Biochimica et Biophysica Acta, 643 (1981) 55—62

BBA 79179

ISOLATION OF FROG HEART SARCOLEMMA POSSESSING (Ca^{2+} + Mg^{2+})-ATPase AND Ca^{2+} PUMP ACTIVITIES

N.C. MORCOS

Departments of Medicine and Physiology, and the American Heart Association, Greater Los Angeles Affiliate Cardiovascular Research Laboratories, U.C.L.A. School of Medicine, Los Angeles, CA 90024 (U.S.A.)

(Received September 10th, 1980)

Key words: (Ca^{2+} + Mg^{2+})-ATPase; Ca^{2+} pump; Sarcolemma; (Frog heart)

Summary

The presence of a (Ca^{2+} + Mg^{2+})-ATPase and Ca^{2+} pump in frog heart sarcolemma was investigated. Frog cardiac sarcolemma was isolated by using a modification of a recent procedure for isolating sarcolemma from dog heart (Morcos, N.C. and Drummond, G.I. (1980) Biochim. Biophys. Acta 598, 27—39). Despite the sparseness of sarcoplasmic reticulum in frog heart, this tissue possessed (Ca^{2+} + Mg^{2+})-ATPase activity which appeared endogenous to the sarcolemma. The purified sarcolemmal vesicles also possessed ouabain-sensitive (Na^+ + K^+)-ATPase, Na^+-Ca^{2+} exchange activity and Ca^{2+} pump activity. Inclusion of ionophore A23187 into the assay medium resulted in an approximately 5-fold increase in (Ca^{2+} + Mg^{2+})-ATPase which may be due to removal of inhibitory concentrations of Ca^{2+} accumulated within the membrane vesicles by an ATP-dependent process. Vesicles pre-loaded with $^{45}Ca^{2+}$, via Na^+-Ca^{2+} exchange, lost 92% of their Ca^{2+} when ATP was present and only 60% in the absence of ATP. The results indicate that frog heart sarcolemma possesses a (Ca^{2+} + Mg^{2+})-ATPase and an ATP-dependent Ca^{2+} pump which may be involved in the outward pumping of Ca^{2+} from the heart cell.

Introduction

In cardiac muscle, the rapid dependence of contractile force on extracellular Ca^{2+} was shown by Shine et al. [1] and others [2,3]. Theories on excitation-contraction coupling suggest that the sarcolemma plays an important role in

Ca^{2+} transport within the cell [4—6]. The necessity of regulation of the cytoplasmic Ca^{2+} pool in heart muscle and the strong concentration gradient at the plasma membrane led several authors [7,8] to propose a transport mechanism for Ca^{2+} linked to a Ca^{2+}-stimulated ATPase at the sarcolemma. Several investigators demonstrated some evidence of a Ca^{2+}-binding and uptake system in cardiac sarcolemma and showed fragmentary evidence that the sarcolemma also contains a $(Ca^{2+} + Mg^{2+})$-ATPase [9—12]. Recently, Morcos and Drummond [13] provided evidence for the existence of a $(Ca^{2+} + Mg^{2+})$-ATPase in purified cardiac sarcolemma. These authors also isolated a purified fraction of the ATPase, allowed it to re-form vesicles, and the capacity of these vesicles to bind and accumulate Ca^{2+} was demonstrated, which thereby suggested the possible role of the enzyme as a Ca^{2+} pump. The aim of the present study was to add further evidence to such a system being present at the sarcolemma by demonstrating its presence in heart muscle which possesses very sparse sarcoplasmic reticulum. Much work has been devoted to the fine structure of heart muscle fibers in various lower vertebrates (e.g. fish [14,15], amphibia [16—19], reptilia [20—22]). The heart cells of these animals are small and devoid of T-transverse tubular system, and the sarcoplasmic reticulum is sparse by comparison with that in most skeletal muscle cells.

