MASTER

BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION
OF LIGHT AND HEAVY SARCOPLASMIC RETICULUM VESICLES

by

Kevin Peter Campbell

Submitted in Partial Fullfillment

of the

Requirements for the Degree

DOCTOR OF PHILOSPHY

Supervised by : Dr. Adil E. Shamoo

Department of Radiation Biology and Biophysics

School of Medicine and Dentistry

The University of Rochester

Rochester, New York

1978

Volume II

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

BIOCHEMICAL AND MORPHOLOGICAL CHARACTERIZATION
OF LIGHT AND HEAVY SARCOPLASMIC RETICULUM VESICLES

by

Kevin Peter Campbell

NOTICE

This report was prepared as an account of work sponsored by the United States Government. Neither the United States nor the United States Department of Energy, nor any of their employees, nor any of their contractors, subcontractors, or their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness or usefulness of any information, apparatus, product or process disclosed, or represents that its use would not infringe privately owned rights.

Submitted in Partial Fullfillment
of the
Requirements for the Degree

DOCTOR OF PHILOSPHY

Supervised by : Dr. Adil E. Shamoo

Department of Radiation Biology and Biophysics
School of Medicine and Dentistry
The University of Rochester
Rochester, New York

1978

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

Volume 11

eb

CHAPTER II

CHLORIDE INDUCED RELEASE

OF ACTIVELY LOADED CALCIUM FROM LIGHT AND HEAVY

SARCOPLASMIC RETICULUM VESICLES

SUMMARY

Light and heavy sarcoplasmic reticulum vesicles isolated from rabbit leg muscle have been used in a study of chloride-induced calcium release. The biochemical and morphological data indicate that light sarcoplasmic reticulum vesicles are derived from the longitudinal reticulum and heavy sarcoplasmic reticulum vesicles are derived from the terminal cisternae of the sarcoplasmic reticulum.

The light and heavy sarcoplasmic reticulum vesicles were both able to accumulate calcium in the presence of ATP to amounts greater than 100 nmoles Ca^{++} per mg of protein in less than one minute. Light and heavy sarcoplasmic reticulum vesicles each had a biphasic time course of calcium uptake. The initial uptake was followed by a rapid release after approximately one minute, of 30-40% of the accumulated calcium, which was then followed by a slower phase of calcium accumulation.

Calcium taken up by the SR vesicles could be released from both the light and heavy SR vesicles by changing the anion outside the vesicles from methanesulfonate to chloride. Due to the difference in permeability between methanesulfonate and chloride, this change should result in a decreased positivity inside the vesicles with respect to

the exterior. It could also result in a swelling of the vesicles. Changing the ionic medium from chloride to methanesulfonate caused no release of calcium. The percentage of accumulated calcium released in 6 seconds by changing the anion outside the vesicles from methanesulfonate to chloride was approximately 40% and 20% for light and heavy SR, respectively.

Sucrose (200mM) caused a slight inhibition of chloride induced calcium release from the heavy SR vesicles but it greatly reduced the release of calcium from the light SR vesicles. The specificity of calcium release was measured using SR vesicles which had been passively loaded with 10 mM $^{22}\text{Na}^+$ or ^{14}C -sucrose. The amount of $^{22}\text{Na}^+$ or ^{14}C -sucrose released during a chloride induced release of cold calcium was then measured. The light SR vesicles released three to five times more $^{22}\text{Na}^+$ than the heavy SR vesicles.

Na dantrolene (20 μM) had no effect on the release of calcium from the light SR vesicles but it inhibited the release of calcium from the heavy SR vesicles.

Our results indicate that the chloride induced release of calcium may be acting by two mechanisms, osmotic swelling and depolarization. The release of calcium from the light SR vesicles is probably due to osmotic swelling and the release of calcium from the heavy SR vesicles is probably due to depolarization.

INTRODUCTION

It is estimated that more than a quarter of the calcium present in the sarcoplasmic reticulum is released by a single stimulus, 210 nmoles of Ca^{++} /ml of muscle in a few milliseconds (Endo, 1977). Calcium release is a graded function of surface membrane depolarization (Constantin and Taylor, 1973), and the release process in the sarcoplasmic reticulum does not seem to be an all-or-none process (Hodgkin and Horowicz, 1960). It is generally accepted that the depolarization of the transverse tubular system of skeletal muscle initiates the release of calcium from the terminal cisternae of the sarcoplasmic reticulum (for reviews see Ebashi and Endo, 1968; Sandow, 1970; Fuchs, 1974 and Endo, 1977). In recent years there have been several mechanisms proposed for the link between the depolarization of the walls of the T-tubule and the release of calcium from the sarcoplasmic reticulum (Ebashi and Endo, 1968; Endo, 1977), but it still remains one of the least understood processes in muscle contraction. The skeletal muscle membranes directly involved in excitation-contraction coupling are the transverse tubular membrane and the junctional sarcoplasmic reticulum membrane (Franzini-Armstrong, 1975). There has been some recent progress in understanding excitation-contraction coupling in terms of electro-

physiological properties of the transverse tubular membrane (Schneider and Chandler, 1973), and in chloride-induced (or 'depolarization' induced) release from the sarcoplasmic reticulum in skinned skeletal muscle fibers, but relatively little has been done on the study of calcium release from isolated sarcoplasmic reticulum vesicles.

The most convincing evidence that depolarization of the sarcoplasmic reticulum causes release of calcium ions comes from the work of Nakajima and Endo (1973) and Endo and Blinks (1973). After loading the sarcoplasmic reticulum in a skinned fiber the bathing medium was changed from sulfate to chloride which resulted in the release of calcium. Stephenson and Poldolsky (1977) in their studies on chloride induced calcium release have shown that the chloride stimulus varies with the concentration gradient of chloride across the internal membranes. Their results support the hypothesis that chloride induced calcium release is due to a membrane depolarization. Kasai and Miyamoto (1976 a and b) demonstrated that the replacement of methanesulfonate with chloride caused a release of calcium from isolated sarcoplasmic reticulum vesicles. Inesi and Malan (1977) have reported that calcium can be released from isolated sarcoplasmic reticulum vesicles when K^+ is exchanged for a less permeable cation. Meissner and McKinley (1977) have studied both anionic and cationic induced calcium release of passively loaded SR vesicles. They concluded that part or all of the ion-induced changes in the sarco-

plasmic reticulum membrane permeability may be due to a massive influx of salt and water into the passively loaded vesicles, thereby causing osmotic swelling and increased membrane permeability.

The purpose of our investigation was four fold: (1) the study of chloride induced calcium release from light and heavy sarcoplasmic reticulum vesicles which had actively accumulated calcium (2) the effects of osmotic buffering on the release of calcium from light and heavy SR vesicles (3) the effects of Na dantrolene on the release of calcium by chloride induced calcium release from light and heavy SR vesicles (4) correlation of the results on skinned fibers with the results on light and heavy sarcoplasmic reticulum vesicles. The data indicate the presence of a chloride induced Na dantrolene inhibited calcium release from the terminal cisternae vesicles. A preliminary report of this work has been presented at the 1977 Biophysical Society Meetings (Campbell and Shamoo, 1977).

Experimental Procedures

Materials

Ultra pure sucrose was obtained from Schwarz/Mann. Acrylamide and N,N'-methylene-bis-acrylamide were obtained from Bio-Rad. Adenosine 5'-[gamma- 32 P] triphosphate (20Ci/mmol) and calcium-45 were obtained from Amersham/Searle. 3 H-water and Omniflour were obtained from New England Nuclear. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and PIPES (piperazine-N,N'-bis-2 ethane sulfonic acid) were obtained from Calbiochem. Calcium and magnesium atomic absorption standards (1000 ppm) were obtained from Fischer Scientific. Ethyleneglycol-bis-(beta amino ethyl ether) N,N'-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), TRIS (hydroxymethyl aminoethane), N,N,N',N'-tetramethyl ethylenediamine (TEMED), disodium adenosine 5' triphosphate, and 2-mercaptoethanol were all obtained from Sigma. Millipore filters HAWP 025(.45u) and GSWP 025(.22u) were obtained from Millipore. All other reagents used were of analytical grade. All solutions were made with deionized distilled water.

Isolation of Light and Heavy Sarcoplasmic Reticulum Vesicles

Briefly, approximately 1000 grams of white rabbit leg muscle was homogenized in a Waring blender in 0.25 M sucrose for 30 seconds. The homogenate was centrifuged twice to remove cell debris and mitochondria and was centrifuged a third time to sediment the SR vesicles. The SR vesicles were placed on a linear sucrose gradient, 26-45% (w/w), and centrifuged at 100,000x g for 16 hours. Fractions containing 30-32.5% sucrose (light SR), 33.5-38% sucrose (intermediate SR) and 38.5-42% sucrose (heavy SR) were combined and then diluted with 1.0 M KCl, 1 mM HEPES (pH 7.4). After two hours incubation in 0.6 M KCl at 0 C the purified SR vesicles were pelleted, homogenized in 0.25 M sucrose, 1 mM HEPES (pH 7.4) and stored in liquid nitrogen. For the detailed procedure see Campbell, Armstrong and Shamo (1978).

Assays

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Percent sucrose (w/w) or

density (grams/cc) measurements of sucrose solutions were determined at 25° C using an Abbe refractometer. Calcium and magnesium were determined by atomic absorption spectroscopy using a Perk and Elmer Atomic Absorption Spectrometer. Internal water volume of the SR vesicles was measured as previously described, which involves the method of Johnson and Scarpa (1976). Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) using 8.75% acrylamide gels. Densitometric tracings of gels were made on a Gilford spectrophotometer with a linear gel scanner at 560 nm. Passive calcium efflux was measured as previously described.

Ca^{++} + Mg^{++} ATPase Activity

Ca^{++} + Mg^{++} ATPase activity was measured in 0.1 M KCl, 5 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mM EGTA, 10 mM PIPES (pH 6.8) and 5 mM ATP (γ - ^{32}P -ATP, 500 cpm/nmole ATP) at a protein concentration of 50-80 micrograms/ml (MacLennan, 1974). All reactants except ATP were incubated at 37 C for one minute. The reaction was initiated by the addition of 100 mM (γ - ^{32}P) ATP and stopped at 1 or 2 minutes with ice cold 30% trichloroacetic acid (TCA). Liberated phosphate (^{32}P) was isolated by the method of Martin and Doty (1949). Mg^{++} ATPase activity was measured in the same manner except calcium was omitted and EGTA was increased to 1 mM. Ca^{++} stimulated ATPase activity was

calculated as the difference between the total and the Mg^{++} ATPase activities. ATPase activities are expressed as micromoles of inorganic phosphate released per mg protein per minute.

Calcium Uptake

$^{45}Ca^{++}$ uptake was measured by the Millipore filtration method of Martonosi and Feretos(1964a and b) using 0.45 micron filters. ATP-dependent calcium uptake was measured at room temperature at a protein concentration of 0.2 mg/ml in a medium containing 0.1 M KCl, 5 mM $MgCl_2$, 0.05 mM $CaCl_2$, (9000 cpm/nmole), 10 mM HEPES (pH 7.1) and 5 mM ATP (Meissner and Fleischer, 1974). The reaction mixture except ATP was incubated at room temperature for 30 minutes. Uptake was initiated by the addition of ATP and terminated after 8 minutes by passing the solution through a 0.45 micron filter. This pore size had already been shown to be suitable for calcium uptake studies (Martonosi and Feretos, 1964a). The amount of accumulated Ca^{++} is determined by measuring the $^{45}Ca^{++}$ content of the complete medium and of the Millipore filtrate by scintillation counting. Ca^{++} binding to the vesicles and filter was measured in the same manner except ATP was omitted from the reaction mixture. Ca^{++} uptake was calculated as the difference in ATP-dependent Ca^{++} accumulation and Ca^{++} binding. Calcium uptake is given as nmoles of Ca^{++} accumulated per mg of

protein.

Chloride Induced Calcium Release

Sarcoplasmic reticulum vesicles (2 mg/ml) were preincubated in 150 mM K methanesulfonate(MS), 5 mM Mg sulfate, 10 mM HEPES (pH 6.8) and 50 μ M $^{45}\text{CaCl}_2$ for 30-60 minutes at room temperature. Calcium uptake was initiated by the addition of 5 mM Na ATP. At set intervals, aliquots (1.0 ml) were taken and filtered through a Millipore filter (0.45 micron). The filtered vesicles were immediately washed with KMS buffer without calcium to measure uptake or KCl buffer without calcium to measure release. ^{45}Ca remaining on the filter was measured, after drying the filters, by soaking the dried filters in 10 ml of scintillation fluid and counting in a liquid scintillation counter. An alternate method to study release, although not frequently used, involved dilution of the vesicles at set times, filtering an aliquot of diluted vesicles and counting the filtrates for ^{45}Ca . Values given for Ca^{++} uptake and release are the mean $\pm$ the standard error of the mean for at least three determinations.

The effect of sucrose on the chloride induced calcium release from sarcoplasmic reticulum vesicles was studied by the addition of

solid sucrose to the potassium methanesulfonate and potassium chloride buffers. Calcium uptake and release was carried out as described with sucrose in all the solutions.

The effects of Na dantrolene on the chloride induced calcium release by the addition of Na dantrolene to the potassium methanesulfonate and potassium chloride buffers at a final concentration of 20 μ M. Calcium uptake and release was carried out as described with Na dantrolene in all the solutions. The maximum concentration of Na dantrolene in salt solutions is 20 μ M.

The specificity of calcium release from light and heavy SR vesicles was measured by passively loading the vesicles with 10 mM $^{22}\text{Na}^+$ (1 $\mu\text{Ci/ml}$) or 10 mM ^{14}C -sucrose (8 $\mu\text{Ci/ml}$). The light and heavy sarcoplasmic reticulum vesicles were then incubated on ice for 36 hours to equilibrate the sucrose or sodium. Calcium uptake and release were carried out as described except cold calcium replaced $^{45}\text{Ca}^{++}$. The amount of $^{22}\text{Na}^+$ or ^{14}C -sucrose retained in the vesicles was measured in the same way as $^{45}\text{Ca}^{++}$ was measured.

RESULTS

Light and Heavy Sarcoplasmic Reticulum Vesicles

Table I shows the biochemical characterization of the sarcoplasmic reticulum vesicles used in this study on calcium release. The vesicles have been potassium chloride washed so the endogenous Ca^{++} and Mg^{++} contents are less than 15 nmoles per mg of protein. The light SR vesicles have a higher $\text{Ca}^{++} + \text{Mg}^{++}$ ATPase activity than the heavy SR vesicles, which should be expected since it contains more calcium pump protein. Calcium uptake in potassium chloride solutions is approximately 100 nmoles Ca^{++} /mg in all three fractions. The internal water volume of the light SR vesicles is 75% higher than that of the heavy SR vesicles. The rate of passive calcium release from the light and heavy SR vesicles is 0.4 and 1.3 nmoles/mg/min, respectively.

