membrane-perturbing agents to study sarcolemmal proteins, and, further, the effects of membrane-perturbing agents on calcium transport were investigated.

Experimental Section

Materials

$^{45}CaCl_2$ (20 mCi/mg) and [γ-^{32}P]ATP (10 Ci/mmol) were purchased from New England Nuclear. β-Galactosidase, trypsin (11 400 BAEE units/mg), trypsin inhibitor, ouabain, Triton X-100, $NaDodSO_4$, and lecithinase C (type I from *Cl. welchii*) were from Sigma; phosphorylase *a*, bovine serum albumin, ovalbumin, carbonic anhydrase, and β-lactoglobulin were from Polyscience, Inc.; electrophoretic reagents and chemicals were from Bio-Rad Laboratories and Eastman Kodak; lecithin (bovine brain) was from P-L Biochemicals. All glass- and plasticware were thoroughly rinsed with deionized glass-distilled water.

Methods

Isolation of Cardiac Sarcolemmal Fraction. Sarcolemmal membranes (gradient fraction, F_{3C}) were prepared from guinea pig heart ventricles as described by Sulakhe et al. (1976a). Membranes were suspended in 0.25 M sucrose–10 mM Tris-Cl (pH 7.5)–2 mM dithiothreitol (sucrose–TD buffer).

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. This was carried out by the method of Weber and Osborn (1969). Gels were prepared in glass tubes (0.6-cm internal diameter) to give a gel column height of 7 cm. Samples were prepared using the solubilization mixtures described in the original reference. Solubilization was performed at 37 °C for 0.5 h, since results indicated that longer solubilization times did not affect the final electrophoretic protein profile. The reference proteins were solubilized for at least 1 h and were run with each set of gels. The reference proteins used, with their respective molecular weights, were β-galactosidase (130 000), phosphorylase *a* (94 000), bovine serum albumin (68 000), ovalbumin (45 000), carbonic anhydrase (32 000) and β-lactoglobulin (18 000). Electrophoresis was carried out (22 °C) at 8 mA/gel for about 4 h (or until the tracking dye had migrated about 6 cm) in a Pharmacia GE-4 apparatus. Gels were stained with 0.025% Coomassie blue as described by Weber and Osborn (1969) and were destained with 7.5% acetic acid containing 5% methanol. Rapid destaining was achieved by heating to about 70 °C as indicated by Andrew et al. (1975). The amount of protein applied per gel was normally between 40 and 100 μg. Gels were scanned at 555 nm using a Transidine RFT scanning sensitometer with a 2960 computing integrator at a slit size of 0.1 × 1.0 mm. Gels were stained for glycoprotein using periodic acid–Schiff reagent described by Martonosi and Halpin (1971).

Phosphorylation of Sarcolemmal Membranes and Fractionation of Phosphorylated Proteins by $NaDodSO_4$–Gel Electrophoresis at pH 2.4. Membranes (150–200 μg of protein) were incubated for 15 s at 22 °C in 0.4 mL of buffered (50 mM imidazole hydrochloride, pH 7.5) reaction mixture in the presence of 5 mM $MgCl_2$, 0.5 mM EGTA, or 0.5 mM EDTA, and 50 μM [γ-^{32}P]ATP (400–800 cpm/pmol). When present, $CaCl_2$, NaCl, and KCl were 0.51, 100, and 20 mM, respectively (see Figure 2). The reaction was started by [γ-^{32}P]ATP and was terminated by the addition of 1 mL of ice-cold 12% Cl_3AcOH containing 1 mM P_i and 1 mM ATP (nonradioactive). The mixtures were kept on ice for 5 min and then centrifuged (International PR-6 centrifuge, 3000 rpm/10 min). The pellets were washed twice with 1 mL of Cl_3AcOH solution as above and then once with 1 mL of ice-cold deionized water. The washed residues were solubilized for electrophoresis as follows: 50 μL each of 50 mM Tris-maleate (pH 5.5) containing 5 mM EDTA, 5% $NaDodSO_4$, 50% sucrose, and 200 mM DTT were added to the residues. The tubes were incubated for 30 min at 22 °C and 5 μL of pyronin Y (10 μg/mL) was added. Electrophoretic fractionation was carried out using 1% $NaDodSO_4$–5.6% polyacrylamide (tube) gels at pH 2.4 according to the method of Fairbanks and Avruch (1972). Gel columns were 7 × 0.6 cm and electrophoresis was performed at 4 mA/gel for 2.5 h with 50 mM phosphate buffer (pH 2.4) containing 1% $NaDodSO_4$. The gels were then sliced (2-mm slices) and the slices placed in glass scintillation counting vials. The slices were treated at 60 °C for 12 h with 0.5 mL at 60% perchloric acid and 0.5 mL of 30% H_2O_2. Fifteen milliliters of Bray's solution (Bray, 1960) was added to each vial, and the radioactivity was determined by liquid scintillation spectrometry. Duplicate gels were run simultaneously, stained for protein, and destained as described by Fairbanks and Avruch (1972).

Treatment of Sarcolemma with Trypsin. Membranes were treated with trypsin (concentrations as indicated are trypsin/sarcolemma, protein/protein ratio) in the presence of 120 mM KCl for 10 min at room temperature (22 °C). Reactions were terminated by the addition of trypsin inhibitor to an approximately fivefold excess. After a further incubation for 10 min, preparations were centrifuged on a Beckman Microfuge B for 1 min. The pelleted residues were resuspended in sucrose–TD buffer. In some experiments the time of trypsin treatment was varied as indicated.

Detergent Treatment of Sarcolemma. Sarcolemmal fractions were treated with detergents (Lubrol-PX, Triton X-100, and sodium deoxycholate) to a final concentration of 1% (w/v) (equivalent to 6 mg of detergent/mg of sarcolemmal protein) for 30 min on ice. Preparations were then centrifuged on the Microfuge B for 1 min. The supernatants (soluble fraction) were retained, and the pellets (residues) were resuspended in sucrose–TD buffer. Samples of both supernatants and residues were prepared for electrophoresis in the usual manner.

Treatment of Sarcolemma with Phospholipase C. The sarcolemmal fraction was incubated with phospholipase C (concentrations as indicated in various experiments) in the presence of 0.4 mM $CaCl_2$ for 10 min at 30 °C. The reaction was stopped by the addition of EGTA (pH 7.5) to 1.0 mM final concentration. Preparations were either centrifuged immediately or kept on ice until centrifuged (Microfuge B for 30 s). After removal of the supernatants, the residual pellets were washed by resuspension in sucrose–TD buffer and centrifugation and finally suspended in sucrose–TD buffer. In some experiments the time of incubation with phospholipase C was varied as indicated.