The frog ventricle is one such tissue in which the sarcoplasmic reticulum is 15-times smaller than that in rat ventricle [23]. The sparseness of sarcoplasmic reticulum in frog heart was demonstrated by electron microscopy and histochemical experiments [16,17,19]. In the present study sarcolemma from frog heart is isolated and the association of a Ca^{2+} pump and $(Ca^{2+} + Mg^{2+})$-ATPase with frog heart sarcolemma is demonstrated.

Materials and Methods

$^{45}CaCl_2$ (4—30 mCi/mg) was obtained from New England Nuclear. Dithiothreitol, enzyme grade Tris, bovine serum albumin and Tris-ATP were purchased from Sigma Chemical Co., and sucrose from Malinkrodt Co. Bull frogs were purchased from Nasco, Wisconsin.

Preparation of sarcolemma

Sarcolemma from heart ventricles of bull frogs was prepared as described previously [13], with several important considerations for frog heart. $(Ca^{2+} + Mg^{2+})$-ATPase from this tissue was much more labile than that from dog heart and homogenates from frog heart had a high tendency towards aggregation, thereby impeding fractionation. To stabilize the enzyme initial homogenization was performed at high protein concentration in the range of 5—6 mg/ml and the homogenate was filtered through a wire mesh. The pellet that sedimented between 3000 and 200 000 $\times g$ was resuspended in homogenizing buffer at 5—6 mg/ml protein. The suspension was homogenized in an all-glass Potter-Elvehjem homogenizer (30 passes), then diluted to 2—3 mg/ml to minimize aggregation and 3 ml were layered on a discontinuous sucrose density gradient. Fractionation and harvest of bands were as described previously [13]. The 8—28% sucrose fractions ($d_{20°C}$, 1.0680—1.1110) were routinely combined and were termed purified sarcolemma.

Assays

Ouabain-sensitive ($Na^+ + K^+$)-ATPase and ($Ca^{2+} + Mg^{2+}$)-ATPase (in the presence of 0.1 mM ouabain and 5 mM NaN_3) were assayed as described previously [13]. Inorganic phosphate determination was previously described [13].

The Ca^{2+} content of the vesicles was measured by incubating membrane protein at 37°C in a medium containing 5 μM free Ca^{2+} ($^{45}CaCl_2$, (11—28) · 10^5 cpm/nmol) and identical in composition to that used for ($Ca^{2+} + Mg^{2+}$)-ATPase assay.