Figure 1 shows densitometry tracings of light and heavy sarcoplasmic reticulum proteins separated by SDS-gel electrophoresis according to the method of Laemmli (1970). The light sarcoplasmic reticulum vesicles consist predominantly of $\text{Ca}^{++} + \text{Mg}^{++}$ ATPase (80-90%), whereas the heavy sarcoplasmic reticulum vesicles consist of $\text{Ca}^{++} + \text{Mg}^{++}$ ATPase (50%), calsequestrin (25%), high

TABLE 1

Properties of Light, Intermediate and Heavy SR Vesicles*

	Light SR	Inter. SR	Heavy SR
Ca ⁺⁺ ATPase (umoles Pi/min/mg)	2.80 ± .21	1.66 ± .24	1.84 ± .40
Mg ⁺⁺ ATPase (umoles Pi/min/mg)	.16 ± .07	.15 ± .01	.10 ± .04
Ca ⁺⁺ Content (nmoles/mg)	16 ± 2	15 ± 2	15 ± 1
Mg ⁺⁺ Content (nmoles/mg)	10 ± 2	7 ± 2	8 ± 1
Ca ⁺⁺ Uptake (nmoles/mg)	100 ± 10	100 ± 15	91 ± 12
Water Volume (microliters/mg)	3.2 ± .5	2.5 ± .6	2.4 ± .4
Passive Ca ⁺⁺ Efflux (nmoles/mg/min)	0.4	-	1.3

*The values given are the mean ± the standard error for at least three determinations.

affinity calcium binding protein (5%), and an intrinsic 30,000 dalton protein (3%).

Chloride Induced Calcium Release

Table II shows the change in calcium retained by intermediate SR vesicles, when methanesulfonate is exchanged for chloride and when chloride is exchanged for methanesulfonate. It should be noted, that the amount of calcium accumulated by the vesicles is independent of the anion in the medium. The amount of calcium released by the methanesulfonate to chloride change is 22.7 nmoles/mg which is 27% of the accumulated calcium. The amount of calcium released by the chloride to methanesulfonate change is 1.6 nmoles/mg which is within the error on the measurement of calcium uptake. It should be noted that including 0.5 mM EGTA in the washing solution caused an increase in the amount of calcium release by chloride induced release from 44% to 57% of the accumulated calcium.

Figure 3 and 4 show the results of changing the ionic conditions for light and heavy SR vesicles, respectively. The amount of calcium retained in the vesicles is plotted against time of calcium accumulation for the methanesulfonate to methanesulfonate change and for methanesulfonate to chloride change. The left hand axis is

Figure 1.

Densitometry tracings of light and heavy sarcoplasmic reticulum proteins separated by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970).

(1) Light Sarcoplasmic Reticulum Vesicles

(2) Heavy Sarcoplasmic Reticulum Vesicles

Figure 2.

Calcium upake for light and heavy SR vesicles versus time. The ordinate is nmoles Ca^{++} /microliter of water space, which was obtained by dividing the nmoles/mg by microliters/mg for each type of SR vesicle. The initial fast uptake of calcium is followed by a fast release of 30-40% of the accumulated calcium, which is followed by a slow phase of Ca^{++} accumulation. The closed circles represent the light SR vesicles and the open circles represent the heavy SR vesicles.

TABLE II

Effect of Changing the Ionic Environment on Calcium
Release from Intermediate SR Vesicles*

	nmoles Ca ⁺⁺ /mg	nmoles Ca ⁺⁺ released	% Ca ⁺⁺ released
KMS to KMS	83.8 ± 0.8		
KMS to KCl	61.1 ± 0.5	22.7	27.0
KCl to KCl	84.4 ± 2.8		
KCl to KMS	82.8 ± 0.9	1.6	1.9

* The values given are the mean ± the standard error of the mean for three determinations.

Figure 3.

The amount of calcium retained in light SR vesicles versus time of accumulation for calcium uptake and chloride induced calcium release. The left hand axis is nmoles Ca^{++} /mg of protein and the right hand axis is nmoles Ca^{++} /mg of ATPase in the light SR vesicles.

Figure 4.

The amount of calcium retained in heavy SR vesicles versus time of accumulation for calcium uptake and chloride induced calcium release. The left hand axis is nmoles Ca^{++} /mg of protein and the right hand axis is nmoles Ca^{++} /mg of ATPase in the heavy SR vesicles.

right hand axis is nmoles Ca^{++} / mg of ATPase. This was calculated by dividing the nmoles Ca^{++} /mg of protein by the percentage of ATPase in the vesicles. The difference between the potassium methanesulfonate to potassium methanesulfonate and potassium methanesulfonate to potassium chloride curves is the amount of calcium released. In the absence of ATP there is practically no difference in the Ca^{++} retained by the vesicles under uptake or release conditions. At early times the amount of calcium released by the light SR vesicles is much larger than that released by the heavy SR vesicles but after 8 minutes both types of vesicles release approximately the same amount of calcium per mg of membrane protein or ATPase (30-35 nmoles Ca^{++} /mg of ATPase or membrane protein). Figure 5 shows the amount of calcium released per mg of membrane protein versus the time of calcium accumulation. Both the light SR and heavy SR have a biphasic response, which is similar to the uptake curves.

Figure 6 shows the percent of accumulated calcium released by light and heavy SR vesicles versus the time of calcium accumulation. The percent of accumulated calcium released by the light SR is practically double that released by the heavy SR vesicles. The percentage of calcium released from the light and heavy SR vesicles is relatively independent of the time of calcium accumulation or the amount of calcium accumulated.

Figure 5.

A plot of nmoles Ca^{++} released by chloride induced release versus uptake time for light and heavy SR vesicles.

Figure 6.

A plot of the percent calcium released from light and heavy SR vesicles versus time of calcium accumulation.

Effect of Sucrose on Calcium Release

Table III shows the effect of sucrose on the accumulation and release of calcium from intermediate SR vesicles. Sucrose (100 mM) caused a 66% increase in the amount of calcium accumulated. The amount of chloride induced calcium release went from 25 to 17.2 nmoles/mg and the percent of calcium released went from 35% to 14.5 % in the presence of sucrose. Figure 7 shows the effect of 200 mM sucrose on the percentage of calcium release from light and heavy SR vesicles. The chloride induced release of calcium from the light SR vesicles is substantially reduced, whereas, the release from the heavy SR vesicles seem to be unaffected by the presence of sucrose.

$^{22}\text{Na}^+$ and ^{14}C -Sucrose Release

Table IV shows the results of chloride induced release on the amount of $^{22}\text{Na}^+$ or ^{14}C -sucrose retained by the light and heavy SR vesicles. The control values obtained for the amount of $^{22}\text{Na}^+$ or ^{14}C -sucrose retained by the light and heavy SR vesicles can be converted into Na space/mg or sucrose space/mg by dividing by 10 mM, which was the concentration at which the vesicles were equilibrated. The results of the conversion are 4.1 and 1.3 microliters/mg for the

TABLE III

Effect of Sucrose on Chloride Induced Calcium Release

	nmoles Ca^{++} /mg	nmoles Ca^{++} released	% Ca^{++} released
5 mM Sucrose			
KMS to KMS	71.6 ± 1.0		
KMS to KCl	46.6 ± 0.3	25.0	35.0
100 mM Sucrose			
KCl to KCl	118.4 ± 1.6		
KCl to KMS	101.2 ± 1.0	17.2	14.5

Values given are the mean $\pm$ the standard error of the mean for at least three determinations.

Figure 7.

Percent of calcium released in the absence and presence of 200 mM sucrose for light and heavy SR vesicles.

Figure 8.

Effect of Na dantrolene on the percent of calcium released from light and heavy SR vesicles. Two experiments are shown for light SR and two for heavy SR .

TABLE IV

$^{22}\text{Na}^+$ and ^{14}C -Sucrose Retained by
Light and Heavy SR Vesicles

	Light SR (nmoles)	Heavy SR (nmoles)
$^{22}\text{Na}^+$		
KMS to KMS	41.0 ± 1.0	13.0 ± 0.1
KMS to KCl	26.0 ± 0.7	10.0 ± 0.3
Amount released	15	3
Percent Released	37%	23%
^{14}C -Sucrose		
KMS to KMS	35.2 ± 4.6	19.8 ± 2.1
KMS to KCl	27.5 ± 1.6	17.0 ± 1.2
Amount released	7.7	2.8
Percent Released	22%	14%

Values given are the mean $\pm$ the standard error of the mean for at least three determinations.

and heavy SR vesicles, respectively. The sucrose space was 3.5 and 1.9 microliters/mg for the light and heavy SR vesicles, respectively. These values are in agreement with those obtained for the internal water space of the light and heavy SR vesicles.

The amount of $^{22}\text{Na}^+$ released by the change from methanesulfonate to chloride was five times greater from the light SR vesicles as from the heavy SR vesicles. There was no significant difference in the amount of ^{14}C -sucrose released from light or heavy SR. The percent of retained $^{22}\text{Na}^+$ which was released was 37% for the light SR vesicles and 23% for the heavy SR vesicles.

Effect of Na Dantrolene on Calcium Uptake and Release

The binding of calcium to light and heavy SR vesicles was unaffected by the presence of 20 μM Na dantrolene. Calcium uptake by light SR vesicles was the same in the presence or absence of Na dantrolene (70.4 vs 69.6 nmoles/mg). Na dantrolene did cause a 20% increase in the calcium accumulated by the heavy SR vesicles. The increase in calcium uptake was in the amount of calcium accumulated not in the rate of calcium accumulation.

Figure 5 shows the results of several experiments on the percent calcium released in the presence and absence of Na dantrolene. The drug does not effect the percent or amount of calcium released by the light SR vesicles upon chloride induced calcium release, whereas, the release of calcium from the heavy SR vesicles is inhibited by Na dantrolene.

DISCUSSION

Light and heavy sarcoplasmic reticulum vesicles have been shown to be derived from the longitudinal and terminal regions of the sarcoplasmic reticulum, respectively. They are both able to accumulate calcium in the presence of ATP which makes them ideally suitable to study the mechanism of calcium release. It is not certain that the terminal cisternae is the sole source of calcium during release. It is clear from the morphological studies that the depolarization of the transverse tubular system initiates calcium release through a mechanism involving the junctional membrane of the terminal cisternae.

Therefore, in the study of calcium release, the light and heavy SR vesicles are useful because the light SR vesicles should not have the ability to release calcium through the physiological mechanism or stimulus. The heavy SR vesicles should have both the mechanism of calcium release and the ability to respond to the physiological stimuli of excitation-contraction.

The time course of calcium uptake by light and heavy SR vesicles has been shown to be biphasic. It has an initial rapid phase of accumulation, followed by a fast release of calcium, followed by a slow phase of accumulation. The cause of the release of calcium is unknown. It is probable that the second phase of accumulation is due to the formation of inorganic phosphate by hydrolysis of ATP, which offers a sink for calcium during the accumulation of calcium by SR vesicles.

The fact that the percent of calcium released from both light and heavy SR vesicles is a constant, is consistent with the skinned fiber results of Thorens and Endo (1975). They showed that the amount of calcium released is a constant fraction of the amount of calcium within the sarcoplasmic reticulum immediately before chloride induced calcium release. The inhibition of the release of calcium from the light SR vesicles by 100 mM sucrose is also in agreement with the work of Thorens and Endo (1975), but the fact that the heavy SR vesicles are unaffected by sucrose suggests that Thorens and Endo (1975) were looking at release of calcium from the longitudinal SR and not the terminal cisternae. It is known that in a resting muscle fiber the calcium is localized in the terminal cisternae but in skinned muscle fibers after loading the SR with buffered calcium solutions it seems probable that both the longitudinal and terminal cisternae are filled with calcium. In addition, Na dantrolene does not seem to affect the release of calcium in skinned muscle fibers

(Endo, 1976) but it does effect the release of calcium from the heavy sarcoplasmic reticulum vesicles. Therefore, the ability of sucrose to inhibit the release of calcium from light SR vesicles and the ability of Na dantrolene to inhibit the release of calcium from heavy SR vesicles suggests that in skinned fiber experiments, where the SR is loaded from buffered solutions with calcium, the chloride induced release could be localized in the longitudinal sarcoplasmic reticulum. The fact that chloride induced release can function under conditions of low calcium uptake supports the hypothesis that the terminal cisternae can release calcium upon chloride depolarization.

The release of calcium induced by the change from methane-sulfonate to chloride seems to be caused by both osmotic swelling and depolarization. After the solution change, chloride diffuses faster into the vesicle than the larger anion, methanesulfonate, diffuses out. This produces a net positive charge on the outside of the vesicle and thus depolarization. Komentani and Kasai (1978) were able to show that chloride is approximately 50 times as permeable to the SR membrane as methanesulfonate or potassium.

Osmotic swelling of the SR vesicles is probably caused by the influx of potassium along with chloride into the vesicle which is accompanied by water. The swelling can then cause the membrane to become leaky. Vesicles, which have an internal content, such as the heavy SR vesicles, can probably resist the swelling and do not become

leaky.

Although an actual membrane potential change in the SR has not been measured upon chloride depolarization, there are some indications that one does take place. Fabiato and Fabiato (1977) have shown an increase of light absorption in a merocytine dye by the replacement of K propionate with tris chloride in the bathing fluid of a skinned muscle fiber. McKinley and Meissner (1977) have shown that a change in the ionic environment of isolated SR vesicles from K gluconate to tris gluconate causes a rapid decrease in fluorescence for diO-C5-(3).

An estimate of the specificity of calcium release from light and heavy SR vesicles can be calculated by dividing the amount of Ca^{++} released/internal Ca^{++} concentration by the amount of $^{22}\text{Na}^{+}$ released/internal $^{22}\text{Na}^{+}$ concentration. The results of these calculations give a $\text{Ca}^{++}/\text{Na}^{+}$ ratio of 0.6 and 2.5 for the light and heavy SR vesicles, respectively. The release of calcium from the heavy SR vesicles is slightly more specific for calcium than the release from the light SR vesicles but in general the heavy SR vesicles seem to be more permeable to several different ions and sucrose than the light sarcoplasmic reticulum vesicles.

The release of calcium from the light SR, which is affected by external sucrose and which shows a larger release of $^{22}\text{Na}^{+}$

is probably due to osmotic swelling. This is also consistent with the morphology of the light SR vesicles which show various shapes and have no internal structure capable of protecting them from osmotic swelling.

Release from the heavy SR vesicles, which is not affected by external sucrose and which shows a smaller amount of $^{22}\text{Na}^+$

release, is probably due to depolarization. The release from the heavy SR vesicles has the additional quality of being inhibited by Na dantrolene. Na dantrolene also seems to decrease the passive calcium permeability of the heavy SR vesicles since it can increase the calcium accumulated by the heavy vesicles. These results agree with the work of Desmedt and Hainaut (1977) on barnacle muscle fibers injected with aequorin. They have shown that dantrolene inhibits rather selectively the Ca^{++} release mechanism for the sarcoplasmic reticulum. In addition, the resting calcium efflux from the sarcoplasmic reticulum is decreased by dantrolene which is consistent with the increased uptake by the heavy SR vesicles.

Finally, it is important to point out the chloride induced release experiments were all performed on SR vesicles in which calcium was taken up using ATP. This is important for two reasons (1) Endo and Kitazawa (1976) have shown that the amount of calcium released by the SR from chloride depolarization was less in the absence of ATP than in its presence (2) the response of the SR to

chloride depolarization will probably be different whether the SR has been passively loaded or actively loaded with calcium due to the possible electrogenicity of the Ca^{++} pump (Zimniak and Racker, 1978).

The results of these experiments suggest that the heavy SR vesicles, which are derived from the terminal cisternae, are able to release calcium upon depolarization, that this release is more specific in the heavy SR than in the light SR and that the heavy SR release is inhibited by Na dantrolene. Future experiments using ionic depolarization of heavy SR vesicles where osmotic effects are minimized seem to be a promising approach to the study of calcium release.