Lecithin (phosphatidylcholine from bovine brain; P-L Biochemicals, 1.5 mg, in solution in benzene) was evaporated to dryness under nitrogen, and to this, 1.0 mL of 10 mM Tris-Cl (pH 7.5) was added. After mixing, the preparation was sonicated (4 × 15 s, on ice) using a Polytron probe. The sus-

TABLE I: Effect of Sodium Deoxycholate on (Na^+,K^+)ATPase, *p*-Nitrophenylphosphatase, and Ouabain Binding Activities of Heart Sarcolemma:[a] Estimation of the Sidedness of the Membrane Fraction.

parameter assayed units of act.:	control prep *A*	detergent-treated prep *B*	diff $B - A$	inside-out vesicle (%) $\frac{B-A}{B} \times 100$
ouabain-sensitive (Na^+,K^+)ATPase (nmol of P_i min^{-1} mg^{-1})	88.60 ± 18.33[b]	115.00 ± 20.60	26.40 ± 6.00	24.55 ± 4.77[c]
ouabain-sensitive (*p*-nitrophenylphosphatase) (nmol of *p*-NP min^{-1} mg^{-1})	13.54 ± 1.88	17.70 ± 2.17	4.16 ± 0.68	24.00 ± 3.61
ouabain binding (pmol/mg)	2.59 ± 0.23	5.03 ± 0.25	2.44 ± 0.40	48.33 ± 6.34
$\frac{\text{ouabain-sensitive } (Na^+,K^+)\text{ATPase}}{\text{ouabain-sensitive } p\text{-nitrophenylphosphatase}}$	6.54 ± 0.80	6.27 ± 0.30	6.35 ± 0.65	

[a] Sarcolemmal membranes (2 mg of protein) were incubated at 22 °C for 15 min in the reaction mixture (1 mL) containing sucrose-TD buffer in the absence and presence of 2 mg of sodium deoxycholate. The membranes were centrifuged at 10 000*g* for 10 min, and the pellet was washed and then resuspended in 1 mL of sucrose-TD buffer. Ouabain-sensitive (Na^+,K^+)ATPase and *p*-nitrophenylphosphatase as well as ouabain-binding activities were determined as indicated under Methods. [b] Results are mean ± standard error of mean of three experiments. [c] Refer to the text for the explanations and the calculations used to estimate the content of inside-out vesicles in the membrane fraction.

pension so obtained was clarified by centrifugation (25 000*g* for 20 min), and the supernatant fluid was used after gassing with nitrogen.

Assay for Calcium Accumulation. Fractions (40–100 μg of protein) were incubated at 30 °C in the reaction mixture (1.0 mL) which contained 25 mM Tris-maleate (pH 6.8), 5 mM $MgCl_2$, 100 mM KCl, 2.5 mM ATP, and 0.1 mM $^{45}CaCl_2$ (5000–20 000 cpm/nmol) in the presence of 5 mM potassium oxalate. At the desired times, 0.3-mL aliquots of the incubation mixture were filtered through Millipore disks (0.45-μm pore size) and the disks were washed with 3 mL of 10 mM Tris-maleate (pH 6.8) and processed as previously described (Sulakhe et al., 1973a; Sulakhe et al., 1976a).

Other Assays. Mg-ATPase and (Mg,Ca)ATPase activities were determined as reported earlier by Sulakhe et al. (1973b). Assays for (Na,K)ATPase (ouabain sensitive) and [^{3}H]ouabain binding have been described previously (St. Louis and Sulakhe, 1976b). *p*-Nitrophenylphosphatase activity was determined as described by Sulakhe et al. (1973b). Protein estimation was carried out according to the method of Lowry et al. (1951) and inorganic phosphate according to the method of Taussky and Shorr (1953).

Results

Sidedness of the Membrane Fraction. It is conceivable that during homogenization of the cardiac tissue plasma membranes would fragment into membrane vesicles of varying size and shape and that the membrane fraction studied would contain both right-side-out and inside-out vesicles. If one were to assume resealing of the membrane vesicle by a random process, then the fraction should comprise approximately 50% inside-out and 50% right-side-out membrane vesicles. To ascertain this, we carried out experiments in which sidedness of the (Na,K)ATPase system of cardiac sarcolemmal membranes was evaluated. Certain assumptions had to be made in these experiments, although supportive evidence for these is available in the literature [see Schwartz et al. (1975) for a detailed review]. The K^+ binding and ouabain binding sites of the system are exposed to the extracellular side of sarcolemma, whereas the catalytic site and the Na^+ binding site are exposed to the cytoplasmic side. This would imply that right-side-out vesicles, but not inside-out vesicles, present in the fraction are capable of specific [^{3}H]ouabain binding, since in these vesicles the binding sites are readily accessible under the in vitro assay condition. Following treatment of the membrane fraction with sodium deoxycholate, the situation is quite different. In this case, both right-side-out and inside-out vesicles contribute toward the binding of [^{3}H]ouabain, since the detergent treatment would render the ouabain binding sites of inside-out vesicles accessible for interaction. Therefore, the binding capacity following the detergent treatment reflects the total capacity of the membrane fraction. It follows then that the difference between the measured binding capacity with and without detergent represents the content of inside-out vesicles of the preparation. This estimation is valid, provided the detergent treatment is such that it does not cause significant activation or inactivation of the binding sites but rather allows accessibility of these sites (of inside-out vesicles) which were otherwise not available for the binding. Therefore, the difference between the binding capacity of the membrane fraction with and without detergent treatment provides an estimation of the inside-out vesicle content of the preparation.

In our study, we treated cardiac sarcolemma with varying concentrations of sodium deoxycholate. Membranes were then centrifuged, and the pellet was washed and assayed for enzyme activities and ouabain binding. The data presented in Table I show that (Na^+ plus ATP)-supported ouabain binding to sarcolemma increased from 2.59 ± 0.23 to 5.03 ± 0.25 pmol/mg of sarcolemmal protein following exposure of the membrane fraction to sodium deoxycholate (mg of detergent/mg of membrane protein). In these experiments, when varying amounts of the detergent were used in the presence of a fixed (2 mg of protein) amount of membrane, the maximal increases in ouabain binding as well as in enzyme activities (see below) were observed at the detergent–protein ratio (w:w) of 1 (results not shown). Therefore, the manifest (without detergent) and the total (with detergent) ouabain binding activity indicated that about 48% of the vesicles in the preparation was inside-out (see Table I). This estimation is quite close to the anticipated value of 50%. However, the estimations based on ouabain-sensitive (Na^+,K^+)ATPase and *p*-nitrophenylphosphatase activities indicated that 24% of the vesicles were inside out. These differences in estimation could be explained in part by assuming that the membrane vesicles are leaky (i.e., they are not permeability intact) which permitted various ligands (such as Na^+, K^+, ATP, and *p*-nitrophenyl phosphate) to interact with their respective sites in both right-side-out and inside-out vesicles under the assay conditions. Ouabain, on the other hand, is less permeable compared to other ligands. Therefore, ouabain binding activity, amongst the assays carried

out, represented a more reliable index of the sidedness of the membrane fraction. Nevertheless, the ratio of (Na^+,K^+)-ATPase activity to *p*-nitrophenylphosphatase activity remained essentially the same (i.e., about 6-7) in membranes with and without detergent treatment; in many reported studies, similar ratios for these activities have been observed [see Schwartz et al. (1975) for a review]. This indicates that the accessibility of ligands (such as Na^+, K^+, ATP, and *p*-nitrophenyl phosphate) toward their respective sites did not differ significantly in control and detergent-treated membrane fraction. If this were not to be true, then the observed ratios should have been different (i.e., following detergent treatment the ratio would have increased or decreased). The fact that this did not occur supports the view that membrane vesicles (right-side-out and inside-out) were permeable to small ligands which would account for the lower estimation (based on the data on enzyme activities) of the contribution of inside-out vesicle in the membrane preparation.