To measure $^{45}Ca^{2+}$ efflux from vesicles of frog heart sarcolemma, the vesicles were first pre-loaded with $^{45}Ca^{2+}$ via Na^+-Ca^{2+}-exchange by a procedure similar to that described by Reeves and Sutko [24] as follows: Vesicles (1 mg/ml) were pre-loaded with Na^+ by incubating with 140 mM NaCl at 4°C for 30 min. To load with $^{45}Ca^{2+}$, 8 μl were transferred to 192 μl of an equimolar medium containing 280 mM sucrose/24 μM $^{45}CaCl_2$ (13 000 cpm/nmol) at 37°C and loading was allowed to proceed for 2 min. An aliquot of 90 μl was filtered through Millipore filter (HAWA 0.45 μm, 25 mm) and washed with 5 vol. equimolar ice-cold buffer containing 140 mM KCl in 50 mM Tris-maleate, pH 7.0. This sample was taken to represent maximum $^{45}Ca^{2+}$ loaded at zero-time efflux. Another 90 μl aliquot was added to 1 ml containing 5 mM $MgCl_2$, 5 mM NaN_3 and 120 mM KCl in the absence or presence of ATP in 50 mM Tris-maleate, and $^{45}Ca^{2+}$ efflux was allowed to proceed. To study the effect of K^+ on efflux, this ion was replaced with 280 mM sucrose in efflux medium. The efflux was stopped by filtering 1 ml on Millipore filters and washing with 5 vol. ice-cold buffer as above. All filters were dried and assayed for radioactivity in a scintillation spectrometer. Control vesicles pre-loaded with KCl instead of NaCl were run in parallel to distinguish the component of Ca^{2+} loaded via the Na^+-Ca^{2+} exchange process. The amount of Ca^{2+} accumulated by vesicles pre-loaded with KCl represented only 30—40% of the total Ca^{2+} of vesicles pre-loaded with NaCl. Permeability of the membrane vesicles to ATP was determined by two methods as follows. ATP entry into the vesicles was measured by incubating in a medium similar in composition to that used for ($Ca^{2+} + Mg^{2+}$)-ATPase assay. The reaction was stopped by filtering an aliquot through a Millipore filter, and washed with 5 vol. ice-cold Tris-maleate (pH 7.0) to remove trapped and loosely bound ATP. Total ATP content of the vesicles was quantitated by measuring inorganic phosphate (P_i) released by hydrolysis when filters containing protein were placed in 6% trichloroacetic acid at 37°C for 30 min. P_i associated with the vesicles on the filter due to ATPase activity during initial incubation was measured prior to hydrolysis and subtracted from total P_i after hydrolysis. Appropriate ATP standards were treated similarly. In another set of experiments, membrane vesicles were maximally pre-loaded with ATP by performing the initial tissue homogenization and all subsequent steps in the presence of 5.0 mM ATP. Membranes were then sedimented and the pellet washed three times with 10 mM Tris-HCl pH 7.5 to remove trapped and loosely bound ATP. Membranes were incubated in 10 mM Tris-HCl pH 7.5 at 37°C for up to 30 min and an aliquot filtered through Millipore, ATP remaining in the vesicles was quantitated as above by measuring total P_i in the filtrate after hydrolysis in 6% trichloroacetic acid as described above.

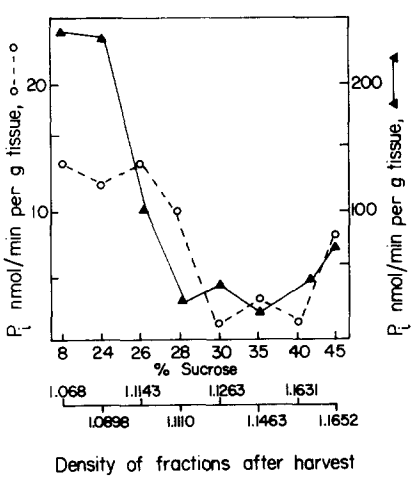
Protein determinations were performed according to Lowry et al. [25], with bovine serum albumin as standard.

Results

Frog heart contained (Ca^{2+} + Mg^{2+})-ATPase activity despite the sparseness of sarcoplasmic reticulum in this tissue. The content of this activity per 100 mg protein in frog heart was similar to that of dog heart (2113 ± 1195 and 2295 ± 625, respectively, from ten different preparations each). The content of the sarcolemmal marker, ouabain-sensitive (Na^+ + K^+)-ATPase was also similar in both tissue (2897 ± 618 and 2583 ± 850, respectively, from five different preparations each).

Fractionation of frog heart suspensions by a discontinuous sucrose density gradient was impeded by the high tendency of particles to aggregate and form one large pellet at the bottom of the gradient. Inclusion of low KCl concentrations (0.05—0.10 M) in the sucrose gradient, lowered the state of aggregation and therefore fractionation based upon distribution of ouabain-sensitive (Na^+ + K^+)-ATPase and protein was satisfactory. However, these conditions were accompanied by severe losses of (Ca^{2+} + Mg^{2+})-ATPase activity. Loss of (Ca^{2+} + Mg^{2+})-ATPase activity associated with membranes in the presence of KCl was previously reported by Morcos and Drummond [13] who also employed high KCl concentrations to aid in solubilizing the enzyme. To avoid aggregation of fractions from frog heart, protein concentration was lowered to 2—3 mg/ml prior to sucrose density gradient fractionation in the absence of any KCl. To help stabilize the labile (Ca^{2+} + Mg^{2+})-ATPase of frog heart, protein concentration was maintained at 5—6 mg/ml during all Potter-Elvehjem homogenizations.