CHAPTER III

INTRINSIC PHOSPHORYLATION OF HEAVY

SARCOPLASMIC RETICULUM VESICLES

SUMMARY

Heavy sarcoplasmic reticulum vesicles, derived from the terminal cisternae of the sarcoplasmic reticulum, have been used in a study of membrane phosphorylation. HSR vesicles were phosphorylated at room temperature for 10 minutes in 5 mM MgCl₂, 1 mM EGTA, 10 mM HEPES (pH 7.4) and 10 μ M [γ -³²P] ATP. ³²P-phosphoproteins were determined by SDS-gel electrophoresis and autoradiography. In the absence of EGTA there was little phosphorylation, but when 1 mM EGTA was present phosphorylation of several proteins could be seen. The phosphorylation was c-AMP independant, hydroxylamine resistant and was seen without the addition of protein kinase. The molecular weights of the phosphorylated proteins which are known not to be involved in glycolysis were 64,000 (E-1), 42,000 (E-2) and 20,000 daltons (E-3). Band E-1 corresponds to the protein calsequestrin in mobility on the Laemmli slab gel while bands E-2 and E-3 have no corresponding bands on the stained gel. The addition of 10 mM EDTA inhibited the phosphorylation of all the proteins except band E-2 (42,000 daltons). In the presence of 2.5 mM HgCl₂ or 1% potassium deoxycholate no phosphorylation was observed. KCl (200 mM) increased the level of phosphorylation of band E-1 and Na dantrolene (20 μ M) caused the decrease of the phosphorylation of the band E-1. Band E-3 was found in both the light SR vesicles and the HSR vesicles whereas

the bands E-1 and E-2 were found only in the HSR vesicles. The band E-2 has the properties of an intrinsic membrane protein while the band E-1 behaves as an extrinsic membrane protein. Isolation of calsequestrin by gel electrophoresis or calcium precipitation reveals a low level of ^{32}P incorporation into calsequestrin. Preliminary experiments with depolarization of the heavy sarcoplasmic reticulum vesicles by changing the ionic medium from K gluconate to tris chloride, indicates that the phosphorylation of band E-1 is increased by depolarization of the HSR vesicles.

INTRODUCTION

It is generally accepted that the depolarization of the transverse tubular system of skeletal muscle initiates the release of calcium from the terminal cisternae of the sarcoplasmic reticulum (for reviews see Ebashi and Endo, 1968; Sadow, 1970; Fuchs, 1974 and Endo, 1977). In recent years there have been several mechanisms proposed for the link between the depolarization of the walls of the T-tubule and the release of calcium from the sarcoplasmic reticulum (Ebashi and Endo, 1968; Endo, 1977), but it still remains one of the least understood processes in muscle contraction. The skeletal muscle membranes directly involved in excitation-contraction coupling are the transverse tubular membrane and the junctional sarcoplasmic reticulum membrane (Franzini-Armstrong, 1975). There has been some recent progress in understanding excitation-contraction coupling in terms of electrophysiological properties of the transverse tubular membrane (Schneider and Chandler, 1973) and in the morphological description of the triad (Franzini-Armstrong, 1971-75), but relatively little is known biochemically about the membranes or membrane proteins involved in excitation-contraction coupling.

The morphological and chemical differences of the longitudinal sarcoplasmic reticulum and the terminal cisternae are consistent with

the hypothesis that the longitudinal SR consists of predominantly the calcium pump protein and is responsible for calcium uptake resulting in relaxation, and that the terminal cisternae, directly involved in excitation-contraction coupling, contains one or more membrane proteins besides the calcium pump protein and is responsible for calcium release to initiate contraction.

In general, it is becoming increasingly evident that the phosphorylation of membranes by protein kinases (c-AMP dependent or independent) may represent a mechanism for controlling calcium movements across membranes. Rudolph and Greengard (1975) have shown that beta-adrenergic agents and c-AMP regulate the membrane permeability and protein phosphorylation in the avian erythrocyte. Altered phosphorylation of membrane channels has been suggested as a possible mechanism linking c-AMP to the increased slow inward current in the heart surface membrane (Tsien, 1978). Kruger et al. (1977) has shown that depolarization of synaptosomes induces the phosphorylation of specific proteins. The phosphorylation requires the influx of calcium ions and is not affected by cyclic nucleotides. In cardiac sarcoplasmic reticulum there has been shown that beta-adrenergic agonists cause an increase in c-AMP which causes a c-AMP protein kinase to phosphorylate a 22,000 dalton SR membrane protein. This phosphorylation has been connected with increased calcium uptake by cardiac sarcoplasmic reticulum vesicles. It has been reported that skeletal muscle has little or no phosphorylation of sarcoplasmic

reticulum membranes (Kirchberger and Tada, 1976). Schwartz et al. (1977) has shown a physiological effect of c-AMP on calcium uptake by skeletal muscle SR vesicles but they did not look at the phosphorylation of the SR vesicles.

The purpose of our investigation was three fold (1) to characterize the endogenous membrane phosphorylation of heavy sarcoplasmic reticulum vesicles (2) to identify and characterize the heavy SR proteins which are phosphorylated and (3) to study the effect of changing the ionic environment as to cause a depolarization of the heavy SR vesicles on the phosphorylation of heavy SR vesicles. Our investigation has shown the existence of an endogenous membrane phosphorylation system in the heavy SR vesicles, which are derived from the terminal cisternae. This phosphorylation is c-AMP independent and was seen without added protein kinase. One of the major phosphorylated bands is probably calsequestrin. A preliminary report of this work has been presented at the 1978 Biophysical Society Meetings (Campbell and Shamoo, 1978).

Experimental Procedures

Materials

Acrylamide, N,N'-Methylene-bis-acrylamide and Chelex 100 were obtained from Bio-Rad. Ultra pure sucrose was obtained from Schwarz/Mann. Adenosine 5'-[gamma-³²P] triphosphate (20Ci/mmol) was obtained from Amersham/Searle. Omniflour was obtained from New England Nuclear. HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and PIPES (piperazine-N-N'-bis-2 ethane sulfonic acid) were obtained from Calbiochem. Calcium and magnesium atomic absorption standards (1000 ppm) were obtained from Fischer Scientific. Butyl acetate was obtained from Eastman. Ethyleneglycol-bis-(beta amino ethyl ether) N,N'-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), dithiothreitol (DTT), Coomassie brilliant blue R, deoxycholate acid, Triton X-100, sodium dodecyl sulfate (SDS), TRIS (hydroxymethyl aminoethane), N,N,N',N'-tetramethyl ethylenediamine (TEMED), disodium adenosine 5' triphosphate, and 2-mercaptoethanol were all obtained from Sigma. Millipore filters HAWP 025(.45u) and GSWP 025(.22u) were obtained from Millipore.

The following proteins were used as molecular weight standards. Insulin was obtained from Lilly. Parathyroid hormone (PTH) was obtained from Inolex. Egg white lysozyme, rabbit muscle phosphoryl-

ase a, bovine liver catalase, bovine carbonic anhydrase, bovine pancreatic trypsin, soybean trypsin inhibitor, horse heart cytochrome c, porcine adrenocorticotrophic hormone (ACTH), bovine glucagon and glucose-3-phosphate dehydrogenase were all obtained from Sigma. Red blood cell spectrin and rabbit muscle actin were a generous gift from Dr. F. Kirkpatrick.

All other reagents used were of analytical grade. All solutions were made with deionized distilled water. Deoxycholate was purified by the method of MacLennan (1970) and stored as a 10% solution which was brought to pH 8.0 with KOH.

Isolation of Light and Heavy Sarcoplasmic Reticulum Vesicles

Briefly, approximately 1000 grams of white rabbit leg muscle was homogenized in a Waring blender in 0.25 M sucrose for 30 seconds. The homogenate was centrifuged twice to remove cell debris and mitochondria and a third time to sediment the SR vesicles. The SR vesicles were placed on a linear sucrose gradient, 26-45% (w/w), and centrifuged at 100,000x g for 16 hours. Fractions containing 30-32.5% sucrose (light SR), 33.5-38% sucrose (intermediate SR) and 38.5-42% sucrose (heavy SR) were combined and then diluted with 1.0 MKCl, 1 mM HEPES (pH 7.4) or 0.25 M sucrose, 1 mM HEPES (pH 7.4).

After two hours incubation at 0 C in 0.6 M KCl or 20% sucrose the purified SR vesicles are sedimented, homogenized in 0.25 M sucrose, 1 mM HEPES (pH 7.4) and stored in liquid nitrogen. For a more detailed procedure see Campbell Armstrong and Shamoo (1978). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Assays

Percent sucrose (w/w) or density (grams/cc) measurements of sucrose solutions were determined at 25° C using an Abbe refractometer. Calcium and magnesium were determined by atomic absorption spectroscopy using a Perk and Elmer Atomic Absorption Spectrometer. Ca^{++} + Mg^{++} ATPase activity was measured in 0.1 M KCl, 5 mM MgCl_2 , 0.1 mM CaCl_2 , 0.1 mM EGTA, 10 mM PIPES (pH 6.8) and 5 mM ATP (γ - ^{32}P -ATP, 500 cpm/nmole ATP) at a protein concentration of 50-80 micrograms/ml (MacLennan, 1974).

Phosphorylation of Sarcoplasmic Reticulum Vesicles

The standard reaction mixture contained 5 mM MgCl₂, 1 mM EGTA, 10 mM HEPES (pH 7.4), 100-200 micrograms of SR protein and 10 uM [γ -³²P] ATP (1-3 uCi/nmole), in a final volume of 0.1 ml. The SR vesicles were incubated at room temperature for 30 minutes in the reaction buffer prior to initiation of phosphorylation. Phosphorylation was initiated by the addition of the radioactive ATP. The reaction was terminated by the addition of 0.05 ml of the SDS-solubilizing buffer (see SDS-gel electrophoresis) and incubated at 57 C for 2-3 hours. In order to determine the extent of phosphorylation of endogenous substrates, 0.05 ml aliquots from each reaction mixture were subjected to Laemmli SDS-gel electrophoresis.

Characterization of the phosphorylation of the HSR vesicles involved the addition of reagents to the standard reaction mixture to investigate their effects on the phosphorylation. Hydroxylamine treatment was carried out according to the method of Lippman and Tuttle (1944). Deoxycholate treatment was carried out according to the method of MacLennan (1970). Triton treatment was carried out according to the method of Ikemoto (1972) and according to Chapter I.

SDS-Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Laemmli and Favre (1974). Slab gels (1.5 x 120 mm) consisted of a 10% acrylamide running gel with a 3 or 4.5% acrylamide stacking gel plus 0.4% agarose. The ratio of acrylamide to cross-linker, bis-acrylamide, was 37.5:1 for the running and stacking gels. The running gel was formed in 0.375 M tris-HCl (pH 8.8) and 0.1% SDS. The stacking gel was formed 0.125 M tris-HCl (pH 6.8) and 0.1% SDS. Both gels were polymerized by the addition of 0.1% TEMED and 0.1% ammonium persulfate. The buffer system in the upper and lower reservoirs was 0.025 M tris , 0.19 M glycine and 0.1% SDS. The samples to be analyzed by electrophoresis were solubilized in 2% SDS, 62.5 mM tris-HCl (pH 6.8), 1 mM EDTA, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue (SDS-solubilizing buffer) at a protein concentration of 1 mg/ml for 2 minutes at 100 C followed by 2 hours at 37 C. The solubilized protein was electrophoresed for 2 hours at 150 V constant voltage. Gels were stained in 0.1% Coomassie blue, 10% acetic acid, 50% methanol overnight and diffusion destained in 10% acetic acid. The slab gels were dried on Whatman No. 5 paper under vacuum and placed on Kodak NS-2T film for 2-7 days. The resulting autoradiograph revealed those proteins into which ^{32}P had been incorporated. The optical density of the bands on the film was measured on a Beckman densitometer, and the areas under the peaks of

the optical density tracings were used as a quantitative measure of incorporation of ^{32}P into the SR proteins. The films were scanned vertically to compare the phosphorylation of several SR proteins under one condition or horizontally to compare the phosphorylation of a single protein under several conditions.

Stained slab gels were scanned using a Beckman densitometer. Apparent molecular weights were calculated from a graph of relative mobilities versus log of the molecular weight Neville (1974). Molecular weight standards used were spectrin (220,000 and 240,000), phosphorylase a (94,600), bovine serum albumin (68,000), catalase (57,500), actin (43,000), lactate dehydrogenase (35,000), carbonic anhydrase (30,000), trypsin inhibitor (21,500), and lysozyme (14,400).

Polyacrylamide gel electrophoresis was also carried out according to the method of Weber and Osborn (1969). Cylindrical gels consisted of a 7.5% acrylamide running gel. The gel was formed in 28 mM Na phosphate, 78 mM disodium phosphate and 0.1% SDS (pH 7.0) and polymerized with 0.1% TEMED and 0.1% ammonium persulfate. The buffer system in the upper and lower reservoirs was 28 mM Na phosphate, 78 mM disodium phosphate and 0.1% SDS. Samples for electrophoresis were solubilized in 2% SDS, 10 mM Na phosphate (pH 7.0), 1 mM EDTA, 2% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue at a protein concentration of 1 mg/ml for 2 minutes at 100 C followed by

incubation for 2 hours at 37 C. The solubilized protein was electrophoresised for 4 hours at 8 mA/gel. The gels were stained and destained the same as described previously and the same molecular weight standards were used.

Low molecular weight proteins (3000 - 20,000 daltons) were resolved on Swank and Munkres (1971) gel system. Cylindrical gels (5.5 x 125 mm) and slab gels (1.5 X 120 mm) consisted of 10% acrylamide, 1% bis-acrylamide, 8M urea, 0.1 M phosphoric acid, and 0.1% SDS (pH 7.0). Samples were solubilized in 1% SDS, 8M urea, 1% mercaptoethanol, 0.01 M phosphoric acid (pH 6.0). Molecular weight standards used were carbonic anhydrase (30,000), trypsin inhibitor (21,500), cytochrome c (12,600), parathyroid hormone (9800), ACTH (4700) and glucagon (3480).

Calsequestrin Isolation

(1) Phosphorylated HSR vesicles were run on Weber and Osborn (1969) gels (7.5%) and the gels stained and destained as described. The band running at 44,000 daltons, which is calsequestrin, was cut out from the gel and homogenized in Laemmli SDS sample buffer. The homogenate was placed in the well of a Laemmli slab gel and electrophoresis as described. The stained gel reveals a band of

approximately 64,000 daltons.

(2) Phosphorylated HSR vesicles were solubilized with Triton X-100 at a 3:2 ratio (mg/mg) of Triton X-100 to protein in the presence of 10% glycerol, 50 mM tris (pH 8.5) and 5 mM beta-mercaptoethanol (Ikemoto, 1972). After removing the insoluble material by centrifugation the supernatant was made 4 mM CaCl_2 by the addition of concentrated CaCl_2 . After a 24 hour incubation period at 0 C the supernatant was centrifuged and a white precipitated , which contained calsequestrin, was recovered.

(3) Calsequestrin was isolated according to the method of MacLennan and Wong (1971) from phosphorylated SR vesicles. The method involves DOC treatment in the presence of 1 M KCl , followed by extensive dialysis against 5 mM tris HCl pH 7.5, followed by DEAE chromatography.

Depolarization Induced Phosphorylation.

HSR-KCl vesicles were preincubated in 150 mM K gluconate, 5 mM MgSO_4 , 10 mM HEPES (pH 7.4) for 24 hours at 0 C. Initial experiments measured the phosphorylation of HSR-KCl vesicles in the K gluconate buffer plus 1 mM EGTA versus time as described previously.

Depolarization induced phosphorylation was carried out using an aliquot of HSR-KCl vesicles (equilibrated in K gluconate buffer) and diluting it in half with the same buffer plus 2 mM EGTA.