Sidedness of the Membrane Fraction Used in Relation to Calcium Transport Activity Measured. Although (bidirectional) Ca^{2+} fluxes across myocardial sarcolemma are known to occur during the contraction-relaxation cycle, the influx of Ca^{2+} from the extracellular space into the myocardium appears to be regulated by (or dependent on) the permeability of the membrane to Ca^{2+} and the concentration gradient for this cation [cytoplasmic level of ionized Ca^{2+} being lower; see review by Langer (1973)]. However, the efflux of Ca^{2+} from the myocardium is an uphill movement of this cation and so requires a supply of energy. Many investigators (Langer, 1972; Morad and Greenspan, 1973; Reuter, 1974) have suggested that the Na^+-Ca^{2+} exchange system of sarcolemma mediates the Ca^{2+} efflux for which the energy derived from the Na^+ gradient is utilized; in these early studies the participation of the ATP-dependent Ca^{2+} transport system in Ca^{2+} efflux was not evaluated or considered. However, very recently a considerable component of Ca^{2+} efflux from the myocardium (Jundt and Reuter, 1976) as well as axons (Baker and McNaughton, 1976; Requena et al., 1977; DiPolo, 1974, 1977) has been shown to require ATP similar to that already documented for red blood cells [see review by Schatzmann (1975)]. Since highly purified sarcolemmal membranes have been found to possess an ATP-dependent Ca^{2+} "pump" (Sulakhe et al., 1976a; also see below), it is necessary to distinguish between the contributions made by right-side-out and inside-out vesicles in studies that deal with Ca^{2+} binding and accumulation by these membranes. It is implicit that the catalytic site of the sarcolemmal "pump" faces the cytoplasmic side of the membrane. This site would therefore be directly accessible to Ca^{2+} and ATP in inside-out vesicles of the preparation, and, further, ATP-supported Ca^{2+} binding should reflect the activity of inside-out vesicles. The studies shown in Table I indicated that the membrane fraction appears permeable to small ions and ATP. Hence, it seems that right-side-out vesicles may also contribute to the Ca^{2+} binding activity of the sarcolemmal membranes. However, the stimulatory effect of oxalate on ATP-supported Ca^{2+} binding must result due to the translocation of Ca^{2+} from the medium into the lumen of inside-out vesicles. The role of oxalate in these studies is believed to be the same as its proposed role in studies of sarcoplasmic reticulum vesicles from heart and skeletal muscle where it is considered that oxalate (passively) accompanies Ca^{2+} and this leads to a trapping of Ca^{2+} inside the lumen of membrane vesicles due to the formation of (insoluble) calcium oxalate (Hasselbach, 1964). In the case of isolated sarcolemmal membranes, even though membrane vesicles of the fraction studied do not appear to be permeability "intact", oxalate

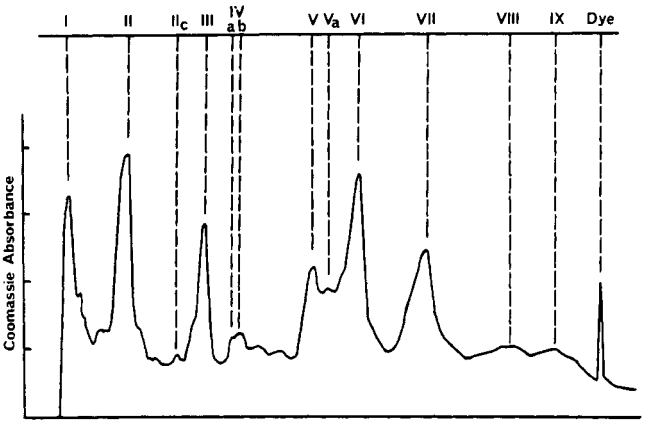

FIGURE 1: Protein profile of cardiac sarcolemmal membranes fractionated on $NaDodSO_4$-polyacrylamide gel. Sarcolemmal membranes (80 μg of protein) were fractionated on 6% polyacrylamide gel, and the gel was stained for protein with Coomassie blue, destained, and scanned. Identifiable polypeptide bands were designated bands I through IX, and their molecular weights are given in Table I.

should not potentiate Ca^{2+} accumulation in right-side-out vesicles, since the "pump" would move Ca^{2+} from their lumen to the medium; in our assay we have measured the amount of Ca^{2+} retained by the membrane following its separation by the Millipore filtration technique (see Methods). Hence, Ca^{2+} accumulation in the presence of oxalate was considered due to transmembrane (in the case of inside-out vesicles) flux for this cation. Both binding and accumulation terms are arbitrarily chosen to represent assay conditions without and with oxalate, respectively. Such terms have been previously used by us and others in studies of Ca^{2+} transport in isolated membranes from heart and muscle. We have previously described some of the characteristics of ATP-dependent Ca^{2+} binding and accumulation by guinea pig cardiac sarcolemma (St. Louis and Sulakhe, 1976b). In the present study, consideration has been given to the effects of membrane perturbing agents on Ca^{2+} accumulation only.

Analysis of Sarcolemmal Proteins on $NaDodSO_4$-Polyacrylamide Gels. Since our preliminary results indicated that gel concentrations higher than 6% did not allow for the effective resolution of high-molecular-weight polypeptides, we therefore routinely used 6% gels in our studies. The electrophoretic protein profiles showed the presence of six major and several minor components which we have designated I-IX as shown in Figure 1. Bands I, II, III, V, VI, and VII represented the six major proteins which together accounted for about 57% of the total protein (Table II). Bands IIc, IV(a + b), Va, VIII, and IX represented a lesser percent of the total protein but were consistently identifiable. In addition, a variable number of minor bands could be detected. Because of these minor bands, the total number of proteins resolved varied between 19 and 22. One possible reason for the variable number of minor bands, as suggested by Fairbanks et al. (1971), is that they may represent overlapping minor components of the membrane with differential responses to subtle changes in the fractionation procedure. Since a relatively high salt concentration (1.25 M KCl) was used in the isolation of cardiac sarcolemma (Sulakhe et al., 1976a; St. Louis and Sulakhe, 1976a), it is very likely that the proteins identified in Table I are intrinsic membrane proteins (Singer, 1974).

Band I remained close to the origin but it is unlikely that it represents unsolubilized material. Varying the conditions for solubilization of samples, including the use of a prolonged time of solubilization and higher (2%) concentrations of $NaDodSO_4$ did not result in its disappearance. However, this band may be

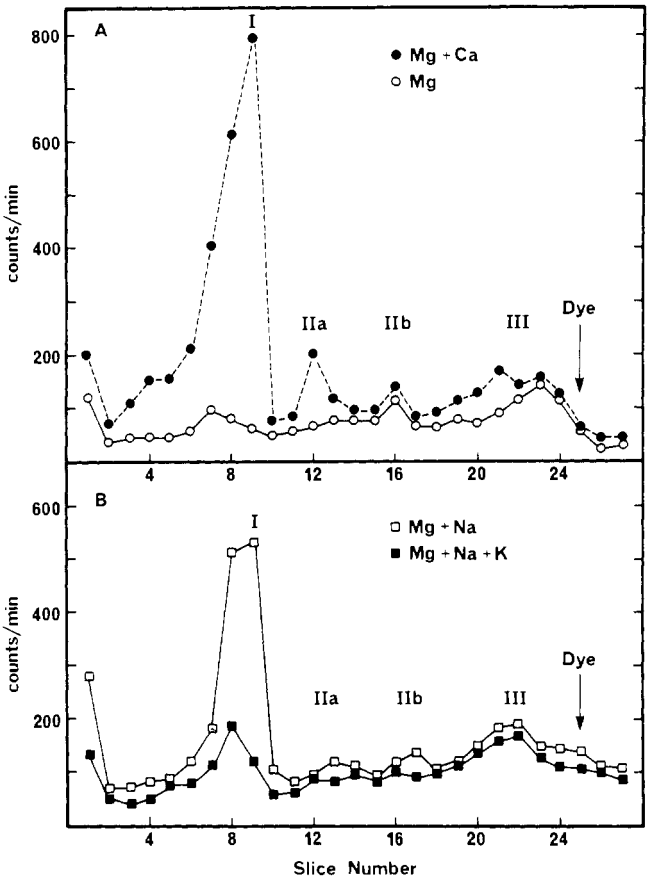

FIGURE 2: Pattern of [γ-^{32}P]ATP labeling of cardiac sarcolemmal membranes. (A) Membranes (184 μg of protein) were incubated at 22 °C for 15 s in the presence of 5 mM $MgCl_2$, 0.5 mM EGTA, and 45 μM [γ-^{32}P]ATP (640 cpm/pmol) (○). When present, $CaCl_2$ was 0.51 mM in addition to above ingredients (●). (B) Membranes (180 μg of protein) were incubated at 22 °C for 15 s in the presence of 5 mM $MgCl_2$, 0.5 mM EDTA, 44 μM [γ-^{32}P]ATP (490 cpm/pmol), 100 mM NaCl with (■) and without (□) 20 mM KCl. Following phosphorylation, membranes were solubilized with $NaDodSO_4$ and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis at pH 2.4 according to the method of Fairbanks and Avruch (1972). The gels were then sliced (2 mm/slice) and the slices counted for radioactivity. For other details, see Methods. The results are from a typical experiment; similar findings were observed in four separate experiments.

composed of more than one high-molecular-weight component unresolved at this gel concentration.