Fig. 1 shows the distribution of ouabain-sensitive (Na^+ + K^+)-ATPase and (Ca^{2+} + Mg^{2+})-ATPase on sucrose density gradients after 2 h centrifugation. It is clear that the low density fractions ($d_{20°C}$, 1.0680—1.1110) are particu-



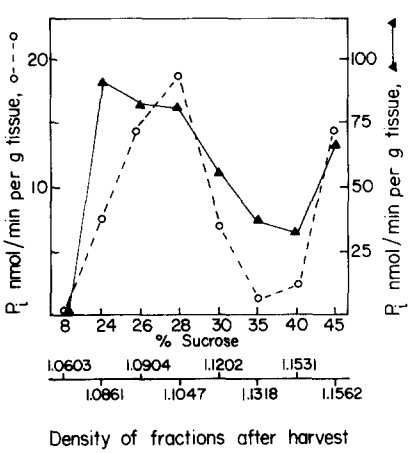


Fig. 1. Distribution of frog heart (Ca^{2+} + Mg^{2+})-ATPase (○) and ouabain-sensitive (Na^+ + K^+)-ATPase (▲) on 2-h sucrose density gradient. Fractions were harvested as described under Materials and Methods. Protein concentration was in the range of 20—200 μg/ml in the reaction medium.

Fig. 2. Distribution of frog heart (Ca^{2+} + Mg^{2+})-ATPase (○) and ouabain-sensitive (Na^+ + K^+)-ATPase (▲) on 18-h sucrose density gradient. Conditions of assay are similar to those in Fig. 1.

larly rich in the plasma membrane marker, ouabain-sensitive ($Na^+ + K^+$)-ATPase. The distribution of Ca^{2+}-stimulated MgATPase on the gradient parallels that of the plasma membrane marker. There was no second peak of ($Ca^{2+} + Mg^{2+}$)-ATPase activity in the density range of $d_{20°C} = 1.1263—1.1631$ where sarcoplasmic reticulum usually appears [26,27]. Some activity was, however, associated with these higher density fractions, the source of which may be sparse amounts of sarcoplasmic reticulum in this tissue. Fig. 2 shows that if fractionation by the sucrose density gradient was allowed to proceed for 18 h, the distribution of enzymes remains essentially the same. There was some density change at several points of the gradient because of diffusion. However, the pattern of distribution of the ouabain-sensitive ($Na^+ + K^+$)-ATPase and ($Ca^{2+} + Mg^{2+}$)-ATPase was similar to that of the 2 h gradient with a large fraction of the total activity in the range $d_{20°C} = 1.0861—1.1202$. The data clearly demonstrates the presence of ($Ca^{2+} + Mg^{2+}$)-ATPase in the lowest density fractions, suggesting the presence of this enzyme in plasma membrane.

Ionophore A23187 caused a significant stimulation of ($Ca^{2+} + Mg^{2+}$)-ATPase activity. Fig. 3 shows P_i release by this ATPase as a function of time. The ionophore caused an approximately 3—5-fold stimulation of activity. Under these conditions the vesicles do accumulate Ca^{2+} in both ATP-independent and ATP-dependent manners. Total Ca^{2+} content of the vesicles measured on an aliquot of the same samples in the ATPase assay was 40% lower in the presence of ionophore. It is suggested that the stimulation produced by the ionophore may be due to removal of an inhibitory Ca^{2+} concentration within the vesicles. Ex-

