Primary Role of Sarcoplasmic Reticulum in Phasic Contractile Activation of Cardiac Myocytes with Shunted Myolemma

M. CHIESI, M. M. HO, G. INESI, A. V. SOMLYO, and A. P. SOMLYO
Department of Biological Chemistry, University of Maryland Medical School, Baltimore, Maryland
21201, and Pennsylvania Muscle Institute and Departments of Physiology and Pathology, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104

ABSTRACT Homogeneous populations of single myocytes showing good preservation of ultrastructure were obtained by enzymatic digestion of rabbit and rat hearts, and maintained in a relaxed state in the presence of free Ca\(^{2+}\) concentrations <10\(^{-7}\) M. Ultrastructural details such as a cytoskeleton of 100-Å filaments connected to the sarcolemma at the Z lines were demonstrated especially well in these preparations. In spite of seemingly normal structure, electron probe analysis of cryosections reveals similar concentrations of electrolytes in the medium and in the cytoplasm, indicating the presence of electrochemical shunting across the external membrane. The dissociated myocytes display Ca uptake and phasic contractions that are apparently dependent on mitochondrial respiration, but are not affected by mitochondrial uncouplers when ATP and phosphocreatine are added. The uptake is augmented by oxalate and, based on identification of calcium oxalate crystals by electron microscopy and electron probe analysis, is localized to the sarcoplasmic reticulum (SR).

An advantageous feature of the dissociated myocytes is that they are suitable for experiments using large numbers of cells in suspension. Thereby, velocities of calcium transport were measured directly by isotopic tracer and filtration methods. It was then found that the lowest Ca\(^{2+}\) concentrations (5 x 10\(^{-7}\) M for the rabbit and 1 x 10\(^{-7}\) M for the rat) sustaining Ca transport also induce phasic contractile activity in all myocytes, even though the external membrane is electrochemically shunted. A stepwise rise in the Ca\(^{2+}\) concentration of up to one order of magnitude, increases transport velocities in parallel with the rates of phasic contractions. Both these parameters are affected by Mg\(^{2+}\), temperature, cyclic-AMP, and methylxanthines, even though the Ca\(^{2+}\) concentration is maintained constant in the medium. Therefore, Ca transport by SR is a requirement and a rate limiting factor for the occurrence of phasic contractile activation in dissociated cardiac cells retaining an electrochemically shunted external membrane. It is suggested that transient Ca release required for phasic contractile activation is due to equilibrium oscillations across the SR membrane. The sequential pattern of sarcomere activation is consistent with a self propagating mechanism of calcium release. SR in dissociated skeletal muscle cells sustains a greater Ca transport activity than in dissociated heart cells. However, the heart cells display a much higher phasic contractile activity, indicating that cardiac SR has a greater tendency to release accumulated calcium. If free Ca\(^{2+}\) in the medium is raised above 10\(^{-6}\) M, both cardiac and skeletal myocytes undergo contractures and degenerative phenomena, accompanied by Ca, Mg, and phosphate accumulation in cardiac mitochondria.

The sarcoplasmic reticulum (SR) plays an important role in the control of intracellular Ca\(^{2+}\) concentration and contractile activity in muscle (15, 46, 88). Vesicular fragments of SR accumulate Ca\(^{2+}\) in the presence of ATP (16, 47), and their ability to lower the Ca\(^{2+}\) concentration in the medium is comparable to that required to induce relaxation of myofibrils in muscle cells (15, 46, 88). Release phenomena (51, 61) and modulation effects (54, 96) observed in isolated vesicles, how-
ever, cannot be evaluated satisfactorily for their functional relevance to activation and regulation of contractile phenomena in muscle. Even greater shortcomings are encountered in the study of cardiac SR which, following isolation in the form of vesicles, presents lower activity and greater instability than that obtained from skeletal muscle (69). Therefore, we have chosen enzymatically dissociated cardiac cells (4, 33, 42, 56, 63, 64, 68, 71, 86, 97) as an experimental system in which the SR retains native structure and relationships with tubular membranes, myofibrils, and other cellular components, and is not subjected to homogenization and centrifugation procedures. Although preparation of dissociated cells with electrochemically competent myolemma has been reported (6, 68), we prefer preparations with a hyperpermeable myolemma to study intracellular Ca fluxes and contractile activation in the presence of an electrochemically shunted myolemma.

In contrast with studies on single fibers or bundles, our experimental observations are conducted on samples containing large number of cells in suspension. Thereby, time resolution of Ca fluxes by measurements of isotopic tracer is rendered easier. On the other hand, the validity of these observations requires that structural and functional features be shared homogeneously by the population of dissociated cells. We describe here a detailed study of structure and function with an emphasis on features which are relevant to Ca$^{2+}$ fluxes and contractile activation in dissociated cardiac cells. Comparative studies on skeletal muscle preparations are also described.