It is noteworthy that, using the procedure of Fairbanks et al. (1971), essentially similar protein profiles were obtained for sarcolemmal membranes fractionated on 5.6% polyacrylamide gels (Sulakhe and St. Louis, 1978). The only major difference, which may well be related to the lower gel concentration used, was the appearance of two closely migrating high-molecular-weight protein bands (M_r > 200 000).

Identification of ATPase by Analysis of ^{32}P-Labeled Sarcolemmal Membranes. Earlier, we have reported that cardiac sarcolemma contained (Mg,Ca)ATPase, Ca-ATPase, and (Na,K)ATPase (St. Louis, 1975; Sulakhe et al., 1976a). The apparent molecular weight reported for monomeric Ca-ATPase and (Na,K)ATPase from various mammalian sources, including muscle, varies between 120 000 and 93 000 (see, for example, MacLennan, 1970; Kyte, 1971; Martonosi and Halpin, 1971; Collins and Albers, 1972; Alexander and Rodnight, 1974; Dowd et al., 1976; Reddy et al., 1976; Stekhoven et al., 1976; Yu et al., 1976). Using [γ-^{32}P]ATP as a substrate, the active site of membrane ATPase can be labeled due to the formation of the acyl [^{32}P]phosphate intermediate; subsequent analysis on polyacrylamide gels can then be used to identify

TABLE II: Protein Composition of Cardiac Sarcolemmal Membranes.[a]

designation	M_r	% of total protein
I	>200 000	5.2 ± 0.8[b]
II	150 000	10.2 ± 3.1
IIa	135 000	1.2 ± 0.5
IIb	130 000	2.6 ± 0.9
IIc	115 000	3.1 ± 0.8
III	105 000	6.5 ± 2.4
IVa	90 000	3.5 ± 1.0
IVb	85 000	2.0 ± 0.4
IVc	76 000	2.3 ± 0.6
IVd	68 000	3.4 ± 1.2
V	58 000	6.9 ± 1.3
Va	55 000	2.9 ± 0.6
VI	47 000	13.5 ± 3.4
VII	33 000	14.7 ± 2.1
VIII	22 500	4.4 ± 1.1
IX	18 000	2.8 ± 0.60

[a] Sarcolemmal membranes were fractionated on $NaDodSO_4$–polyacrylamide (6%) gels, stained for protein, and destained as described under Methods. Gels were then scanned at 555 nm, and the integrated peak values were converted to relative percent. The values for percent total protein are the means ± standard error of the mean computed from results for six different preparations. The peaks were arbitrarily designated as shown, and the relative molecular weights (M_r) were obtained from a standard curve for proteins of known molecular weights. [b] Unresolved polypeptides migrating between bands I and II constituted 6.4 ± 0.8% of total protein.

the peptide locus of the active site (e.g., see Drickamer, 1975; Froehlich and Taylor, 1976; Reddy et al., 1976; also see review by Glynn and Karlish, 1975). Accordingly, sarcolemmal fraction, phosphorylated in the presence of different cationic compositions of the reaction mixture, was fractionated on $NaDodSO_4$ (1%)–polyacrylamide (5.6%) acid gels according to the method of Fairbanks and Avruch (1972). The use of acidic pH (pH 2.4) during electrophoretic fractionation helped considerably in minimizing the degradation of labile acyl [^{32}P]phosphoenzyme intermediates. As shown in Figure 2 (upper panel), peak I (M_r 105 000) contained the Ca-ATPase active site, since Ca^{2+} markedly enhanced the incorporation into peak I while with Mg^{2+} alone the incorporation was barely detectable. Although it is known that Mg^{2+} is required for dephosphorylation of the acyl phosphate intermediate of sarcoplasmic reticulum (Mg,Ca)ATPase (see review by MacLennan and Holland, 1975), the experiment described was designed only to identify the peptide locus of the sarcolemmal enzyme. In agreement with the numerous reports of the sarcoplasmic reticulum ATPase, the cardiac sarcolemmal ATPase also shows the dependence on Ca^{2+} for the formation of the phosphoenzyme intermediate. The results shown in the lower panel of this figure indicated that this region also contains sarcolemmal (Na,K)ATPase, a conclusion based on the observation that Na^+ promoted the incorporation of radioactivity, whereas K^+ caused dephosphorylation of the Na^+-promoted phosphoenzyme. Radioactivity incorporated into peak I was readily removed by exposure to 0.8 M hydroxylamine, suggesting that the terminal [^{32}P]phosphate of ATP was incorporated into an acyl-phosphate bond. Drickamer (1975) suggests that the specific ATPases in red cell membranes are manifestations of three distinct molecular species. Whether or not cardiac sarcolemmal ATPase activities are due to a single or different proteins is not known and is being investigated in this laboratory. Radioactivity into peak III (Figure 2) was observed under all ionic conditions tested and corre-

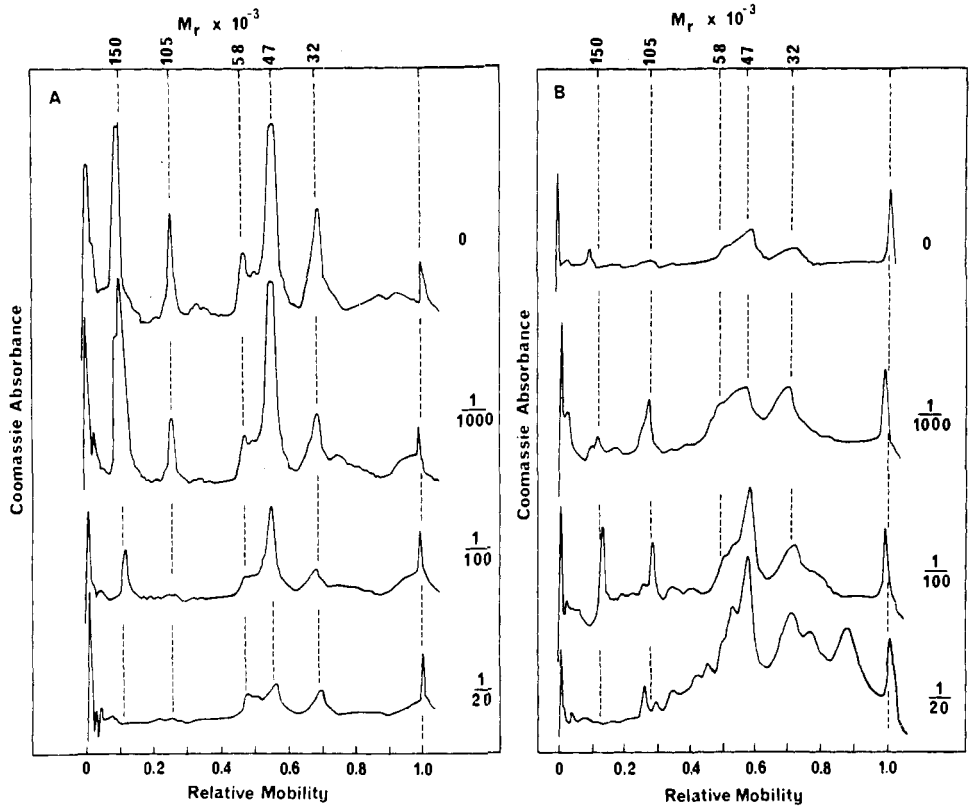

FIGURE 3: Protein profiles of cardiac sarcolemmal membranes: Effect of trypsin. Sarcolemma (200 μg of protein) was treated with trypsin at the protein ratios (trypsin/sarcolemma) indicated (0, 1:1000, 1:100, 1:20) as described under Experimental Section. The solubilized (supernatant) fractions and pelleted residues were fractionated on 6% gels: (A) pellets; (B) supernatants. Twenty to thirty micrograms of protein was applied per gel.