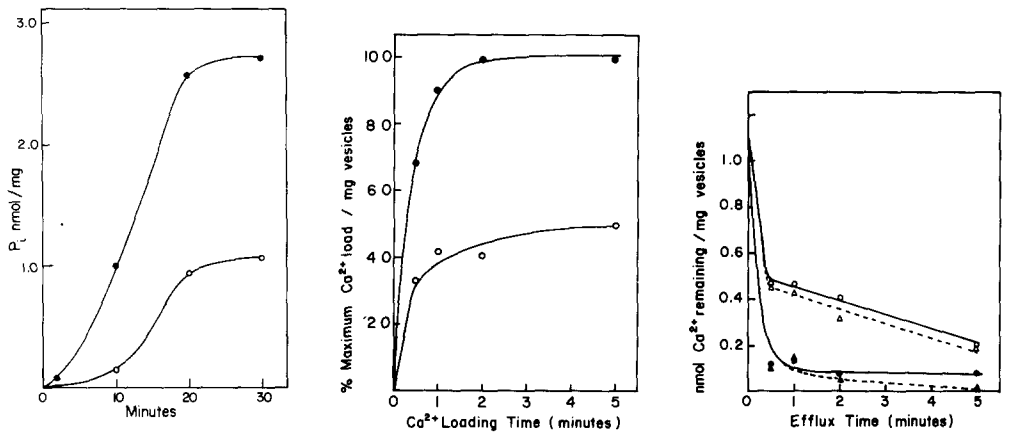


Fig. 3. (left) Effect of presence (•) or absence (○) of ionophore A23187 on ($Ca^{2+} + Mg^{2+}$)-ATPase activity. Assays were performed on purified sarcolemma from pooled fractions in the 8—28% sucrose region on the gradient. Protein concentration in the reaction medium was 25 μg/ml. Samples with A23187 contained 5 μl ionophore solution (0.33 mM in dimethylsulfoxide) to a final concentration of $1.65 \cdot 10^{-9}$ M. Dimethylsulfoxide by itself had no effect on enzyme activity.

Fig. 4. (centre) Ca^{2+} loading in sarcolemma: •, vesicles were pre-loaded with 140 mM NaCl; ○, vesicles were pre-loaded with 140 mM KCl.

Fig. 5. (right) Efflux of Ca^{2+} from sarcolemmal vesicles in a medium containing 5 mM $MgCl_2$/5 mM NaN_3 in 50 mM Tris-maleate and the following variations: ○, 140 mM KCl, no ATP; •, 140 mM KCl, 5 mM ATP; △, no KCl, no ATP; ▲, no KCl, 5 mM ATP.

periments on the permeability of the membrane vesicles to ATP in the incubation medium indicate that ATP content of the vesicles (155 μg protein/ml) increased rapidly within 1 min to 12.90 nmol/mg and reached 19.4 nmol/mg after 30 min. The amount of ATP associated with the membranes in the first minute represents 25% of their maximal ATP capacity in the presence of 5 mM ATP. Membranes pre-loaded with ATP (40.8 mmol/mg) and incubated in an ATP-free medium (143 μg protein/ml) lost 60% their ATP content to the incubation medium within the first minute. The results indicate that the membrane vesicles are permeable to ATP movement in either direction and may support $(Ca^{2+} + Mg^{2+})$-ATPase activity of sites facing the inside of the vesicles as well.

Fig. 4 shows that the sarcolemmal vesicles load Ca^{2+} via Na^{+}-Ca^{2+}-exchange process. The Ca^{2+} content of vesicles pre-loaded with Na^{+} was 60% higher than those pre-loaded with K^{+}. Loading of the vesicles with Ca^{2+} via Na^{+}-Ca^{2+} exchange was complete within 2 min. To test the possibility that a portion of the ATPase and Ca^{2+} pump sites face the inside of the vesicles, Na^{+}-Ca^{2+} exchange was utilized to load these vesicles with ^{45}Ca. The efflux of $^{45}Ca^{2+}$ from these vesicles was then measured when the ATP-dependent Ca^{2+} pump and $(Ca^{2+} + Mg^{2+})$-ATPase were inactive (in the absence of ATP) and when they were active (in the presence of ATP) as shown in Fig. 5. The $^{45}Ca^{2+}$-pre-loaded vesicles lost 60% of their $^{45}Ca^{2+}$ within 2 min in the absence of ATP. In the presence of ATP, however, 92% of the $^{45}Ca^{2+}$ was lost within the same time interval. This suggested that the vesicles from frog heart sarcolemma do indeed possess an ATP-dependent Ca^{2+} pump, a portion of which may be oriented such that it faces the inside and pumps Ca^{2+} to the outside of the vesicles. The presence or absence of KCl in the efflux medium did not influence the amount of Ca^{2+} remaining in the vesicles. Although K^{+} stimulated the activity of $(Ca^{2+} + Mg^{2+})$-ATPase in the range of 5 mM Ca^{2+}, it may be that the local concentration of Ca^{2+} within the vesicles is in the range were the presence or absence of K^{+} does not alter ATPase activity significantly [13].