Phosphorylation was initiated with the addition of cold ATP (10 μ M) and after 2 minutes the vesicles were diluted (1:10) into K gluconate buffer (with 1 mM EGTA) or tris chloride buffer (with 1 mM EGTA) containing 32 P-ATP (10 μ M). The reaction was then terminated after 30 seconds with SDS. In order to see if the depolarization was causing an increased phosphorylation or dephosphorylation followed by a phosphorylation, the experiment was carried out with the addition of 32 P-ATP first and cold ATP was used in the dilution buffer.

RESULTS

Characterization of Heavy SR Phosphorylation

Table I gives a summary of the biochemical characteristics of KCl diluted heavy SR vesicles (HSR-KCl) and sucrose diluted HSR vesicles (HSR-sucrose). The most important difference in the two types of vesicles for phosphorylation studies is the Ca^{++} and Mg^{++} contents, which are high in the HSR-sucrose and low in the HSR-KCl vesicles.

An autoradiogram illustrating the incorporation of ^{32}P into proteins of KCl and sucrose washed HSR vesicles is shown in Figure 1. The HSR vesicles had been preincubated in 5 mM MgCl_2 and 10 mM HEPES (pH 7.4), plus any added reagent for 30 minutes at room temperature. The phosphorylation was initiated by ^{32}P -ATP and terminated after 10 minutes by the addition of a SDS-solubilizing solution. A picture of the resulting stained slab gel is shown in Figure 1 (a). KCl and sucrose HSR samples alternate across the gel. Ca^{++} + Mg^{++} ATPase and calsequestrin can be seen to be the major proteins in the HSR vesicles. The KCl HSR vesicles are also rich in a 30,000 dalton

TABLE I

Properties of KCl and Sucrose Diluted Heavy SR Vesicles*

	HSR-KCl Vesicles	HSR-Sucrose Vesicles
Ca ⁺⁺ ATPase (umoles Pi/min/mg)	1.84 ± .40	0.72 ± .13
Mg ⁺⁺ ATPase (umoles Pi/min/mg)	.10 ± .04	.09 ± .03
Ca ⁺⁺ Content (nmoles/mg)	15 ± 1	114 ± 12
Mg ⁺⁺ Content (nmoles/mg)	8 ± 1	38 ± 8
Water Volume (microliters/mg)	2.4 ± .4	2.1 ± .3
Passive Ca ⁺⁺ Efflux (nmoles/mg/min)	1.3	1.4

*The values given are the mean ± the standard error for at least three determinations.

Figure 1.

Effect of several reagents on the phosphorylation of HSR-KCl and HSR-sucrose vesicles. The SR vesicles were preincubated with 5 mM MgCl₂, 10 mM HEPES (pH 7.4) plus any additional reagent for 30 minutes at room temperature. Phosphorylation was initiated by the addition of 10 μ M ³²P-ATP, terminated by the addition of SDS and the samples subjected to SDS gel electrophoresis, as described under Experimental Procedures. The gel was stained (a), dried and exposed to x-ray film to produce the autoradiogram (b).

(a) Coomassie blue stained slab gel containing 50 micrograms of SR protein per sample. Odd samples contain HSR-KCl proteins and even samples contain HSR-sucrose proteins. Arrow points to calsequestrin.

(b) Autoradiogram of (a) [1,2] control, [3,4] EGTA (1 mM), [5,6] NaF (25 mM), [7,8] NaN₃ (5 mM), [9,10] NEM (1 mM), and [11,12] EGTA and NaF. Phosphorylated bands E-1, E-2 and E-3 are indicated. The high molecular weight proteins that are phosphorylated are probably glycogen debranching enzyme (160,000 daltons), phosphorylase kinase (126,000 and 145,000 daltons), phosphorylase a (94,000 daltons) and glycogen synthetase (88,000 daltons).

A

B

sucrose HSR vesicles are rich in a 34,000 dalton protein. When the HSR vesicles were incubated in the standard reaction mixture there was very little phosphorylation in the HSR vesicles and a small amount of phosphorylation in the sucrose HSR vesicles. The higher molecular weight proteins (greater than 88,000 daltons) that are phosphorylated in the HSR vesicles have been tentatively identified as glycogen debranching enzyme (160,000 daltons), phosphorylase kinase (128,000 and 145,000 daltons), phosphorylase a (94,000 daltons) and glycogen synthetase (88,000 daltons) (Michalak et al. ,1977, and Horl et al. ,1978).

The addition of 1 mM EGTA to the reaction buffer dramatically stimulates the phosphorylation of three specific proteins, E-1 (64,000 daltons), E-2 (42,000 daltons) and E-3 (20,000 daltons). It is these three proteins which we will be discussing in this chapter. Quantitative results of the phosphorylation of band E-1 in HSR-KCl and HSR-sucrose vesicles are given in Figure 2. In HSR-KCl vesicles, which have a low calcium and magnesium content, over a six fold increase in ^{32}P incorporation is observed when 1 mM EGTA or 1 mM EGTA plus 25 mM NaF are added to the standard reaction mixture. This is also true for bands E-2 and E-3. NaF (25 mM) or Na dantrolene (10^{-5} M) cause little change in the level of phosphorylation of band E-1 in HSR-KCl vesicles. In HSR sucrose vesicles, which contain a high calcium and magnesium content, the level of E-1 phosphorylation in 5 mM MgCl_2 , 10 mM HEPES (pH 7.4) and 10 μM ^{32}P -ATP is relatively high

Figure 2.

Bar graph of the ^{32}P incorporated in 10 minutes into the band E-1 (64,000 daltons) for HSR-KCl and HSR-sucrose vesicles in presence of 5 mM MgCl_2 , 10 mM HEPES (pH 7.4), 10 μM ^{32}P -ATP and the added reagent (indicated).

when compared to HSR-KCl vesicles. EGTA (1 mM) or EGTA (1 mM) plus 25 mM NaF cause a 45% and 50% increase in 32 P incorporation into band E-1, respectively. NaF (25mM) has no affect on the level of phosphorylation of E-1 and 10^{-5} M Na dantrolene reduces the phosphorylation by 60% in HSR-sucrose vesicles. It should be noted that no phosphorylation is observed when the vesicles are solubilized with SDS prior to the addition of 32 P-ATP.

Figure 3 (a) shows the results of HSR-KCl and HSR-sucrose phosphorylation in the presence of 1 mM EGTA, 5 mM MgCl₂, 10 mM HEPES (pH 7.4) and 10 μ M 32 P-ATP. Band E-1 and E-3 are phosphorylated faster in the HSR-KCl vesicles but after 10 minutes the phosphorylation of band E-1 is approximately the same for HSR-KCl and HSR-sucrose vesicles. Band E-2 is more predominant in the HSR-KCl vesicles but is present in HSR-sucrose vesicles after 10 minutes. Light SR vesicles (KCl or sucrose diluted) have been shown in the absence of EGTA to contain no phosphorylated proteins. In the presence of EGTA (1 mM) and after 10 minutes of phosphorylation, LSR sucrose vesicles contain a small amount of band E-1 (which corresponds with the small amount of calsequestrin in the LSR sucrose vesicles), no visible band E-2, and a roughly equal amount of E-3 phosphorylation when compared to HSR vesicles.

HSR-sucrose vesicles which were subsequently KCl washed contain less of the high molecular weight phosphorylated proteins. The phos-

Figure 3.

(a) Autoradiogram of HSR-KCl (1,3,5) and HSR-sucrose (2,4,6), 15 second phosphorylation (1,2), 1 minute (3,4), 10 minutes (5,6), Light SR 10 minute phosphorylation (7), HSR-sucrose KCl washed 10 minute phosphorylation (8), Intermediate SR phosphorylation (9), Triton pellet of intermediate SR phosphorylation (10). Note that band E-1 appears as double band in this autoradiogram.

(b) Autoradiogram of HSR-sucrose vesicles phosphorylation control (1), 0.1 mM EGTA (2), 1 mM EGTA (3), 25 mM EDTA (4), 1 mM EGTA plus 25 mM NaF (5), stained HSR-sucrose sample (6), 200 mM KCl plus EGTA (7), 1% KDOC plus EGTA (8), 10^{-6} M c-AMP plus EGTA (9), 10^{-5} M c-AMP plus EGTA (10), 10^{-5} M c-AMP no EGTA (11), 1 mM ZnCl₂ plus EGTA (12), 2.5 mM HgCl plus EGTA (13), and 0.3 mM MeHg plus EGTA (14).

A

B

phorylation of intermediate SR vesicles (sucrose diluted) is very similar to the hSR sucrose vesicles with slightly less E-2 phosphorylation. The triton insoluble material of the phosphorylated intermediate SR vesicles is enriched in the glycolytic enzymes, bands E-1 and E-2. Band E-3 is solubilized by the triton treatment and a new band of 28,000 daltons appears in the triton insoluble material.

In Figure 3 (b) we can see that 25 mM EDTA abolishes all the phosphorylation except that of band E-2. KDOC (1%) and 2.5 mM HgCl₂ completely inhibit the phosphorylation of all proteins. KCl (200 mM) increases the phosphorylation of band E-1. When the stained gel is compared to the autoradiogram band E-1 appears at the same position as calsequestrin on the stained gel .

The effect of 10^{-6} M to 10^{-5} M c-AMP in the presence of 1 mM EGTA can be seen in Figure 3 (b). The phosphorylation of phosphorylase kinase (128,000 and 145,000 daltons), phosphorylase a (94,000 daltons) and glycogen synthetase (68,000 daltons) is stimulated by c-AMP in the presence of EGTA but not in its absence. The phosphorylation of band E-1 is not affected by c-AMP but the band right below E-1 is highly phosphorylated in the presence of c-AMP (possible protein kinase). The phosphorylation of band E-2 and E-3 are not affected by c-AMP but a band at 34,000 daltons is increased by c-AMP and a band around 28,000 daltons is stimulated in the presence of c-AMP.

Figure 4.

Bar graph of the ^{32}P incorporated in 10 minutes into the band E-1 (64,000 daltons) for HSR-sucrose vesicles in presence of 5 mM MgCl_2 , 10 mM HEPES (pH 7.4) 10 μM ^{32}P -ATP and the added reagent (indicated).

Treatment of the SDS solubilized samples with 0.8 M hydroxylamine at 30 C for 30 minutes (Lipmann and Tuttle, 1944) caused no decrease in the level of phosphorylation. This result was expected since the method of determining ^{32}P incorporation using slab gel electrophoresis followed by staining and destaining in 10% acetic acid, should hydrolyze acyl phosphate bonds and should leave ester phosphate bonds intact.

Figure 4 shows the quantitative results of the autoradiogram in figure 3 (b) for band E-1. NaF (25 mM) and KCl (200 mM) in the presence of EGTA increase the ^{32}P incorporated in to band E-1 by 50% and 150%, respectively. Na dantrolene as in the absence of EGTA causes an inhibition of the phosphorylation of E-1 by over 50%. Cyclic-AMP (10^{-6} , 10^{-5} M) leaves the phosphorylation unchanged.

Figure 5 shows the quantitative results of phosphorylation of E-1 in the presence 150 mM KCl, 5 mM MgCl_2 , 1 mM EGTA, 10 mM HEPES and 10 μM ^{32}P -ATP. Alkaline phosphatase, which can be seen on the slab gel and in the autoradiogram has no affect on the phosphorylation of band E-1 although it does reduce the ^{32}P incorporated into bands E-2 and phosphorylase kinase and phosphorylase a. NaF (25 mM) increases the level of phosphorylation of band E-1 along with band E-2 in the presence of KCl and EGTA. Caffeine (5 mM) increases slightly the phosphorylation of bands E-1 and E-2. Triton X-100 (0.2%) stimulates the phosphorylation of E-1 and E-2. The addition of ATP to the

Figure 5.

Bar graph of the ^{32}P incorporated in 20 minutes into the band E-1 (64,000 daltons) for HSR-sucrose vesicles in presence of 150 mM KCl, 5 mM MgCl_2 , 10 mM HEPES (pH 7.4) and 20 μM ^{32}P -ATP. Control (1), alkaline phosphatase (2), NaF 25 mM (3), caffeine 5 mM (4), and 0.2% Triton X-100 (5).

solubilizing buffer causes no change in the level of phosphorylation of HSR vesicles. Finally, in the presence of KCl (200 mM) and 25 mM EDTA only band E-2 is phosphorylated.

Figure 6 shows the ^{32}P incorporated into band E-1 versus the time of incubation in 150 mM KCl, 5 mM MgCl_2 , 10 mM HEPES (pH 7.4) and 10 μM ^{32}P ATP. The initial fast phosphorylation of band E-1 is followed by a slower dephosphorylation with a half time of approximately 15 minutes.

Figure 7 shows the ^{32}P incorporated versus time in the presence of NaF (25 mM). The phosphorylation of band E-1 is maximum after 2 minutes for HSR-KCl vesicles and after 5 minutes for HSR-sucrose vesicles. The maximum level of phosphorylation of band E-1 in the HSR-KCl vesicles is double that of the HSR-sucrose vesicles. The dephosphorylation in the presence of NaF is very slow.

Figure 8 shows the effect of free calcium on the phosphorylation of band E-1 from HSR sucrose vesicles. At a free calcium concentration of 10^{-6} M the phosphorylation of band E-1 is inhibited by over 50%. It should be noted that when the total calcium concentration is above 5 mM calsequestrin and band E-1 ran at a lower molecular weight (56,000 daltons).

Figure 6.

^{32}P incorporated into band E-1 versus time of incubation (HSR-sucrose vesicles) in 150 mM KCl, 5 mM MgCl_2 , 10 mM HEPES (pH 7.4), 1 mM EGTA and 20 μM ^{32}P -ATP.

Figure 7.

^{32}P incorporated into band E-1 versus time of incubation for HSR-KCl and HSR-sucrose vesicles in the 150 mM KCl, 5 mM MgCl_2 , 10 mM HEPES (pH 7.4), 25 mM NaF and 10 μM ^{32}P -ATP.

Figure 8.

^{32}P incorporated into band E-1 for 10 minutes versus the free calcium concentration in the presence of 150 mM KCl, 5 mM MgCl_2 10 mM HEPES (pH 7.4), 1 mM EGTA and 20 μM ^{32}P -ATP plus calcium to the desired concentration.

Figure 8.

^{32}P incorporated into band E-1 for 10 minutes versus the free calcium concentration in the presence of 150 mM KCl, 5 mM MgCl_2 10 mM HEPES (pH 7.4), 1 mM EGTA and 20 μM ^{32}P -ATP plus calcium to the desired concentration.

Table II summarizes the conditions required for maximum phosphorylation of band E-1 and lists some stimulants and inhibitors of E-1 phosphorylation.

Figure 9 shows the results of an optical density scan of the Comassie blue stained gel and the corresponding autoradiogram. Band E-1 has the same mobility as the protein calsequestrin. Bands E-2 and E-3 do not correspond to bands on the gel scan.

The results of deoxycholate (DOC) treatment of HSR vesicles reveals that band E-1 can be removed by DOC in the presence of 1 M KCl, while bands E-2 and E-3 remain in the membrane. Therefore band E-1 is an extrinsic membrane protein and bands E-2 and E-3 are intrinsic membrane proteins. Triton treatment in the absence of salt leaves an insoluble complex which contains bands E-1 and E-2 while E-3 is solubilized. Triton treatment (3:2, Triton:protein) in the presence of 10% glycerol, 50 mM tris (pH 8.5), 5 mM beta-mercaptoethanol (Ikemoto, 1972) reduces the amount of calsequestrin and reduces the level of band E-1 on the autoradiogram.