sponds to protein band VIII (Figure 1). Interestingly, we have found that protein band VIII (M_r 22 500) was phosphorylated in the presence of cAMP with and without exogenous protein kinase (Sulakhe and St. Louis, 1978; St. Louis and Sulakhe, submitted for publication). Whether or not this protein is similar to phospholamban (Tada et al., 1975) present in cardiac sarcoplasmic reticulum remains to be determined. The identity of peaks IIa and IIb is still in doubt. It is likely that peaks II (a + b) and III were phosphorylated by endogenous protein kinase present in these membranes (Sulakhe et al., 1976a); both peaks II and III were hydroxylamine insensitive and also failed to show any significant dependence in the degree of incorporation of phosphate on the cationic compositions of the reaction mixtures. A detailed analysis of the phosphorylation of cardiac sarcolemmal enzymes by protein kinase will be the subject of another report from this laboratory.

Treatment of Sarcolemma with Trypsin. As seen in Figure 3A, at a trypsin/sarcolemma protein ratio of 1:1000, the principal change noted in the electrophoretic profile of cardiac sarcolemma was the appearance of two closely migrating bands in the region of band II (M_r 150 000). Together these two bands represented approximately the same amount of total protein as did band II in the control (see Table II), and the amount of band I was not significantly less than that found in the control. This finding suggests that the two new bands are derived from band II, probably by the removal of a small peptide, and would imply that the orientation of band II protein in the membrane is such that a small peptide segment of this protein is readily accessible to trypsin. The relative percent of band III (M_r 105 000) material was slightly decreased (6%) compared to that in control preparations (7.0%), and material of similar M_r appeared in the solubilized (supernatant) fraction obtained after trypsin treatment (Figure 3B). Also, the supernatant fraction contained increased amounts of material of M_r 58 000–32 000.

Increasing the trypsin/sarcolemma ratio to 1:100 resulted in increased solubilization of band I material, while band II now represented 6.5% of the total compared to about 13% in the control (untreated) sarcolemma (Figure 3A). Coincident with the disappearance of band II material from the residue there was the appearance in the supernatant (Figure 3B) of a sharp band of similar M_r (150 000). However, the relative amount of this band did not account entirely for the amount of band II which had been lost from the insoluble residue. Band III (M_r 105 000), which contained ATPase, was about 60% solubilized and was probably fragmented, as evidenced by the reduced amount of M_r 105 000 material recovered in the supernatant. Bands of M_r 85 000 and 70 000 were now observed in the supernatant (soluble fraction), and these bands may well have been derived from bands I, II, and/or III. It appears, therefore, that up to a 1:100 trypsin/sarcolemma ratio, there was solubilization of high-molecular-weight material but only limited degradation occurred. At a higher ratio (1:20), there was extensive solubilization of proteins as well as degradation of high-molecular-weight proteins (Figure 3). In particular, the 150 000 protein (band II) and the 105 000 protein (containing ATPase) were less prominent in both the residue and in the supernatant fractions. The bulk of the protein in the supernatant was represented by broad bands with M_rs of 48 000 and 32 000. We were unable to detect the presence of lower molecular weight peptides (M_r < 32 000) in the supernatant fraction from 1:20 trypsin treatment because of the relatively broad peaks due to trypsin and trypsin inhibitor (M_rs of 24 000 and 18 000, respectively).

Critical examination of these results indicated that sarcolemmal protein bands II and III were particularly susceptible to proteolysis by trypsin. Band I material, which occurs close to the origin, was also influenced by trypsin. This view was

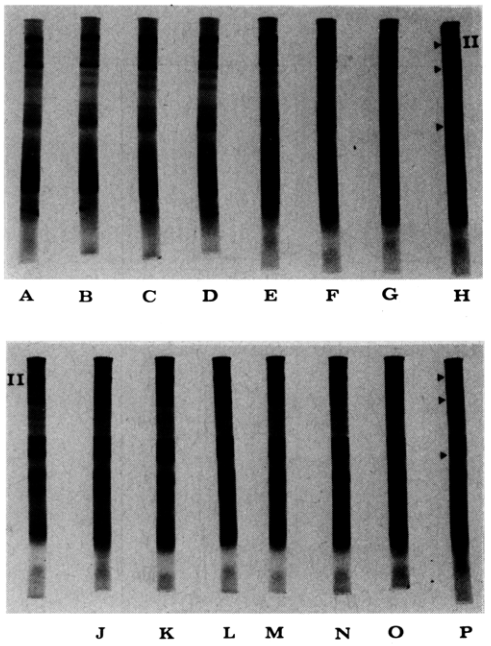

FIGURE 4: Exposure of sarcolemma to trypsin for different times. Sarcolemmal membranes were incubated for varying times in the presence of trypsin (trypsin/sarcolemma ratio of 1:125) for 0 (A, I), 0.5 (B, J), 1 (C, K), 2 (D, L), 5 (E, M), 12 (F, N), 20 (G, O), and 30 (H, P) min. Gel patterns A to H are for the supernatant fractions and I to P are for the pelleted residues. Arrow heads indicate the location of the polypeptides which were decreased in amount from the pelleted residues and which appeared in the supernatant fractions following trypsin exposure for varying times. For other details, see Experimental Section.

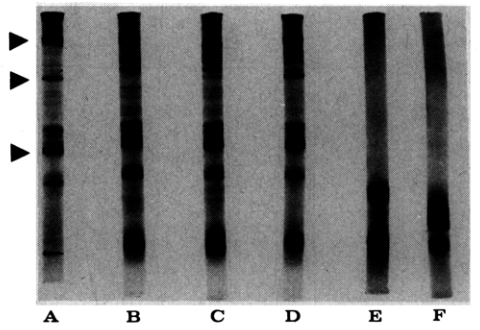

FIGURE 5: Protection by sucrose against the tryptic proteolysis. Sarcolemma were incubated with trypsin (sarcolemma/trypsin ratio of 20) for 5 min in the absence (gel B) and presence of 1 M sucrose (gel C). Control untreated sarcolemmal protein profile (gel A), sarcolemma incubated with trypsin and excess trypsin inhibitor (gel D), trypsin alone (gel E) and trypsin inhibitor alone (gel F) are also shown. Arrow heads indicate the location of protein components protected by sucrose against the tryptic proteolysis. For other details, see Experimental Section.

further supported by the results obtained when membranes were treated with trypsin for various times. For example, examination of the $NaDodSO_4$–polyacrylamide gel profiles for sarcolemmal membranes (pelleted residues) treated with 1:1000 trypsin for various times indicated that: band I was rapidly solubilized (about 85% in 10 min); band II appeared as split bands within 5 min but was represented by a single band after 20 min, the extent of solubilization being 20%; band III (M_r 105 000) protein, which represented 7.4% of the total protein in untreated sarcolemma, represented 7.2, 6.7, 6.2, and 6.0% of the total protein after 1, 2, 5, and 10 min of trypsin treatment, respectively, and appeared as a doublet after 20 min; band VI (M_r 47 000) was decreased by 20% after 20 min, but no significant difference in the profile of lower molecular weight proteins could be detected up to 40 min (results not shown).