Discussion

The present communication demonstrates the presence of a $(Ca^{2+} + Mg^{2+})$-ATPase and ATP-dependent Ca^{2+} pump in frog heart despite the sparseness of sarcoplasmic reticulum in this tissue. Sucrose density gradient fractionation indicated that these activities are associated with the sarcolemmal fraction. The possibility of the existence of such a system in the sarcolemma of cardiac tissue has received much consideration in recent years [9—12,28, 29]. One difficulty in trying to establish the existence of a specific $(Ca^{2+} + Mg^{2+})$-ATPase and Ca^{2+} pump in cardiac sarcolemma is the possibility of contamination with fragments of sarcoplasmic reticulum. It has been shown by several authors that sarcoplasmic reticulum from various sources bands at a density range of 1.1263—1.1631 in sucrose density gradients [28,30]. Morcos and Drummond [13] showed that when dog heart suspensions were fractionated on sucrose density gradient there were two peaks of $(Ca^{2+} + Mg^{2+})$-ATpase activity. One portion of this activity was associated with a light sarcolemmal fraction in the density range of 1.0591—1.1083. The other portion was associated with a more dense fraction where sarcoplasmic reticulum usually appears at a density range of 1.1263—

1.1631. In the present study, the sparseness of sarcoplasmic reticulum in frog heart is further supported by the absence of a second major peak at the higher density range on the sucrose gradient. Only one major peak of $(Ca^{2+} + Mg^{2+})$-ATPase appeared in association with the sarcolemmal fraction isolated from this tissue. The isolated frog sarcolemma was purified 7—10-fold over the crude homogenate, based on ouabain-sensitive $(Na^+ + K^+)$-ATPase activity. This is in agreement with the results reported previously on dog heart employing the same method of sarcolemmal preparation [13]. $(Ca^{2+} + Mg^{2+})$-ATPase showed a 5-fold purification over the crude homogenate. The extreme lability of this enzyme from frog heart resulted in the loss of a portion of the activity during the purification procedure.

The ability of the vesicles to accumulate as well as to extrude Ca^{2+} by ATP-dependent processes suggests that the preparation may contain a mixed population of inside-out and right-side-out vesicles. The large stimulation of total $(Ca^{2+} + Mg^{2+})$-ATPase activity in the presence of ionophore A23187 was accompanied by a lower Ca^{2+} content of the vesicles. This may suggest that a portion of the vesicles are oriented such that the ATPase and Ca^{2+} pumps face the outside. Removal of high concentrations of accumulated Ca^{2+} within the vesicles by ionophore A23187 allows continued Ca^{2+} pump activity and, hence, higher ATPase activity.

There is no direct evidence at present that the $(Ca^{2+} + Mg^{2+})$-ATPase activity in the frog sarcolemma is a Ca^{2+} pump, although it had been demonstrated in dog heart sarcolemma [13]. However, the findings reported here indicate a possible relation between the ATPase and pump activity especially in view of the sparseness of other sites (e.g., sarcoplasmic reticulum) for transport of intracellular Ca^{2+} to the exterior.