TABLE II

CONDITIONS FOR PROTEIN E-1 PHOSPHORYLATION

<u>Requirements</u>	<u>Stimulatory</u>
MgATP (10 μ M)	NaF (25 mM)
K ⁺ , Na ⁺ (100 mM)	Triton X-100 (0.2%)
10^{-8} to 10^{-7} Ca ⁺⁺	Caffeine (5 mM)
<u>Inhibitory</u>	<u>No Effect</u>
HgCl (2.5 mM)	Alkaline Phosphatase
Na Dantrolene (10^{-5} M)	c-AMP (10^{-6} to 10^{-5} M)
EDTA (25 mM)	hydroxylamine (0.8M)
KDCC (1%)	Me Hg
Ca ⁺⁺ (greater than 10^{-6} M)	

Figure 9.

Optical density scans of the Comassie blue stained slab gel and of the autoradiogram. Band E-1 has the same mobility as calsequestrin, while bands E-2 and E-3 do not have corresponding bands on the gel scan.

Calsequestrin Isolation

In an attempt to conclusively identify band E-1 as calsequestrin we have tried to purify calsequestrin from phosphorylated SR vesicles using three different methods. First, we isolated calsequestrin from Weber and Osborn stick gels (44,000 daltons) and then ran the calsequestrin on a Laemmli slab gel. The results are seen on Figure 10 (a) which shows a single band for calsequestrin. The autoradiograph of the gel reveals a faint but visible band corresponding to calsequestrin.

Second, we have isolated calsequestrin from phosphorylated HSR vesicles after Triton treatment using calcium precipitation method of Ikemoto (1972). The calsequestrin is at least 80% pure. The autoradiogram of the gel reveals a faint band corresponding to calsequestrin.

Finally, we have tried to purify calsequestrin using the method of MacLennan (1972) from phosphorylated vesicles but we have had no success in obtaining phosphorylated calsequestrin, probably due to dephosphorylation of calsequestrin during the long isolation.

Effect of Depolarization on Phosphorylation

Figure 11 shows the phosphorylation of HSR sucrose vesicles in the presence of 150 mM K gluconate and 5 mM MgSO_4 versus time. If the phosphorylation is instead carried out in the presence of cold ATP and the vesicles are diluted with K gluconate buffer with ^{32}P -ATP after 2 minutes of phosphorylation one obtains little phosphorylation of the HSR vesicles. But if the vesicles are diluted with tris chloride buffer plus ^{32}P -ATP then the level of phosphorylation of band E-1 is stimulated. The ratio of ^{32}P incorporated into band E-1 when the vesicles are depolarized to the control ^{32}P incorporation is approximately four. This seems to be due to a phosphorylation instead of a dephosphorylation since if the vesicles are phosphorylated with ^{32}P first and then diluted into tris chloride buffer with cold ATP, one finds that the band E-1 is the same as the K gluconate dilution.

Figure 12 shows the results of ^{32}P phosphorylation of band E-1 of HSR sucrose vesicles which were initiated with ^{32}P ATP and at the same time diluted into K gluconate buffer or tris chloride buffer. In the case of the tris chloride dilution the level of ^{32}P

Figure 10.

- (a) gel electrophoresis purified calsequestrin (arrow)
- (b) autoradiogram of (a), ^{32}P -calsequestrin indicated by an arrow
- (c) calcium precipitated calsequestrin (arrow)
- (d) autoradiogram of (c), ^{32}P -calsequestrin indicated by an arrow

Figure 11.

^{32}P incorporated into HSR sucrose vesicles in the presence of 150 mM K gluconate, 5 mM MgSO_4 , 10 mM HEPES (pH 7.4) and 10 μM ^{32}P ATP. 0.5, 1.0, 2.0, 3.0 minutes incubation (1-4)

Phosphorylation with cold ATP, 0-2 minutes, followed by dilution into K gluconate with ^{32}P -ATP (5)

Phosphorylation with cold ATP, 0-2 minutes, followed by dilution into Tris Cl with ^{32}P -ATP (6)

Phosphorylation with ^{32}P -ATP, 0-2 minutes, followed by dilution into K gluconate with cold ATP (7)

Phosphorylation with ^{32}P -ATP, 0-2 minutes, followed by dilution into Tris Cl with cold ATP (8)

All reactions were terminated 30 seconds after the dilution with SDS.

Figure 12.

^{32}P incorporated into HSR sucrose vesicles in the presence of 150 mM K gluconate, 5 mM MgSO_4 , 10 mM HEPES (pH 7.4) and 10 μM ^{32}P ATP. Addition of ^{32}P ATP is made at the same time as the vesicles are being diluted into K gluconate buffer or Tris Cl buffer.

band E-1 is greater initially and after 120 seconds.

DISCUSSION

The evidence described in the present chapter indicates that sarcoplasmic reticulum vesicles, in particular heavy SR vesicles, have endogenous protein kinase activity and associated substrate proteins. Previous studies of isolated SR preparations have been unsuccessful in measuring phosphorylation of skeletal muscle sarcoplasmic reticulum vesicles (Kirchberger and Tada, 1976). In this study we were successful probably due to the use of heavy SR vesicles, which have a high level of phosphorylation, and because the technique of autoradiography is much more sensitive than counting slices of gels to determine ^{32}P incorporation. All our studies have been done without adding soluble protein kinase because soluble protein kinase will have access to substrates that a particulate or membrane bound kinase might not, and phosphorylations that do not occur in vivo possibly could result. Such a problem is reduced, however, if only endogenous phosphorylation is measured.

In previous work we have shown that the preparation of heavy SR vesicles is derived from the terminal cisternae of the sarcoplasmic reticulum and it contains the junctional sarcoplasmic reticulum membrane. The HSR-sucrose vesicles have been shown to have intact

'SR feet' on the surface of their membrane, whereas the 'SR feet' are absent in the HSR-KCl vesicles. The main biochemical difference between the HSR-KCl and HSR-sucrose vesicles is the content of endogenous calcium and magnesium. The sucrose diluted vesicles have 38 and 114 nmoles of Mg^{++} and Ca^{++} per mg of protein, respectively. In the HSR-KCl the Mg^{++} and Ca^{++} have been reduced to 8 and 15 nmoles per mg of protein, respectively. The HSR-sucrose vesicles also have a larger amount of membrane associated proteins which are absent in the HSR-KCl vesicles. Therefore, in the phosphorylation studies the HSR-KCl and HSR-sucrose vesicles could differ in their level of phosphorylation due to the amount of endogenous calcium and magnesium or to the effect that the membrane associated proteins have on the phosphorylation.

The phosphorylated proteins in the SR vesicles can be broken up into three classes. The first class are the proteins which are involved in glycolysis. They have been identified as glycogen debranching enzyme (160,000 daltons), phosphorylase kinase (128,000 and 145,000 daltons), phosphorylase a (94,000 daltons) and glycogen synthetase (88,000 daltons) (Michalak et al. ,1977, and Horl et al. 1978). They are predominantly located in the heavy SR vesicles except phosphorylation of phosphorylase a is also seen in the light SR vesicles. The addition of c-AMP to the reaction mixture in the presence of 1 mM EGTA causes the increase in the ^{32}P incorporation into phosphorylase kinase, phosphorylase a and glycogen synthetase.

Finally, washing the HSR-sucrose vesicles with KCl lowers the amount of phosphorylation seen in the above proteins. KCl washing has been previously used to remove the glycolytic enzymes from SR vesicles (MacLennan, 1970). Recently, Horl et al. (1978) have found that phosphorylase a and phosphorylase kinase are involved in the regulation of the Ca^{++} transport ATPase of the sarcoplasmic reticulum using an endogenous protein kinase and protein phosphatase system.

The second class of phosphorylated proteins are those whose phosphorylation is c-AMP dependent or stimulated by c-AMP. The phosphorylation of the glycolytic enzymes is stimulated by the presence of 10^{-6} to 10^{-5} M c-AMP. The only other proteins affected by c-AMP is a protein of molecular weight 28,000 daltons, which is seen only in the presence of c-AMP and 1 mM EGTA, and a protein of 60,000 daltons whose phosphorylation is stimulated in the presence of c-AMP (10^{-5} M) and 1 mM EGTA. The 28,000 dalton protein is also seen in the Triton X-100 treated HSR vesicles. The 60,000 dalton protein is probably protein kinase.

Whether these proteins are involved in controlling calcium uptake like the protein phospholamban in cardiac SR vesicles remains to be seen. Their presence in skeletal muscle SR does lend support to the work of Fabiato and Fabiato (1978), who showed c-AMP response by the SR in skinned skeletal muscle fibers.

The third class of phosphorylated proteins are those which are c-AMP independent and not involved in glycolysis. This class consists of three proteins, E-1, E-2 and E-3, whose molecular weights are 64,000 daltons , 42,000 daltons and 20,000 daltons. Their phosphorylation is extremely dependent on the presence of 1 mM EGTA in the reaction mixture.

The phosphorylation of band E-3, 20,000 daltons, has been found in both the light and heavy SR vesicles. Its phosphorylation is extremely fast and within one minute it reaches its maximum level of phosphorylation. The phosphorylation of E-3 in HSR-sucrose vesicles lags behind the phosphorylation of E-3 in HSR-KCl vesicles but after 10 minutes the levels are equal. The phosphorylated band E-3 is present in DOC treated HSR vesicles and is absent in the Triton treated SR vesicles.

The phosphorylation of band E-2, 42,000 daltons, is only found in the heavy SR vesicles. It has the unique property of being phosphorylated in the presence of 25 mM EDTA and in the absence of EGTA there is very little phosphorylation of this band. The level of phosphorylation of this band in the HSR vesicles is much higher than in the HSR sucrose vesicles. It appears in both the DOC and Triton treated HSR vesicles, which indicates it is an intrinsic membrane protein. Treatment of phosphorylated HSR vesicles with alkaline phosphatase completely removes the phosphorylation of band E-2. The

data indicate that this protein is an intrinsic protein with the following properties (1) an external phosphorylation site and (2) its phosphorylation is extremely inhibited by external or internal calcium.

The phosphorylated band E-1, 64,000 daltons, is predominantly found in the HSR vesicles. It is the only phosphorylated protein which corresponds to a major protein constituent of the HSR vesicles. The rate of phosphorylation of E-1 is faster in HSR-KCl vesicles and after 30 minutes the ^{32}P incorporation into HSR KCl vesicles is approximately double that of the HSR sucrose vesicles. DOC treatment indicates that it is an extrinsic protein while Triton treatment in the absence of salt indicates that it is probably part of the electron dense content within the SR vesicles. Alkaline phosphatase has been shown not to affect the phosphorylation of band E-1. Naloxone in the presence or absence of EGTA causes an inhibition of E-1 phosphorylation. The indications we have that E-1 is calsequestrin are the following (1) both bands have the same mobility on a Laemmli slab gel (2) E-1 and calsequestrin are extrinsic membrane proteins (3) E-1 seems to be part of the electron dense content of the HSR vesicles, as is calsequestrin and (4) the protein calsequestrin and the Band E-1 are precipitated by high calcium after the membranes have been solubilized by Triton.

Our attempts on the isolation of phosphorylated calsequestrin have been partially successful but the level of ^{32}P incorporated in the isolated calsequestrin is very low. We believe this is due to dephosphorylation during the isolation procedure. In a long isolation procedure such as MacLennan and Wong (1971), we were able to find no ^{32}P remaining on the purified calsequestrin. When calsequestrin was isolated using Weber and Osborn (1972) gel electrophoresis a small amount of phosphorylation was found in the purified calsequestrin. Finally, when calsequestrin was isolated from HSR vesicles using the calcium precipitation method a small amount of ^{32}P remained with the calsequestrin. Although we have not completely proven that the band E-1 is calsequestrin we do believe that all the evidence points to that fact.

It should also be noted that the band E-1 sometimes appears as a doublet. Calsequestrin also appears as a doublet and this has been described by MacLennan (1974) when he showed that the lower molecular weight band is a mutant form of calsequestrin.

Preliminary experiments in studying the effects of depolarization on the phosphorylation of HSR vesicles has given us some promising results. The band E-1 appears to be phosphorylated when the HSR vesicles, which have been previously phosphorylated with cold ATP in K gluconate buffer, and are diluted into tris chloride buffer with ^{32}P -ATP. This additional phosphorylation is due to a

phosphorylation and not to a dephosphorylation since when the same experiments are carried out using ^{32}P -ATP first and cold ATP in the dilution buffer, one still sees an increase in phosphorylation by depolarization. This additional phosphorylation occurs within the first 15 seconds after the addition of ^{32}P -ATP and the depolarization, and it is still present after 120 seconds.

The findings presented here have shown the existence of an endogenous membrane protein kinase and substrate proteins in heavy sarcoplasmic reticulum vesicles. Whether the phosphorylation and dephosphorylation of these proteins is involved in the mechanism of excitation-contraction coupling is an interesting possibility. The location of the phosphorylation in the heavy SR vesicles which are derived from the terminal cisternae lends support to this possibility. Our preliminary data on the effect of Na dantrolene, an inhibitor of calcium release, and the effect of depolarization, a possible initiator of calcium release, leads us to believe that phosphorylation may be involved in the mechanism of calcium release or in its control. Combined studies on calcium release from SR vesicles and phosphorylation of specific proteins may lead to identification of the proteins involved in calcium release, which could be small in quantity and not identified by other means.

BIBLIOGRAPHY

- Adrian, R.H., Chandler, W.K., and Rakowski, R.F. Charge movement and mechanical repriming in skeletal muscle. *J. Physiol.*, London 254: 361-388 (1976)
- Almers, W., And Best, P.M. (1976) Effects of Tetracaine on contraction and gating current in frog skeletal muscle. *Biophys. J.* 16: 152a
- Andrew and Appel (1973) Macromolecular Characterization of Muscle membranes. *J. Biol. Chem.* 248:5156-5163
- Andrew C.G., Almon and Appel. (1974) Macromolecular Characterization of Muscle Membranes. *J. Biol. Chem.* 250:3972-3980
- Armstrong, C.M., Bezanilla, F.M., Horowicz, P. (1972). Twitches in the presence of ethylene glycol bis (B-aminoethylether) -N,N'-tetraacetic acid. *Biochim. Biophys. Acta* 267:605-8.
- Ashley, C.C., Ridgaway, E.B. (1970). On the relationships between membrane potential, calcium transient and tension in single barnacle muscle fibers. *J. Physiol.* 209: 105-130.
- Baker, P.F. (1972). Transport and metabolism of calcium ions in nerve. *Prog. Biophysics. Mol. Biol.* 24: 177:222.
- Baylor, S.M. and Oetliker, H. (1975). Birefringence experiments on isolated skeletal muscle fibres suggest a possible signal from the sarcoplasmic reticulum. *Nature* 253: 97-101.
- Bendall, J.R. (1953) Further observations on a factor (the Marsh factor) effecting relaxation of ATP-shortened muscle-fibre models, and the effect of Ca and Mg ions upon it. *J. Physiol.*, London 121: 232-254
- Bennett and Porter (1953). An electron microscope study of sectioned breast muscle of the domestic fowl. *Am J. Anat.* 93:61-105
- Bezanilla, F., and Horowicz, P. (1974). Fluorescence changes in frog muscle stained with Nile blue associated with excitation-contraction coupling. *Fed. Proc.* 33 : 1259.