Figure 4 shows that at a trypsin/sarcolemma ratio of 1:125 a marked reduction in the amount of three protein bands (band II, M_r 150 000; band III, M_r 105 000; band VI, M_r 47 000) had occurred in a time-dependent manner; protein bands of the respective molecular weights appeared in the supernatant fraction. That band II is composed of more than one protein of rather similar molecular weights can be suggested from pattern P as well as from patterns B–H (Figure 4). In fact, it appeared that at this trypsin concentration band II is clearly split within 1 min. Although it may well be that a small peptide (or peptides) was removed from band II by trypsin, resulting in the formation of polypeptides of very similar mass, we cannot rule out the possibility that the effect of trypsin was simply to "resolve" the preexisting proteins. Band III also appears as a doublet after a 5-min incubation, and band VI is clearly reduced (from 15.4% of total protein to 5.4%, time = 0 and 20 min, respectively).

Interestingly, the accessibility of bands II, III, and VI to trypsin can be blocked by the addition of 1 M sucrose during trypsin treatment. As shown in Figure 5, even at high trypsin concentration (sarcolemma/trypsin ratio of 20), very little degradation of bands II, III, and VI was evident in the presence of sucrose.

Effects of Trypsin Treatment on Sarcolemmal ATPase and Ca^{2+}-Accumulation Activities. Having found that trypsin exerted well-defined effects on sarcolemmal proteins, particularly on the ATPase (band III), we examined the effects of trypsin on sarcolemmal ATPase and ATP-dependent Ca^{2+} accumulation. Cardiac sarcolemmal "extra" ATPase and Ca^{2+}-accumulating activities were reduced by treatment with trypsin in a concentration-dependent manner, although at 1:1000 trypsin there was definite enhancement of the (Mg,Ca)- and ouabain-sensitive (Na,K)ATPase activities (Table III). At 1:100 trypsin no "extra" ATPase could be detected. Ca^{2+}-accumulating activity was also markedly reduced by trypsin treatment (Table III).

When sarcolemma was treated for different times with trypsin (1:1000), (Mg,Ca)ATPase activity (18.24 μmol of P_i mg^{-1} h^{-1}) was increased with increasing time of trypsin-treatment (26.97 μmol of P_i mg^{-1} h^{-1} after 40 min). However the increase in (Mg,Ca)ATPase activity was due specifically to enhancement of Mg-ATPase ("basal" ATPase) and, in fact, "extra" ATPase was slightly inhibited (about 25%) up to 20 min of treatment and was not detectable thereafter. Sarcolemmal Ca^{2+}-accumulating activity was steadily diminished with increasing time of treatment with trypsin; about 70% reduction in activity was observed after 20 min of trypsin treatment. The apparently greater degree of reduction of sarcolemmal "extra" ATPase compared to Ca^{2+}-accumulation activity, as well as the enhancement of Mg-ATPase, by treatment with trypsin for various times was also observed at a trypsin/sarcolemma ratio of 1:100. For example, in a representative experiment, "extra" ATPase was not detectable after 5 min of trypsin treatment, while Ca^{2+} accumulation activity was reduced about 57%.

It may well be significant that the disappearance of "extra" (Ca^{2+}-stimulated) ATPase activity after treatment of sarcolemma with trypsin for various times coincides with the appearance of doublet peptide bands in the ATPase region of the membrane protein profile (see earlier). We are, however, unable, at this time, to speculate further on this point. It is interesting, nonetheless, to note that Inesi and Scales (1974) have shown that the initial cleavage of the ATPase in skeletal-muscle sarcoplasmic reticulum membranes by trypsin was not

TABLE III: Effect of Trypsin Treatment on ATPase and Ca^{2+}Accumulating Activities of Sarcolemma.[a]

trypsin sarcolemma ratio	(Mg,Ca)ATPase[b,e]	"extra" ATPase[c,e]	ouabain-sensitive (Na,K)ATPase[e]	Ca^{2+} accum[d,f]
none	22.82	4.58	7.44	33.35
1:1000	28.62	2.58	10.68	23.39
1:100	22.74		6.30	12.81
1:20	12.30		0.78	2.10

[a] Sarcolemmal membranes (500 μg of protein) were incubated (22 °C, 10 min) in the absence and presence of trypsin as indicated. For other details, see Experimental Section. [b] ATPase activities (30–60 μg of protein/mL of assay) were determined in the presence of 5 mM ATP, 5 mM Mg^{2+}, and 0.05 mM Ca^{2+}. [c] "Extra" ATPase refers to the Ca^{2+}-stimulated component of the (Mg,Ca)ATPase, i.e., [(Mg,Ca)ATPase] − Mg-ATPase]. [d] Determined in the presence of 2.5 mM ATP, 5 mM oxalate, and 100 μM $^{45}CaCl_2$ (13 000 cpm/nmol); protein was approximately 100 μg/mL of assay. [e] In μmol of P_i (mg of protein)$^{-1}$ h^{-1}. [f] In nmol of ^{45}Ca (mg of protein)$^{-1}$ (5 min)$^{-1}$.

TABLE IV: Effects of Phospholipase C Treatment on Sarcolemmal ATPase and Ca^{2+}-Accumulating Activities.[a]

	phospholipase C sarcolemma ratio	(Mg,Ca)-ATPase[b,d]	"extra" ATPase[d]	Ca^{2+} accum[c,e]
untreated:		14.49	1.36	49.89
treated:	1:250	12.57	0.91	33.10
	1:50	12.07	0.60	28.48
	1:10	8.70		5.36

[a] Sarcolemmal membranes (484 μg of protein) were incubated (30 °C, 10 min) with and without phospholipase C at the protein ratios indicated, and membranes were recovered as described under Experimental Section. [b] ATPase activities (30–50 μg of protein/mL of assay) were determined in the presence of 5 mM ATP. [c] Determined in the presence of 2.5 mM ATP, 5 mM oxalate, and 100 μM $^{45}CaCl_2$ (20 000 cpm/nmol); protein was 50–75 μg/mL of assay. [d] In μmol of P_i (mg of protein)$^{-1}$ h^{-1}. [e] In nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$.

accompanied by any reduction in enzyme activity.

Effects of Phospholipase C on Sarcolemma. Treatment of cardiac sarcolemmal membranes with phospholipase C did not significantly affect the $NaDodSO_4$–polyacrylamide gel protein profile of sarcolemma. This is not unexpected, since the enzyme could have hydrolyzed essentially surface-located phospholipids without disrupting the membrane proteins themselves. Phospholipase C, however, caused a reduction of sarcolemmal ATPase activities, and the extent of this effect was directly related to the concentration of phospholipase C (Table IV). In addition, calcium accumulation in sarcolemma was reduced; the degree of reduction depended on the concentration of phospholipase C. Using a fixed concentration of phospholipase C (1:50, phospholipase C/sarcolemma) there was only a slight decrease in (Mg,Ca)ATPase activity (about 17% after 20 min), whereas "extra" ATPase declined by about 50%. At the same time, Ca^{2+}-accumulating activity was almost completely abolished [27.89 nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$ at time = 0 to 2.59 nmol mg^{-1} (5 min)$^{-1}$ after 20 min]. These results indicate that, as shown for muscle microsomal membranes (Martonosi, 1968; MacLennan et al., 1971; Meissner et al., 1973; Warren et al., 1974), the specific lipid environment is critical for optimal sarcolemmal ATPase and Ca^{2+}-accumulating activities.

It has been shown that a phospholipase C induced decrease in the Ca^{2+}-transport activity of sarcoplasmic reticulum was restored by the addition of lecithin (Martonosi et al., 1968). Hence, it was of interest to investigate the effect of adding a lecithin suspension to phospholipase C treated cardiac sarcolemma. Our results indicated that there was only a slight enhancement of Ca^{2+} uptake by lecithin. In a representative experiment, values for Ca^{2+} accumulation by phospholipase C (1:50 ratio) treated membranes were 25.95 and 31.18 nmol (mg of protein)$^{-1}$ (5 min)$^{-1}$ in the absence and presence, respectively, of 40 μg of lecithin in the assay. With higher amounts of added lecithin there appeared to be a reversal of stimulation of Ca^{2+} accumulation. It is probable that the degree of perturbation of the sarcolemmal membranes was too severe to allow for recovery of Ca^{2+}-accumulating activity. Alternatively, we may not have achieved optimal conditions for the interaction of the lecithin suspension with the phospholipase C treated sarcolemma, even though varying amounts of lecithin had been tried (results not shown).