The isolated sarcolemmal fraction possessed Na^+-Ca^{2+} exchange activity characteristics of these surface membranes [11,24,31]. The stimulation of Ca^{2+} efflux from the inside to the outside of these Ca^{2+}-pre-loaded vesicles upon inclusion of ATP demonstrates that sarcolemma possess ATP-dependent Ca^{2+} pump properties in a population of vesicles that are oriented such that the pump faces the inside. The presence of an ATP-dependent Ca^{2+}-transport system in the sarcolemma may be an important consideration in describing models of excitation-contraction coupling in the heart.

Acknowledgements

This work was done during the tenure of a Research Fellowship No. 581 of the American Heart Association, Greater Los Angeles Affiliate. This work was also supported by a grant from USPHS HL 11351-13.

References

1 Shine, K.I., Serena, S.D. and Langer, G.A. (1971) Am. J. Physiol. 221, 1408—1417
2 Ringer, S. (1883) J. Physiol. 4, 29—43
3 Bailey, L.E. and Dresel, P.E. (1968) J. Gen. Physiol. 52, 969—982
4 Langer, G.A. (1968) Physiol. Rev. 48, 708—757
5 Sonnenblick, E.H. and Stam, A.C. (1969) Annu. Rev. Physiol. 31, 647—674
6 Stam, A.C., Weglicki, W.B., Jr., Feldman, O., Shelburne, J.C. and Sonnenblick, E.H. (1970) J. Mol. Cell. Cardiol. 1, 117—130

7 Okita, G.T., Richardson, F. and Roth-Schecter, B.F. (1973) J. Pharmacol. Exp. Therapeut. 185, 1—11
8 Skou, J.C. (1965) Physiol. Rev. 45, 596—617
9 Hui, C.W., Drummond, M. and Drummond, G.I. (1976) Arch. Biochem. Biophys. 173, 415—427
10 St. Louis, P.J. and Sulahke, P.V. (1976) Can. J. Biochem. 54, 946—956
11 Morcos, N.C. and Jacobson, A.L. (1978) Can. J. Biochem. 56(1), 1—6
12 Morcos, N.C. and Jacobson, A.L. (1979) Can J. Physiol. Pharmacol. 57, 529—534
13 Morcos, N.C. and Drummond, G.I. (1980) Biochim. Biophys. Acta 598, 27—39
14 Kilarski, W. (1964) Acta. Biol. Cracov. (Ser. Zool.) 7, 75—87
15 Kilarski, W. (1964) Acta. Biol. Cracov. (Ser. Zool.) 7, 235—240
16 Staley, N.A. and Benson, E.S. (1968) J. Cell. Biol. 38, 99—114
17 Sommer, J.S. and Johnson, E.A. (1969) Z. Zellforsch. 98, 437—468
18 Baldwin, K.M. (1970) J. Cell Biol. 46, 455—476
19 Page, S.G. and Niedergerke, R. (1972) J. Cell. Sci. 11, 179—203
20 Slautterbach, D.B. (1963) J. Cell Biol. 19, 66A
21 Leak, L.V. (1967) Am. J. Anat. 120, 553—582
22 Forbes, M.S. and Sperelakis, N. (1975) J. Ultrastruct. Res. 34, 439—451
23 Page, E., McCallister, L.P. and Power, B. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 1465—1466
24 Reeves, J.P. and Sutko, J.L. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 590—594
25 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
26 Lau, Y.H., Caswell, A.H. and Brunschwig, J.P. (1977) J. Biol. Chem. 252, 5565—5574
27 Meisner, G. (1975) Biochim. Biophys. Acta 389, 51—68
28 Caroni, O. and Carafoli, E. (1980) Nature 283, 765—767
29 Dhalla, N.S., Arand, M.P. and Harrow, J.A.C. (1976) J. Biochem. 79, 1345—1350
30 Barchi, R.L., Bonilla, E. and Wong, M. (1977) Proc. Natl. Acad. Sci. U.S.A. 74(1), 34—38
31 Pitts, B.J.R. (1979) J. Biol. Chem. 254, 6232—6235