- Bezanilla, F and Horowicz, P. (1975). Fluorescence intensity changes associated with contractile activation in frog muscle stained with Nile Blue A. *J Physiol.* 246: 709-735
- Bianchi, C.P. and Bolton, T.C.. (1967). Action of local anesthetics on coupling systems in muscle. *J. Pharmacol. Exptl. Ther.* 157: 300-388
- Bianchi, C.P. (1968) Pharmacological actions on excitation - contraction coupling in striated muscle. *Federation Proc.* 27: 126-131
- Boland, de A.R., Jilka, Martonosi (1975) Passive permeability of phospholipid vesicles and sarcoplasmic reticulum membranes. *J. Biol. Chem.* 250:7501-7510
- Boldyren, A.A., Tkachuk, V. A. and Titanji, P. V. K. (1974). Activation energy of skeletal muscle sarcolemma Na^+ , K^+ -adenosine triphosphatase. *Biochem. Biophys. Acta.* 357: 319-324
- Bozler, E. (1954) Relaxation in extracted muscle fibers. *J. Gen. Physiology* 38:149-159
- Bozler, E. (1954) Relaxation of extracted muscle fibers. *J. General Physiology* 38:149-159
- Bray, D.F., Rayns, D.G., Wagenaar, E.B. (1978). Intramembrane particle densities in Freeze-Fracture sarcoplasmic reticulum. *Can. J. Zool.* 56:1. 140-145
- Campbell, K.P. and A.E. Shamoo (1977) Depolarization induced calcium release from light and heavy sarcoplasmic reticulum vesicles. *Biophysical J.* 17:200a
- Campbell, K.P. and A.E. Shamoo (1977) Identification of two proteins uniquely associated with the terminal cisternae of the sarcoplasmic reticulum. *Proc. Internal. Sympos. on 'Calcium Binding proteins and calcium function in health and disease'* ed. Wasserman, North-Holland, 185-187
- Campbell, K.P., Franzini-Armstrong, C. and A.E. Shamoo Biochemical and Morphological characterization of light and heavy sarcoplasmic reticulum vesicles. (in preparation).
- Campbell, K.P. and A.E. Shamoo (1978) Intrinsic Phosphorylation of heavy sarcoplasmic reticulum vesicles. *Biophysical J.* 1978 abstract

- Caputo, C. (1976) The effect of caffeine and tetracaine on the time course of potassium contractures of single muscle fibres. *J. Physiol.*, London 255: 191-207
- Carvalho, A.P. (1972) Binding and release of cations by sarcoplasmic reticulum before and after removal of lipid. *European J. Biochem.* 27: 491-502
- Carvalho, A.P. (1967) Effects of ATP on the interaction of Ca^{++} , Mg^{++} and K^+ with fragmented sarcoplasmic reticulum isolated from rabbit skeletal muscle. *J. Gen. Physiol.* 50: 1327-1352
- Casswell, A.H., Y.H. Lau and J.P. Brunschwig (1976). Ouabain Binding Vesicles from skeletal muscle. *Archives of Biochem and Biophys.* 176:417-430
- Caswell, A. H. , Baerwald, R.J. and Brunschwig, J.P. (1975). Abstract, Isolation of transverse tubule vesicles from rabbit skeletal muscle. *Biophys. J.* 15: 254a.
- Caudwell, B., J. Antoniow and P. Cohen (1978). Calsequestrin, Myosin and the components of the protein-glycogen complex in rabbit skeletal muscle. *Eur. J. Biochem.* 86:511-518
- Chandler, W.K., Rakowski, R.F., and Schneider, M.F. (1976) A non-linear voltage dependent charge movement in frog skeletal muscle. *J. Physiol.*, London 254: 245-283
- Chandler, W.K., Rakowski, R.F., and Schneider, M.F. (1976) Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol.*, London 254: 285-316
- Chen P. S. Toribara, T.Y. and Warner, H. (1956). Microdetermination of phosphorus. *Analy. Chem.* 28: 1756-1758
- Cohen, L.B., Salzberg, B., Davila, H.V., Ross, W.N., Landowne, D., Waggoner, A.S., and Wang, C.H. (1974) Changes in axon fluorescence during activity: molecular probes of membrane potential. *J. Memb. Biol.* 19:1-36
- Cohen, L. B., Hille, B. and Keynes, L.D., (1970). Changes in axon birefringence during the action potential. *J. Physiol. Lond.* 211:495-515
- Constantin, L.L., Podolsky, R.J. (1967). Depolarization of the internal membrane system in the activation of frog skeletal muscle. *J. Gen. Physiol.* 50:11

- Constantin, L.L., Franzini-Armstrong, C. and Podolsky, R.J. (1965). Localization of calcium-accumulating structures in striated muscle fibres. *Science* 147: 158-159.
- Constantin, L.L., and Podolsky, R.J. (1966) Evidence for depolarization of the internal membrane system in activation of frog semitendinosus muscle. *Nature* 210: 483-486
- Constantin, L.L., and Taylor, S.R. (1973) Graded activation in frog muscle fibres. *J. Gen. Physiol.* 61: 424-443
- Crowe, L.M., and Baskin, R.J. (1978). Freeze-fracture of intact Sarcotubular membranes. *J. Ultra. Res.* 62:147-154
- Desmedt, J.E. and K. Hainaut (1978) Excitation-contraction coupling in single muscle fibers and the calcium channel in the SR. *Annals of NYAS, Vol 70, Calcium Transport and Cell Function*, pg 433-435
- Desmedt, J.E. and K. Hainaut (1977) Inhibition of the intracellular release of calcium by Dantrolene in barnacle giant muscle fibres. *J. Physiol.* 265:565-585
- Duggan, P.F. and Martonosi, A. (1970). Sarcoplasmic reticulum.-IX. The permeability of sarcoplasmic reticulum membranes. *J. Gen. Physiol.* 56:147 light and heavy sarcoplasmic reticulum vesicles. (in preparation).
- Eastwood, A.B., Grundfest, H., Brandt, P.W., and Reuben, J.P. (1975) Sites of action of D20 in intact and skinned crayfish muscle fibres. *J. Membrane Biol.* 24: 249-263
- Ebashi, S. (1976) Excitation-contraction coupling. *Ann. Rev. Physiol.* 38: 293-313
- Ebashi, S., and Endo, M. (1968) Ca ion and muscle contraction. *Progr. Biophys. Mol. Biol.* 18: 123-183
- Ebashi, S., and Lipmann, F. (1962) Adenosine triphosphate-linked concentration of calcium ions in a particulate fraction of rabbit muscle. *J. Cell Biol.* 14: 389-400
- Ebashi, S. (1958) A granule bound relaxation factor in skeletal muscle. *Arch. Biochem. Biophys.* 76:410-423
- Ebashi, S. and Endo, M. (1968). Calcium ions and muscle contraction. *Progr. Biophys. Mol. Biol.* 18: 123-183.
- Ebashi, S. (1965). The sarcoplasmic reticulum and excitation-contraction coupling. In *Molecular Biology of Muscular Contraction*, Ebashi, S., Oosawa, F., Sekme, T., and Tonomura, Y.,

Igaku-shon, Tokyo.

- Ebashi, S. (1960). Calcium binding and relaxation in the actomyosin system. *J. Biochem.* 48: 150-151
- Ebashi, S. (1961). The relaxing factor and the contraction-relaxation cycle of skeletal muscle. *Tokyo J. Med. Sci.* 69: 65-78.
- Ebashi, S., Endo, M., Ohtsuki, I., (1969). Control of Muscle Contraction. *Quart. Rew. Biophys.* 2: 351-384.
- Ebashi, S. (1961). Calcium binding activity of vesicular relaxing factor. *J. Biochem. (Tokyo)* 50: 236-247.
- Eisenberg, B. R. and Kuda, A.M. (1975). Stereological analysis of Mammalian Skeletal Muscle. II. White vastus muscle of the adult guinea pig. *J. Ultrastructure Res.* 51: 176-187.
- Eisenberg, B., Kuda, A. and Peter, J. (1974). Stereological Analysis of Mammalian Skeletal Muscle. I Soleus muscle of the adult guinea pig. *J. Cell Biol.* 60: 732-754.
- Eisenberg, B.R. and A.M. Kuda (1975). Stereological Analysis of mammalian skeletal muscle II White Vastus muscle of the adult guinea pig. *J. Ultrastructure Res.* 51:176-187
- Eisenberg, B.R., A.M. Kuda and J.P. Peter (1974) Stereological Analysis of mammalian skeletal muscle I. Soleus Muscle of the adult guinea pig. *J. Cell Biol.* 60:732-754
- Ellis, K.O., and Carpenter, F. (1972) Studies on the mechanism of action of dantrolene sodium. A skeletal muscle relaxant. *Arch. Exptl. Pathol. Pharmacol.* 275: 83-94
- Ellis, K.O., Butterfield, F.L. Wessels and Carpenter. (1976) A comparsion of skeletal, cardiac and smooth muscle actions of Dantrolene sodium- a skeletal muscle relaxant. *Arch Pharmacod. and Therapie* 224:118-132
- Endo, M. and Blinks, J.R. (1973). Inconstant association of aequorin luminescence with tension during calcium release in skinned muscle fibres. *Nature New Biol.* 246: 218-220.
- Endo, M., Tanaka, M., Ogawa, Y. (1970). Calcium induced release of calcium from the sarcoplasmic reticulum of skinned skeletal muscle fibres. *Nature* 228: 34-36.

- Endo, M. (1975). 101. Mechanisms of the action of caffeine on the sarcoplasmic reticulum of skeletal muscle. Proc. Japan Acad. 51: 479-484.
- Endo, M. (1975). 99. Conditions required for calcium-induced release of calcium from the sarcoplasmic reticulum. Proc. Japan Acad. 51: 467-472.
- Endo, M. (1977). Calcium release from the sarcoplasmic reticulum. Physiol. Rev. 57: 1. 71-108.
- Endo, M. (1972) Stretch-induced increase in activation of skinned muscle fibres by calcium. Nature New Biol. 237: 211-213
- Endo, M. (1975) Conditions required for calcium-induced release of calcium from the sarcoplasmic reticulum. Proc. Japan Acad. 51: 467-472
- Endo, M. (1975) Mechanism of action of caffeine on the sarcoplasmic reticulum of skeletal muscle. Proc. Japan Acad. 51: 479-484
- Endo, M. and Kitazawa, T. The effect of ATP on calcium release mechanisms in the sarcoplasmic reticulum of skinned muscle fibres. Proc. Japan Acad. In press.
- Endo, M. and Nakajima, Y. (1973) Release of calcium induced bydepolarisatio of the sarcoplasmic reticulum membrane. Nature New Biol. 246: 216-218
- Endo, M., Takagi, A., and Ebashi, S. (1975) Calcium released by the halothane from the sarcoplasmic reticulum of skeletal muscle. Abstr. Intern. Congr. Pharmacol. 6th. p.565
- Endo, M. and T. Kitazawa (1978) The effect of ATP on the calcium release mechanisms in the sarcoplasmic reticulum of skinned skeletal muscle. Proc Japan Acad. (in press).
- Entman, M.L., Bornet, Van Winkle, Goldstein and Schwartz (1977). Association of Glycogenolysis with cardiac SR II. Effect of glycogen depletion, deoxycholate solubilization and cardiac ischemia: Evidence for a phosphorylase kinase membrane complex. J. Molecular and Cellular Cardiology 9:515-528
- Fabiato, A., and Fabiato, F. (1977). Calcium release from the sarcoplasmic reticulum. Circ. Res. 40: 2. 119-129.
- Fabiato, A., and Fabiato, F. (1978). Cyclic AMP-induced enhancement of calcium accumulation by the sarcoplasmic reticulum with no modification of the sensitivity of the myofilaments to calcium in skinned fibres from a fast skeletal muscle. Biochim. Biophys.

Acta. 539: 2. 253-260.

- Fabiato, A. and F. Fabiato (1977). Variations of the membrane potential of the sarcoplasmic reticulum of skinned cells from cardiac and skeletal muscle detected with a potential-sensitive dye. *J. General Physiology* 70:6a
- Fabiato, A. and F. Fabiato (1978). Cyclic AMP induced enhancement of calcium accumulation by SR with no modification of the sensitivity of the myofiliaments to calcium in skinned fibers from fast skeletal muscle. *B.B.A.* 539:253-260
- Fleischer, S and Fleischer B. (1967) In *Methods of Enzymology* Estabrook, R.W. and Pullman, M.E. (eds), Vol. 10 406-433, Acad. Press, N.Y.
- Ford, L. E., Podolsky, R.J. (1970). Regenerative calcium release within muscle cells. *Science* 167: 58-59.
- Ford, L.E., Podolsky, R.J. (1972). Calcium uptake and force development by skinned muscle fibers in EGTA buffered solutions. *J. Physiol.* 223:1-19
- Ford, L.E., and Podolsky, R.J. (1970) Regenerative calcium release within muscle cells. *Science* 167: 58-59
- Ford, L.E., and Podolsky, R.J. (1972) Calcium uptake and force development by skinned muscle fibres in EGTA buffered solutions. *J. Physiol., London* 223: 1-19
- Franzini-Armstrong, C. (1970). Studies of the triad. I. Structure of the junction in frog twitch fibres. *J. Cell. Biol.* 47: 488-499.
- Franzini-Armstrong, C. (1971). Studies of the triad. II. Penetration of tracers into the junctional gap. *J. Cell. Biol.* 49: 196-203.
- Franzini-Armstrong, C. (1972). Studies of the triad. III. Structure of the junction in fast twitch fibers. *Tissue Cell.* 4: 469-478.
- Franzini-Armstrong, C. (1973). Studies of the triad. IV. Structure of junction in frog slow fibers. *J. Cell. Biol.* 56: 120-128.
- Franzini-Armstrong, C. (1974). Freeze-fracture of skeletal muscles of the *Trantula* spider. Structural differentiations of sarcoplasmic reticulum and transverse tubular system membranes. *J. Cell. Biol.* 61:501-513

- Franzini-Armstrong, C. (1975). Membrane particles and transmission at the triad. *Fed. Proc.* 34: 1382-1389.
- Franzini-Armstrong, C. Membraneous Systems in Muscle Fibers. In *The Structure and Function of Muscle*, 2nd Edition, Jeoffrey Bourne(ed.), Chap. 9, Vol II, Part 2. 1975
- Fuchs, F. (1974). Striated Muscle. *A. Rev. Physiol.* 36: 461-502.
- Gesema, W., Lingny, C.L., Luten, J.B. and Vossenberg, F.G. (1975). The use of cerenkov effect in counting of B-and Y-emitting radionuclides. *Inter. J. Applied Radiation Isotopes* 26: 443-450.
- Gilbert, D.L., and Fenn, W.O. (1957) Calcium equilibrium in muscle. *J. Gen Physiol.* 40: 393-408
- Gilbert, D.L. and W.O. Fenn. (1957) Calcium equilibrium in muscle. *J. General Physiology* 40:393-408
- Goldin, S.M. and Tong s.w. (1974). Reconstitution of active transport catalyzed by the purified sodium and potassium ion-stimulated adenosine triphosphatase from canine renal medulla. *J. Biol. Chem.* 249: 5907-5915.
- Hasselbach, W. and Makinose, N. (1961). Die calcium pumpe der Erschlaffungsgrana des muskels und ihre Abhangigke it von der ATP-spaltung. *Biochem. Z.* 333: 518-528.
- Hasselbach, W. and Makinose, M. (1962). ATP and Active Transport. *Biochem. Biophys. Res. Commun.* 7: 132.
- Hasselbach, W., and Heimberg, K. (1970). The interaction of Ans with the membranes of the sarcoplasmic reticulum. *J. Memb. Biol.* 2: 341.
- Hasselbach, W. and Makinose, M. (1963). Ueber der mechanismus der calcium transportes durch die membranen des sarkoplasmatischen reticulums. *Biochem. Z.* 339: 94-111.
- Hasselbach, W. (1964). Relaxing factor and the relaxation of muscle. *Prog. Biophys. Mol. Biol.* 14: 167-222.
- Hasselbach, W. (1964) Relaxing factor and the relaxation of muscle. *Progr. Biophys. Mol. Biol.* 14: 167-222
- Hasselbach, W. (1978). The reversibilty of the SR Calcium Pump. *B.B.A.* 515:23-53