Detergent Treatment of Sarcolemma. Various detergents have been used to selectively solubilize membrane proteins, thereby allowing for the analysis of the organization of the proteins within these membranes (see, for example, Martonosi, 1968; Robinson and Tanford, 1975; Clark, 1976; Le Maire et al., 1976). We have, therefore, investigated the influence of different detergents on the electrophoretic protein profile of cardiac sarcolemma.

We observed that Lubrol-PX did not preferentially solubilize any specific protein (Figure 6) and this is in agreement with the observations of Louis et al. (1974) on skeletal-muscle microsomes. On the other hand, with Triton X-100 a greater degree of protein solubilization was noted (30% compared to 13% with Lubrol-PX). Further, there appeared to be preferential solubilization of band IV (a + b) and band Va proteins (M_r 90 000, 87 000, and 57 000, respectively). Quantitative analysis of the results indicated that band IV (a + b) represented 1% of the total protein (3.5% in the control) and band Va was not detectable (3.0% in the control). Triton X-100 has been shown to solubilize a protein kinase enzyme/substrate macromolecular complex from synaptic membrane fractions (Ueda et al., 1975). Sarcolemma contains endogenous cAMP-stimulable protein kinase (Sulakhe et al., 1976a). Further, it has been shown that fractionation on $NaDodSO_4$–polyacrylamide gels can resolve purified protein kinases from heart into their regulatory and catalytic subunits which exhibit M_rs of about 48 000–55 000 and 40 000 respectively (see, for example, Hoffman et al., 1975; Rosen et al., 1975). It was thus considered possible that a portion of band Va represented a regulatory subunit of endogenous protein kinase.

When sarcolemmal membranes were treated with deoxycholate, 40% of the total protein was solubilized and bands I and II protein were both decreased by about 50% (Figure 6). Although the band III material in the pellet still represented about the same percent of total protein (8.2%) as in the control pellet, polypeptide corresponding to band III (M_r 105 000) was

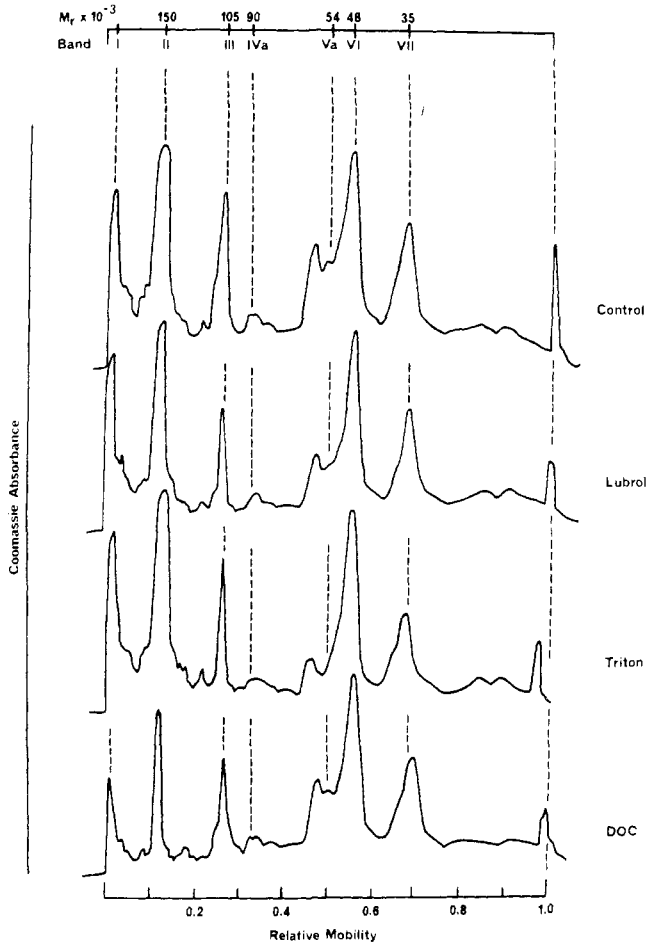

FIGURE 6: Protein profiles of cardiac sarcolemmal membranes after treatment with detergents. Cardiac sarcolemmal membranes (290 μg of protein) in a final volume of 200 μL were treated with Lubrol-PX, Triton X-100, and sodium deoxycholate to 1% (w/v) final concentration. Following treatment, membranes were centrifuged. Aliquots of the pelleted residues were fractionated on 6% gels (40 μg of protein/gel). Control, (untreated) membranes; Lubrol, residue after Lubrol treatment; Triton, residue after Triton treatment; DOC, residue after deoxycholate treatment.

identified in the deoxycholate-solubilized (supernatant) fraction; this is likely to represent solubilized ATPase protein. Bands IV (a + b) and Va did not appear to be solubilized by deoxycholate, in contrast to the effect of Triton X-100 (see above). The percent protein in bands V and VI, and especially band VII (M_r 35 000), in the deoxycholate pellet was increased relative to the control pellet. Thus, it appeared that bands V–VIII were relatively resistant to solubilization by deoxycholate.

Discussion

In contrast to a relatively simple protein composition of fast muscle sarcoplasmic reticulum, sarcolemma from guinea pig heart ventricles showed numerous protein components.[2] This was not an unexpected finding. We have already found that cardiac sarcolemma contains numerous enzymes such as adenylate and guanylate cyclase, cyclic nucleotide phosphodiesterases, protein kinase, protein phosphatase, ATPases, *p*-nitrophenylphosphatase, and other proteins such as β-adrenergic receptors and muscarinic receptors (Sulakhe et al., 1976a,b; St. Louis and Sulakhe, 1976a,b; Ma et al., 1978). It is thus more difficult to study the organization of proteins in sarcolemmal membranes than is possible with muscle sarcoplasmic reticulum.

Interestingly, under limited proteolysis (such as at 1:1000 trypsin) only bands II and III were evidently affected, suggesting that parts of their polypeptide chains are not completely buried in the lipid bilayer. However, because of the presence of numerous protein bands in these membranes, it was difficult to precisely quantitate the degree of proteolysis of individual bands, especially of minor components. When trypsin concentration and the treatment time were increased, extensive solubilization of proteins, particularly bands II, III, and VI, occurred. Our results, so far, do not allow us to infer whether or not these proteins are specifically exposed in right-side-out or inside-out vesicles. Thus, they may well be transmembrane proteins with susceptible sites on both aspects of the membrane. Indeed, this is quite likely, since, for example, sarcolemmal ATPase(s), represented by band III protein, would be expected to have portions of the protein exposed to (or readily accessible to) both the cytoplasm and the extracellular space. Clarification of this issue will be dependent on the successful resolution of the two types of sarcolemmal vesicles.

At 1:1000 trypsin, (Mg,Ca)ATPase as well as (Na,K)-ATPase activities were moderately elevated. Our results further show that, for any given concentration of trypsin, Mg-ATPase (basal ATPase) increases with increasing time of incubation. These stimulatory actions could well reflect a further change in membrane permeability, resulting either in greater accessibility of ATP to the catalytic site(s) of ATPase(s) or in the exposure of new catalytic sites normally unaccessible to substrate. Thus, trypsin may well act to promote the accessibility or exposure of sites in right-side-out (and inside-out) vesicles, thereby enhancing total ATPase activity. It is well documented that a controlled tryptic cleavage of sarcoplasmic reticulum ATPase does not destroy its enzymatic activity (see MacLennan and Holland, 1976). However, there are some differences in the trypsin effect reported on sarcoplasmic reticular ATPase protein and the effect observed in this study on heart sarcolemmal ATPase protein. For example, at 1:1000 trypsin, Louis et al. (1974) observed significant proteolysis of the SR ATPase protein (A_2 band) even at early exposure times, whereas we did not in cardiac sarcolemma. Instead, the M_r 150 000 component appeared more susceptible under these conditions. In the light of the foregoing, the marked decrease in "extra" ATPase, as a result of trypsin-catalyzed proteolysis, is interesting. This would suggest that the "extra" ATPase activity is dependent on the integrity of the ATPase or of the membrane itself. For example, the activity may be dependent on the interaction of intact (monomers of) ATPase protein. At the same time, the possibility cannot be ruled out that trypsin may have effected the removal of a fragment of the ATPase(s) which is essential for the expression of "extra" ATPase activity.

The reduction of sarcolemmal Ca^{2+}-accumulating activity caused by trypsin was to be expected, since trypsin would render the vesicles more permeable. It is worthwhile to mention at this point the apparent lack of reasonable stoichiometry between sarcolemmal "extra" ATPase and Ca^{2+}-accumulating activity. Results from numerous experiments give a value of 2–3 mol of ATP hydrolyzed/mol of Ca^{2+} translocated (accumulated). As discussed under Results (Sidedness of Membranes), it is apparent that sarcolemmal membrane vesicles are not permeability intact, being permeable to, at least, small ions and ATP. The implication of this is twofold. First, ATP

[2] Periodic-Schiff staining indicated the presence of two glycoprotein bands with M_r 160 000 and 110 000.

and Ca^{2+} would be accessible to the catalytic sites of ATPase in right-side-out vesicles, thereby permitting ATP hydrolysis. At the same time, these vesicles, as we have indicated, do not contribute to net (oxalate-supported) Ca^{2+} accumulation. Secondly, in inside-out vesicles part of the Ca^{2+} transported into the lumen of the vesicles would be lost as a result of the "leakiness" of the membranes, thereby reducing the net Ca^{2+} accumulation. It is anticipated that a more reasonable stoichiometry will be obtained if Ca^{2+} accumulation is examined in a homogeneous population of inside-out sarcolemmal vesicles.

Another important point to note is that we are dealing with ATP-dependent, oxalate-facilitated Ca^{2+} accumulation by the membrane and *not* oxalate pump. This is supported by the fact that the fraction studied did not accumulate [^{14}C]oxalate in the absence and presence of ATP and with and without Ca^{2+} in the reaction mixture. Therefore, the stimulatory effect of oxalate, which we believe is on the Ca^{2+} accumulation by inside-out vesicles of the fraction (see Results), is due to a passive influx of this anion along with an active movement of Ca^{2+} that finally results in trapping the Ca^{2+} inside the lumen of inside-out vesicles.

The effects of phospholipase C on calcium accumulation and ATPase activity of heart sarcolemma were qualitatively similar to those reported on sarcoplasmic reticulum from heart and skeletal muscle. However, under the conditions tested, lecithin supplementation failed to completely restore Ca^{2+} uptake in the lipase-treated sarcolemma, whereas this is reported for the lipase-treated sarcoplasmic reticulum (MacLennan and Holland, 1976). In other studies, lysolecithin caused significant reduction in calcium transport in control (untreated) as well as phospholipase C treated sarcolemma (results not shown). Additional studies are planned to determine if specific membrane lipid(s) is (are) essential for the optimal activities of the ATPase(s) and calcium "pump" of these membranes. Recently, we have noted that phospholipase C treatment resulted in impairment of basal, but not of NaF-stimulated, adenylate cyclase activity (Narayanan and Sulakhe, 1977). Earlier we observed decreased guanylate cyclase following the lipase treatment (Sulakhe et al., 1976c) of sarcolemma. Such observations support the view that membrane lipids play an important role in the regulation of the permeability characteristics as well as the enzyme activities of cardiac sarcolemma.

In a preliminary analysis, Saccomani et al. (1974) reported the polypeptide composition of a plasma-membrane-enriched fraction isolated from dog heart. (Na,K)ATPase content of their membrane preparation was rather low; in fact, Na-DodSO_4-polyacrylamide gel electrophoresis of membranes failed to show a well-defined polypeptide band in the region of M_r 100 000. In Na DodSO_4-gel electrophoretic systems, the catalytic subunit of (Na,K)ATPase is of about 100 000-dalton mass (Reddy et al., 1976). Saccomani et al. (1974) observed 12 polypeptide bands in their membranes ranging from M_r 10 000 to 83 000. Band II (M_r 68 000) was the major polypeptide, constituting 18% of the membrane protein. Clearly, the polypeptide composition of sarcolemma reported by Saccomani et al. (1974) differs markedly from our findings in this study. It is not clear whether these differences are due to differences in species (dog vs. guinea pig) or in the methods of isolation of membranes or electrophoretic conditions (1% Na DodSO_4–7% acrylamide vs. 0.1% Na DodSO_4–6% acrylamide). Also, the membrane fraction employed by Saccomani et al. (1974) was obtained from a crude microsomal fraction, whereas we have isolated sarcolemma in a high degree of purity from a low-gravitational residue (Sulakhe et al., 1976a; St. Louis and Sulakhe, 1976b). Finally, ouabain-sensitive (Na,K)ATPase and adenylate cyclase (plasma-membrane markers) were recovered in good yields in sarcolemma isolated according to our method (Sulakhe et al., 1976a; St. Louis and Sulakhe, 1976b).

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Synthesis of Des(tetrapeptide B^{1-4}) and Des(pentapeptide B^{1-5}) Human Insulins. Two Biologically Active Analogues†

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ABSTRACT: Two analogues of human insulin, des(tetrapeptide B^{1-4})- and des(pentapeptide B^{1-5})-insulin, which differ from the parent molecule in that the N-terminal tetrapeptide and pentapeptide sequences, respectively, have been eliminated, have been synthesized. The des(tetrapeptide B^{1-4})-insulin shows potencies of 13 IU/mg by the mouse convulsion assay method and 7.6 IU/mg by the radioimmunoassay method. The des(pentapeptide B^{1-5})-insulin possesses a potency of 1.2 IU/mg when assayed by the glucose-oxidation method in isolated fat cells and 3.7 IU/mg by the radioimmunoassay technique. The natural hormone has a potency of 23–25 IU/mg by both assay methods.

Current studies in our laboratory are directed toward the determination of possible correlations between chemical structure and the biological activity of insulin. One feature of this work is the evaluation of the relative importance of amino acid residues, located at the amino-terminal regions of the A and B chains, for the biological activity of this protein. We have found that removal of the N-terminal tetrapeptide sequence from the A chain leads to the total loss of activity of insulin (Katsoyannis and Zalut, 1972), and even the replacement of the α-amino group of the N-terminal residue of this chain by a hydrogen causes a ca. 65% loss of the biological activity of the hormone. The significance of the N-terminal region of the A chain for the biological activity is also evident from the work carried out in other laboratories [for a review, see Blundell et al., 1972]. However, a different situation exists regarding the role of the amino-terminal region of the B chain to the biological activity of insulin.

Early studies by Brandenburg (1969) have shown that des(B^1)-insulin, produced by selective Edman degradation of the unprotected molecule, is biologically and immunologically identical with the natural hormone. Subsequently, by stepwise degradation of the B-chain moiety of partially protected natural insulin, using the Edman method, Geiger and Langner (1973) found that des(B^1)- and des(dipeptide B^{1-2})-insulin retained almost the full activity of the natural hormone in lowering rabbit blood glucose level. Attempts to prepare the des(tripeptide B^{1-3})-insulin by the same method, however, led

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