- Hasselbach and Weber (1953) Der Einfluss des MB-factors auf die Kontraktion des Fasermodells B.B.A. 11:160-161
- Heilbrunn, L.V. and Wiercinski, F.C. (1947). The action of various compounds on muscle proteoplasm. J. Cellular Comp. Physiol. 29:15-32
- Heldt, H. W., K. Werdan, M. Melancev, and G.Geller (1973). Alkalization of the chloroplast stroma caused by light-dependent proton flux into the thylakoid space. B.B.A. 314:224-241
- Heuson-Stiennon, J.A., Wanson, J.C., Drochmans, P. (1972). Isolation and characterization of the sarcoplasmic reticulum of skeletal muscle. J. Cell. Biol. 55: 471-488.
- Hidalgo, C., and Ikemoto, N. (1977). Disposition of proteins and aminophospho- lipids in the sarcoplasmic reticulum membrane. J. Biol. Chem. 252: 8446-8454.
- Hidalgo, C. and N. Ikemoto (1977). Disposition of proteins and aminophospholipids in the SR membrane. 252:8446-8454
- Hille, B. (1970) Ionic changes in nerve membranes. Progr. Biophys. Mol. Biol. 21: 3-32
- Hodgkin, A.L. and Horowicz, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibers. J. Physiol. 148: 127-160,
- Hodgkin, A.L., and Horowicz, P. (1960) The effect of nitrate and other anions on the mechanical response of single muscle fibres. J. Physiol., London 153: 404-412
- Hokin, L.E. Dahl, J.L., Derpre J.D., Dixon, J.F., Hackney, J.F. and Perdue, J.F. (1973). Studies on the characterization of the sodium potassium transport adenosine triphosphatase. J, Biol. Chem. 248:2593-2605
- Hoppel, C. and C. Cooper (1968). The Action of Digitonin on Rat Liver Mitochondria. Biochem J. 107:367-376
- Horl, W.H. and Heilmeyer L.M. Jr. (1978). Evidence for the participation of a Ca^{2+} dependent protein kinase and protein phosphatase in the regulation of the Ca^{2+} transport ATPase of the sarcoplasmic reticulum. 2.Effect of Phosphorylase kinase and phosphorylase phosphatase. Biochem. 17:766-
- Horl, W.H., Jennissen, H.P., Heilmeyer, L.M. Jr. (1978). Evidence for the participation of a Ca^{2+} -dependent protein kinase and a protein phosphatase in the regulation of the Ca^{2+} transport ATPase

- of the sarcoplasmic reticulum. 1. Effect of inhibitors of the Ca^{++} Dependent Protein Kinase. *Biochem* 17:759-766
- Howell, J.N. (1969) A lesion of the transverse tubules of skeletal muscle. *J. Physiol.* 201: 515-533.
- Howell, J.N. (1969) A lesion of the transverse tubules of skeletal muscle *J. Physiol., London* 201:515-533
- Huxley, A.F., Taylor, R.E. (1958). Local activation of striated muscle fibers. *J. Physiol.* 144: 426-441.
- Ikemoto, N., J. Cucchiaro and Garcia (1976) A new glycoprotein factor of the sarcoplasmic reticulum. *J. Cell Biol.* 70:290a
- Ikemoto, N., G.M. Bhatnagar, and J. Gergely (1971) Fractionation of Solubilized Sarcoplasmic Reticulum *B.B.A.* 44:1510-1517
- Ikemoto, N., Sreter, Gergely (1971) Structural features of the surface of the vesicles of FSR. *Archives of Biochem and Biophy.* 147:571-582
- Inesi, G. and Scarpa, A. (1972). Fast kinetics of adenosine triphosphate dependent Ca^{++} uptake by fragmented sarcoplasmic reticulum. *Biochem.* 11: 356-359.
- Inesi, G., and Malan, N. (1976) Mechanisms of calcium release in sarcoplasmic reticulum. *Life Sci.* 18: 773-779.
- Jain, M.K., White, F.P., Strickholm, A. and Coades, E.H. (1972). Studies concerning the possible reconstitution of an active cation pump across an artificial membrane. *J. Membrane Biol.* 8: 363.
- Jilka, R.L. and A. Martonosi (1975) Effect of Purified Mg^{++} + Ca^{++} ATPase of SR upon the passive Ca^{++} permeability and Ultrastructure of Phospholipid vesicles. *J. Biol. Chem.* 250:7511-7524
- Jobsis, F. F. and O'connor, M.J. (1966). Calcium release and reabsorption in the sartorius muscle of the toad. *Biochem. Biophys. Res. Commun.* 25:246-252
- Johnson, R. G., and Scarpa, A. (1976). Ion permeability of Isolated Chromaffin Grandules. *J. General Physiology* Vol. 68 , 601-631
- Julian, F.J. (1971) The effect of calcium on the force-velocity relation of briefly glycerinated frog muscle fibres. *J. Physiol., London* 218: 117-145

- Kasai, M. and Miyamoto. (1976). Depolarization-induced calcium release from sarcoplasmic reticulum fragments. II. Release of calcium incorporated with ATP. J. Biochem. (Tokyo) 79: 5. 1053-1066.
- Kasai, M., and Miyamoto, H. (1976). Depolarization-induced calcium release from sarcoplasmic reticulum fragments. I Release of calcium taken up upon using ATP. J. Biochem. (Tokyo) 79: 5. 1053-1066.
- Kasai, M., and Miyamoto, H. (1973) Depolarization induced calcium releas from sarcoplasmic reticulum membrane fragments by changing ionic enviornment Fed. European Biochem. Soc. Letters 34: 299-301.
- Kasai, M., Miyamoto, H. (1976) Depolarization-induced calcium release from sarcoplasmic reticulum fragments. I. Release of calcium taken up upon using ATP. J. Biochem. 79: 1053-1066.
- Kasai, M., and Miyamoto, H. (1976) Depolarization-induced calcium release from sarcoplasmic reticulum fragments. II. Release of calcium incorporated without ATP. J. Biochem. 79: 1067-1076.
- Katz, B. (1966) Nerve, Muscle, and Synapase. McGraw-Hill Book Co., New York.
- Katz, A. M., Repke, D. F., Upshaw, J.E. and Polascik, M.A. (1970). Characterization of dog cardiac microsomes. Biochim. Biophys. Acta 205:473-490
- Katzung, B.G., Reuter, H. and Porzig. H. (1973). Lanthanum inhibits Ca inward current but not Na-Ca exchange in cardiac muscle. Experientia 29: 1073-1075.
- Kirchberger, M.A. and M. Tada (1976) Effects of c-AMP protein kinase on SR isolated from Cardiac and Slow and Fast contracting skeletal muscles. J. Biol. Chem. 251:725-729
- Kirchberger, M.A., M. Tada, and A.M. Katz (1974). C-AMP Protein Kinase catalyzed phosphorylation reaction and its relationship to calcium transport in cardiac SR. J. Biol. Chem. 249:6166-6173
- Kitazawa, T., and Endo, M. Increase in passive calcium influx into the sarcoplasmic reticulum by Depolarizatio and caffine. Proc. Japan Acad. In press.
- Kitazawa, T. and M. Endo. (1978) Increase in passive calcium influx into the sarcoplasmic reticulum by 'depolarization' and caffeine. Proc Japan Acad (in press)

- Kohlhardt, M. Bauer, B., Krause, H. and Fleckenstein A. (1973). Selective inhibition of the transmembrane Ca conductivity of mammalian myocardial fibers by Ni and Mn ions. *Pfluegers Arch.* 338: 115-125.
- Kohlhardt, M., Bauer, M., Krause, M. and Fleckenstein, A. Differentiation of the transmembrane Na and Ca channels in mammalian cardiac fibers by use of specific inhibitors. (1972). *Pfluegers Arch.* 335: 309-322.
- Kometani, T. and M. Kasai (1978). Ionic Permeability of Sarcoplasmic Reticulum Vesicles measured by light scattering method. *J. Memb. Biol.* 41:295-308
- Krolenko, S.A. (1969). Change in the T-system of muscle fibers under the influence of the influx and efflux of glycerol. *Nature* 221: 966-968.
- Krueger, B.K., Forn, J. and Greengard (1977). Depolarization induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Bio. Chem.* 252:2764-2773
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
- Laggner, P. and Graham (1976). The effect of a proteolipid from the SR on the physical properties of artificial phospholipid membranes. *B.B.A.* 433:311-317
- Lau, Y.H., A.H.Caswell, and J. Brunschwig (1977). Isolation of Transverse Tubules by fractionation of Triad junctions of skeletal muscle. *J. Biol. Chem.* 252:5565-5574
- Lee, K., Ladinsky, H., Choi, S. and Kasura, Y. (1966). Studies on the in vitro interaction of electrical stimulation and Ca^{++} movement in sarcoplasmic reticulum. *J. Gen. Physiol.* 49: 691-715.
- Levitsky, D.O., Aliev, Kuzmin, Levchenko, and Chazov (1976) Isolation of Calcium Pump system and purification of the calcium ion dependent ATPase from heart muscle. *B.B. A.* 443:468-484
- Louis, C.F., Budnaffina, R. and Blinks, B. (1974). Effect of trypsin on the proteins of skeletal muscle sarcoplasmic reticulum. *Arch. Biochem. Biophys.* 161: 83-92.
- Louis, C.F. and Holroyd (1978) the effects of deoxcholate and trypsin on the cross-linking of rabbit skeletal muscle SR proteins. *B.B.A.* (in press)

- Louis, C.F., M. Saunders and J.A.Holroyd (1977) The cross-linking of rabbit skeletal muscle SR proteins B.B.A. 493:78-92
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275
- MacLennan, D., Seeman, P., Iles, G.H., Yip, C.C. (1971). Membrane formation by adenosine triphosphatase of sarcoplasmic reticulum. J. Biol. Chem. 246: 2702-2710.
- MacLennan, D. H., Ostwald, T.J. and Stewart, P.S. (1974) Structural components of sarcoplasmic reticulum membrane. Ann. N.Y. Acad. Sci 227: 527-536.
- MacLennan, D.H., Yip, C.C., Iles, G.M., and Seeman, P. (1972). Isolation of sarcoplasmic reticulum proteins. Cold Springs Harbor Symp. Quant. Biol. 37: 469-478.
- MacLennan, D.H., and Holland P.C. (1974). Calcium transport in sarcoplasmic reticulum. Ann. Rev. Biophys. Bioengrn. 4: 377-404.
- MacLennan, D.H., and Wong, P.T.S. (1971) Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. Proc Natl. Acad. Sci. U.S. 68: 1231-1235.
- MacLennan, D.H. (1974). Isolation of a second form of Calsequestrin. J. Biol. Chem. 249:980-984
- MacLennan, D.H. (1975). Resolution of the Calcium Transport System of Sarcoplasmic Reticulum. Cand. J. Biochem 53:251-261
- MacLennan, D.H. (1970) Purification and properties of an ATPase from sarcoplasmic reticulum. J.Biol. Chem 245:4508-4518
- Madeira, V.M. and Antunes-Madeira, M.C. (1973). Chemical Composition of sarcolemma isolated from rabbit skeletal muscle. Biochem. Biophys. Acta 298: 230-238.
- Maderia and Madeira (1973) Chemical Compostion of sarcolemma isolted from rabbit skeletal muscle. B.B.A. 298:230-238
- Makinose, M. (1971) Calcium efflux dependent formation of ATP from ADP and orthophosphate by the membranes of the sarcoplasmic vesicles. Fed. European Biochem. Soc. Letters 12: 269-270.
- Marsh, B. B. (1951) A factor modifying muscle fibre synaeresis. Nature 167: 1065-1066.

- Marsh, B.B. (1952) The effects of ATP on the fibre volume of a muscle homogenate B.B.A. 9:247-260
- Mardu, S. (1975). A method for measuring the Cerenkov radiation induced by ^{32}P in small sample volume. Anal. Biochem. 63: 1-4.
- Martonosi, A. (1972). Biochemical and clinical aspects of sarcoplasmic reticulum function. In Current Topics in Membranes and Transport, F. Bronner, A. Kleinzeller, (eds.), Vol. III, 83-197, New York Academic.
- Martonosi, A. (1971). The structure and function of sarcoplasmic reticulum membranes. In Biomembrane, L. Manson (ed.), Vol. I, 191-256, New York: Plenum.
- Martonosi, A. (1967). The role in phospholipid in the ATPase activity of skeletal muscle microsomes. Biochem. Biophys. Res. Commun. 29: 753.
- Martonosi, A. (1969). Sarcoplasmic Reticulum. VII. Properties of a phosphoprotein intermediate implicated in calcium transport. J. Biol. Chem. 244: 613.
- Meissner, G. (1975). Isolation and characterization of two types of sarcoplasmic reticulum vesicles. Biochim. Biophys. Acta 389: 51-68.
- Meissner, G. and Fleischer, S. (1971). Characterization of sarcoplasmic reticulum from skeletal muscle. Biochim. Biophys. Acta 241: 356-378.
- Meissner, G. and Fleischer, S. (1974). Dissociation and reconstitution of functional sarcoplasmic reticulum vesicles. J. Biol. Chem. 249: 302-309.
- Meissner, G., Conner, G.E. and Fleischer, S. (1973). Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{++} -pump and Ca^{++} -binding proteins. Biochim. Biophys. Acta 298: 246-269.
- Michell, R. H. and Hawthorne, J.N. (1965). The site of diphosphomositide synthesis in rat liver. Biochem. Biophys. Res. Commun. 21: 333-338.
- Mikamoto, N. and Kasai, N. (1973). Reexamination of electrical stimulation on sarcoplasmic reticulum fragments in vitro. J. Gen. Physiol. 62: 775.

- Morad, M. and Goldman, Y. Excitation-contraction coupling in heart muscle: membrane control of development of tension. (1973). *Progr. Biophys. Mol. Biol.* 27: 257-313.
- Mueller, P., Rudin, D.O., Tien, H.T., and Wescott, W.C. (1962). Reconstitution of cell membrane structure in vitro and its transformation into an Excitable system. *Nature* 194: 979.
- Martonosi, A. (1972) Biochemical and clinical aspects of sarcoplasmic reticulum function. *Current Topics Membranes Transport* 3: 83-197.
- McKinley D. and G. Meissner (1977). Sodium and Potassium Permeability of SR vesicles. *Febs Letters* 82:47-50
- Meissner, G., and McKinley, D. (1976). Permeability of Sarcoplasmic Reticulum Membrane. The effect of changed ionic environments on Ca^{2+} release. *J. Membr. Biol.* 30: 1. 79-98.
- Meissner, G, and McKinley, D. Permeability of sarcoplasmic reticulum membrane. The effect of changed ionic environments on Ca^{2+} release. *Biochim. Biophys. Acta*. In press.
- Michaylova, V. and Ilkova, P. (1971). Photometric determination of micro amounts of calcium. *Anal. Chim. Acta* 53: 194-198.
- Michalak, M., Sarzala, M. and Drabikowski, W. (1977). Sarcoplasmic reticulum vesicles and glycogen-protein particles in microsomal fraction of skeletal muscle. *Acta Biochem Polonica* 24:105-115
- Millman, S. and J. Azari (1977) ATP-induced rapid calcium release from fragmented SR. *B.B.R.C.* 78:60-66
- Miyamoto, H., and Kasai, M. (1973) Reexamination of electrical stimulation on sarcoplasmic reticulum fragments in vitro. *J. Gen. Physio.* 62: 773-786
- Morgan K.G. and S.H. Bryant (1977) The mechanism of action of Dantrolene Sodium. *J. Pharmacology and Experimental Therapeutics* vol 201 138-147
- Morton, D., Hoppel and C. Cooper (1968). The Action of Digitonin on Rat liver mitochondria. *Biochem J.* 107:377-380
- Muscatello, U.E. Cedergren and Azzone (1962) The mechanism of muscle fiber relaxation. ATP and the relaxing activity of the sarcotubular system. *B.B.A.* 63:55-74
- Muscatello, U.E. Cedergren, and Azzone (1961) The sarcotubular system of frog skeletal muscle. A morphological and biochemical study. *J. Biophys Biochem Cytol Suppl* 10:201-218

- Nakajima, Y., and Endo, M. (1973). Release of Calcium induced by depolarization of the sarcoplasmic reticulum membrane. *Nature New Biol.* 246:216-218.
- Natori, R. (1965). Effect of Na and Ca ions on the excitability of isolated myofibrils. In *Molecular Biology of Muscular Contraction* Ebashi, S et al. (eds), Igaku-Shoin, Tokyo, An Elsevier, Amsterdam.
- Natori, R. (1954). The property and contraction process of isolated myofibrils. *Jikeikai. Med. J.* 1: 119-126.
- O'Brien, R. and G. Brierley. (1965). Permeability characteristics of isolated beef heart mitochondria. *J. Biol. Chem.* 240:4527-4531
- Ogawa, Y. and Ebashi (1976). Ca-releasing action of beta, gamma methylene ATP on fragmented SR. *J. Biochem.* 80:1149-1157
- Ohnishi, T. and Ebashi, S. (1963). Spectrophotometrical measurement of instantaneous calcium binding of the relaxing factor of muscle. *J. Biochem.* 54: 506-511.
- Ostwald, T.J. and MacLennan, D.H. (1972). Isolation of a high affinity calcium binding protein from sarcoplasmic reticulum. *J. Biol. Chem.* 249: 974-979.
- Peachey, L.D. (1965). The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *J. Cell Biol.* 25: Pt.2, 209-231.
- Pinkett, M.O., and Perlman, R.L. (1974). Phosphorylation of muscle plasma membrane protein by a membrane bound protein kinase. *Biochim. Biophys. Acta.* 372: 379-387.
- Porter, K.R. (1961) The sarcoplasmic reticulum. Its recent history and present status. *J. Biophys. Biochem. Cytol. Suppl.* 10:219-226
- Porter, K.R. and Palade (1957) Studies on the endoplasmic reticulum III Its form and distribution in striated muscle cells. *J. Biochem. Biophys. Cytol. Suppl.* 3:269:299
- Pucell, A.G. and Martonosi, A. (1971). Sarcoplasmic Reticulum XIV Acetylphosphate and carbamylphosphate as energy sources for Ca^{++} transport. *J. Biol. Chem.* 246: 3389.
- Racker, E. (1971). Reconstitution of a calcium pump with phospholipids and a purified Ca^{++} adenosine triphosphatase from sarcoplasmic reticulum. *J. Biol. Chem.* 247: 8198-8200.

- Racker, E. and Ester, E. (1975). A coupling factor from SR required for the translocation of Ca^{++} ions in a reconstituted Ca^{++} -ATPase pump, J. Biol. Chem. 250: 7533-7534.
- Reijngoud, D. J., and J.M. Tager (1973). Measurement of intralysosomal pH. B.B.A. 297:174-178
- Reuter, H. (1973). Divalent cations as charge carriers in excitable membranes. Prog. Biophys. Mol. Biol. 26: 1-43.
- Rouser, G., Kritchevsky, G. and A. Yamamoto (1967) Column chromatographic and associated procedures for separation and determination of phosphatides and glycolipids. Lipid Chromatographic Analysis ed G. Marinetti Vol 1, 99-162
- Rudolph, S.A., and P. Greengard (1975). Regulation of protein phosphorylation and membrane permeability by beta adrenergic agents and c-AMP in the avian erythrocyte. J. Biol. Chem. 249:5684-5687
- Sadow, A. (1970). Skeletal Muscle. A Rev. 17: 87-118.
- Sadow, A. (1965) Excitation - contraction coupling in skeletal muscle. Pharmacol. Rev. 17: 265-320.
- Scales, D. and Inesi (1976). Assembly of ATPase protein in Sarcoplasmic Reticulum membranes. Biophys. J. 16:735-751
- Scales, D., and G. Inesi (1976) Localization of ATPase protein in the SR membrane. Arch. Biochem and Biophys. 176:392-394
- Scarpa, A., Brinley, Tiffert, and Dubyak (1978) Metallochromic Indicators of ionized calcium. Annals of NYAS, Vol 307 Calcium Transport and Cell Function Ed. A. Scarpa and E. Carafoli, pg 86-112
- Schnaitman, C., Erwin, V.G. and Greenaualt, J.W. (1967). The submitochondrial Localization of Monoamine Oxidase. J. Cell Biol. 32: 719-735.
- Schneider, M. F., Chandler, W.K. (1973). Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. Nature 242: 244-246.
- Schwartz, A., Entman, M., Borner, E. (1974). Rapid Kinetic Studies of Cardiac Sarcoplasmic Reticulum. Effect of Inhibitors, Drugs, pH, Dyes, and Ionophores. In Calcium Binding Protein Polish Scientific Publishers, Warsaw

- Schwartz, A., Entman, M., Kaniike, Lane, Van Winkle and Bornet. (1976). The rate of calcium uptake into SR of cardiac and skeletal muscle Effects of c-AMP protein kinase and phosphorylase b kinase. B.B.A. 426:57-72
- Shamoo, A.E. and MacLennan, D.H. (1974). A Ca^{++} -dependent and -selective ionophore as part of Ca^{++} + Mg^{++} -dependent adenosine triphosphatase of sarcoplasmic reticulum. Proc. Nat. Acad. Sci. (USA) 71: 3522-3526.
- Shamoo, A., Thompson, T. Campbell, K. , Scott, T. and Goldstein, D. (1975). Mechanism of action of Ruthenium Red compounds on Ca^{++} -ionophore from sarcoplasmic reticulum Ca^{++} + Mg^{++} -Atpase and lipid bilayer. J. Biol. Chem. In Press.
- Singer, T.P. (1974) Determination of the Activity of Succinate, NADH, Choline, and glycerophosphate dehydrogenases. Method of Biochemical Analysis Vol 22 ed D. Glick John Wiley and Sons, N.Y.
- Smith, D., Baerwald, R. and Hart, M. (1975). The distribution of orthogonal assemblies and other intercalated particles in frog sartorius and rabbit sarcoplasmic muscle. Tissue Cell 7: 369-382.
- Somlyo, A.V., Shuman, H., and Somlyo, A.P. (1976) Chloride compartmentalization in striated muscle: quantitative electron probe analysis. J. Cell Biol. 70: 336a.
- Somlyo, A.P., A.V. Somlyo, Sloane, and A. Scarpa (1978) Electron Probe Analysis of Calcium Compartments in cryo sections of smooth and striated muscle. Annals of NYAS, Vol 70, pg 523-544
- Somlyo, A.V., Shuman, H. and A.P. Somlyo (1977) Composition of sarcoplasmic reticulum in situ by electron probe X-ray microanalysis. Nature 268:556-558
- Somlyo, A.V., H. Shuman and A.P. Somlyo (1977) Elemental Distribution in striated muscle and the effects of hypertonicity. J. Cell. Biol. 74:828-857
- Stephenson, E.W. and R.J. Poldosky (1977) Influence of Magnesium on Chloride-Induced Calcium Release in Skinned Muscle Fibers. J.G.Physiology 69:17-35
- Stewart, P., MacLennan, D.H. and Shamoo, A.E. (1975). Isolation and characterization of tryptic fragments from Ca^{++} -ATPase from sarcoplasmic reticulum. J. Biol. Chem., in press.

- Stewart, P.S. and MacLennan D.H. (1974). Surface particles of sarcoplasmic reticulum membranes. *J. Biol. Chem.* 249: 985-993.
- Sulakhe, P.V. and St. Louis, P.J. (1976) Membrane Phosphorylation and Calcium Transport in cardiac and skeletal muscle membranes. *Gen Pharm.* 7:313-319
- Sularhe, P.V. and Drummonds, G.I. (1974). Protein kinase catalyzed phosphorylation of muscle sarcolemma. *Arch. Biochem. Biophys.* 161:448-455.
- Swank, R., and K. Munkres (1971) Molecular weight analysis of oligopeptides by electrophoresis in SDS-PAGE. *Anal. Biochem.* 39: 462-477
- Tada, M. and M.A. Kirchberger. (1978) Significance of the membrane protein phospholamban in c-AMP mediated regulation of calcium transport by the SR. *Cardiac Structure and Metabolism*, Vol 11 Ed. Dhalla Univ. Park Press, Baltimore 265-271
- Tada, M., M.A. Kirchberger, and A.M. Katz (1975). Phosphorylation of a 22,000 dalton component of the Cardiac SR by c-AMP protein kinase. *J. Biol. Chem.* 250:2640-2647
- Takachuk, V.A. and A.A. Boldyrev (1974). Isolation and Characterization of Highly purified preparations of rabbit skeletal muscle sarcolemma containing Na^+ , K^+ ATPase. *Biokhimiya* 39:869-877
- Thorens, S. and Endo, M. (1975). 100. Calcium-induced calcium release and Depolarization-induced calcium release: their physiological significance. *Proc. Japan Acad.* 51: 473-478.
- Thorens, S., and Endo, M. (1975) Calcium-induced calcium release and depolarization-induced calcium release: their physiological significance. *Proc. Japan Acad.* 51: 473-478.
- Tonomura, Y. (1972) The Ca^{2+} - Mg^{2+} -dependent ATPase and the uptake of Ca^{2+} by the fragmented sarcoplasmic reticulum. In *Muscle Proteins, Muscle Contraction and Cation Transport* by Y. Tonomura. Tokyo: Univ. of Tokyo Press 2. p.305-356.
- Tsien, R.W. (1978) Cyclic AMP and contractile activity in heart. *Advances in Cyclic Nucleotide Research*, Vol 8. ed P. Greengard and G. Robison, Raven Press N.Y. 363-412
- Vale, M.G.P. Localization of the amino phospholipids in the SR membranes revealed by Trinitrobenzene-sulfonate and Fluorodinitrobenzene. (1977) *B.B.A.* 471:39-48

- Van Winkle, W.E. (1976) Calcium release from skeletal muscle SR site of action of Dantrolene sodium. *Science* 193:1130-1131
- Van der klott, W.G.s (1969). Calcium uptake by isolated sarcoplasmic reticulum treated with dithiothreitol. *Science* 164: 1294.
- Warren, G.B., Toon, P.A., Birdsall, N.J.N., Lee, A.G. and Metcalfe, J.C. (1974). REconstitution of a calcium pump using refined membrane components. *Proc. Nat. Acad. Sci. (USA)* 71: 622-626.
- Watanabe, S. (1955) Relaxing effects of EDTA on glycerol treated muscle fibers. *Arch. Biochem and Biophy* 54:559-562
- Weber, A. and Herz, R. (1968). The relationship between caffeine contraction of intact muscle and effect of caffeine on reticulum. *J. Gen. Physiol.* 52:750
- Weber, A. and Murray, J. (1973). Molecular control mechanisms in muscle contraction. *Physiol. Rev.* 53: 612-673.
- Weber, A. (1959) On the role of calcium in the activity of adenosine 5'-triphosphate hydrolysis by J. Biol. Chem. 234: 2764-2769.
- Weber, A. (1966) Energized calcium transport and relaxing factors. In *Current Topics in Bioenergetics* edited by D. R. Sanadi. New York: Academic p. 203-254.
- Weber, A. (1968) The mechanism of the action of caffeine on sarcoplasmic reticulum. *J. Gen. Physiol.* 52: 760-772.
- Weber, A. (1971) Regulatory mechanisms of the calcium transport system of fragmented sarcoplasmic reticulum. I. The effect of accumulated calcium on transport and adenosine triphosphate hydrolysis. *J. Gen. Physiol.* 57: 50-63
- Weber, A. (1971) Regulatory mechanisms of the calcium transport system of fragmented rabbit sarcoplasmic reticulum. II. Inhibition of outflux in calcium-free media. *J. Gen. Physiol.* 57: 64-70.
- Weber, A. and Herz, R. (1968). The relationship between caffeine contracture of intact muscle and the effect of caffeine on reticulum. *J. Gen. Physiol.* 52: 750-759.
- Weber, A., Herz, R. and Reiss, I. (1963) On the mechanism of the relaxing effect of fragmented sarcoplasmic reticulum. *J. Gen. Physiol.* 46: 679-702.

- Weber, A., Herz, R. and Reiss, I. (1966) Study of the kinetics of calcium transport by isolated fragmented sarcoplasmic reticulum. *Biochem Z.* 345: 329-369.
- Weber, K., and M. Osborn (1968) The reliability of Molecular Weight Determinations by SDS-gel electrophoresis. *J. Biol. Chem.* 244:4406-4412
- Weber, A. (1959) On the role of calcium in the activity of ATP hydrolysis by actomyosin. *J. Biol. Chem.* 234:2764-2769
- Winegrad, S. (1970). The intracellular site of calcium activation of contraction in frog skeletal muscle. *J. Gen. Physiol.* 55: 77-88.
- Winegrad, S. (1968).. Intracellular calcium movement of frog skeletal muscle during recovery from tetanus. *J. Gen. Physiol.* 51: 65.
- Winegrad, S. (1965) Autoradiographic studies of intracellular calcium in frog skeletal muscle. *J. Gen. Physiol.* 48: 455-479.
- Winegrad, S. (1965) The location of muscle calcium with respect to the myofibrils. *J. Gen Physiol.* 48: 997-1002.
- Winegrad, S. (1968) Intracellular calcium movements of frog skeletal muscle during recovery from tetanus. *J. Gen. Physiol.* 51: 65-83.
- Winegrad, S. (1970) The intracellular site of calcium activation of contraction in frog skeletal muscle. *J. Gen Physiol* 55: 77-88.
- Wray, H. and R. Gray (1977). Cyclic AMP stimulated membrane phosphorylation and Ca^{++} activated ATPase in cardiac SR. *B.B.A.* 461:441-459
- Yagi, S., and Endo, M. (1976) Effect of deuterium oxide (D_2O) on excitation-contraction coupling of skeletal muscle. *J. Physiol. Soc. Japan* 38: 298-300.
- Yagi, S., and Endo, M. (1976) Effect of dantrolene on excitation-contraction coupling of skeletal muscle. *Japan. J. Pharmacol.* 26: 164P
- Yagi, S. and M. Endo (1976) Effect of dantrolene on excitation-contraction coupling of skeletal muscle. *Japan J. Pharmacol* 26:164P

- Yamada, S., and Tonomura, Y. (1972) Phosphorylation of the Ca^{2+} - Mg^{2+} -dependent ATPase of the sarcoplasmic reticulum coupled with cation translocation. J. Biochem 71: 1101-1104.
- Ca^{2+} - Mg^{2+} -dependent ATPase of the sarcoplasmic reticulum
- Yamamoto, T. and Tonomura, Y. (1967). Reaction mechanism of the Ca^{++} dependent ATPase of sarcoplasmic reticulum from skeletal muscle. II. Intermediate formation of phosphorylprotein. J. Biochem.(Tokyo) 64:137
- Zimniak, P. and E. Racker (1978) Electrogenicity of Ca^{++} Transport Catalyzed by the Ca^{++} ATPase from sarcoplasmic reticulum. J.Biol. Chem. 253:4631-4637