**MATERIALS AND METHODS**

**Preparation of Dissociated Muscle Cells**

Hearts from adult rabbits (New Zealand strain) and adult rats (Long-Evans strain) were used for preparation of dissociated cardiac myocytes. For each preparation, one rabbit or two rats were used. Animals were heparinized and killed by cervical dislocation. Rats were given light ether anesthesia before heparin injection. The heart(s) were excised and mounted on the perfusion chamber as previously described (13) and perfused with a series of solutions saturated with 95% O$_2$–5% CO$_2$ at 37°C as described as follows: (a) Perfusion for 10 min with 200 ml of medium A containing: 118 mM NaCl, 4.7 mM KCl, 12 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgCl$_2$, 50 mM 2-(N-morpholino) propane-sulfonic acid (MOPS), pH 7.4, and 5 mM glucose. (b) Perfusion for 30 min with 100 ml of medium A to which 45 mg of collagenase (Type IV, 165 U/mg; Sigma Chemical Co.) were added. (c) Perfusion for 10 min with 100 ml of ice-cold medium A containing 0.1 mM EGTA. After perfusions, the heart(s) were transferred to a petri dish containing 20 ml (for rabbits) or 10 ml (for rat) of medium A to which EGTA and bovine (Sigma A-4378) albumin were added to final concentrations of 0.3 mM and 10 mg/ml, respectively. The ventricular chamber was cut open and dissociated myocytes were obtained by gentle scraping. The cell suspension was then filtered through gauze and stored on ice (Fig. 1A). Dissociated skeletal muscle cells were prepared from diaphragms from adult rats (Long-Evans strain). For each preparation, two diaphragms were used. Tissues were first rinsed with medium A followed by incubation in 20 ml medium A to which 18 mg of collagenase (Type IV, 165 U/mg; Sigma Chemical Co.) were added. Incubation was carried out by constant shaking at 37°C under a stream of 95% O$_2$–5% CO$_2$ for 30 min. The diaphragms were then rinsed with 20 ml of ice-cold medium A containing 0.1 mM EGTA. Thereafter, they were transferred to a petri dish containing 10 ml medium A to which EGTA and bovine albumin were added to final concentrations of 0.6 mM and 10 mg/ml respectively. Cells were dispersed by gentle scraping. Debris was removed from the cell suspension with the aid of forceps. The cell suspension was filtered through 95% O$_2$–5% CO$_2$ for 30 min. The diaphragms were then rinsed with 20 ml medium containing 0.1 mM EGTA. (d) Perfusion for 10 min with 100 ml of cold medium A containing 0.1 mM EGTA. After perfusions, the heart(s) were transferred to a petri dish containing 20 ml (for rabbits) or 10 ml (for rat) of medium A to which EGTA and bovine (Sigma A-4378) albumin were added to final concentrations of 0.3 mM and 10 mg/ml, respectively. The ventricular chamber was cut open and dissociated myocytes were obtained by gentle scraping. The cell suspension was then filtered through gauze and stored on ice (Fig. 1A).

**Protein Determination**

Stock cell suspensions were sonicated. The protein concentration of the sonicated samples was determined by the Lowry method (59) and the amount of bovine albumin added to the cell suspension was subtracted. The stock suspension prepared by methods described in this report usually contained 6–8 mg cell protein/ml.

**RESULTS**

**General Properties of Dissociated Myocytes**

Enzymatic dissociation of cardiac tissue yields large amounts of myocytes which apparently constitute a homogeneous popu-
The relationship of the sarcolemma with intracellular structures is not disrupted by the process of enzymatic dissociation, and the presence of 100-Å filaments at the Z lines can be demonstrated especially well in the dissociated myocytes (Fig. 3a and b). Bundles of intermediate (100-Å) filaments appear to encircle the Z disks in agreement with Lazarides and Grandier's (58) fluorescence studies on chicken skeletal muscle. When appropriately sectioned, the intermediate filaments appear to be anchored to the internal surface of the sarcolemma at the Z line (Fig. 3a and b), and are also associated with intracellular membrane systems similar to the opossum cardiac muscle anchor-fibers found by Junker and Sommer (52).
In spite of these normal structural features, it is apparent that the native semipermeability properties of the myocytes are not maintained following dissociation. In fact, although not permeable to large dyes such as trypan blue, the dissociated cells are sensitive to manipulations of the outside medium composition which normally do not affect intact tissue (13).

We have used electron probe analysis of freeze-dried cryosections (Fig. 4) to determine the concentrations of the inor-
FIGURE 3  A, Longitudinal section of two isolated cardiac cells fixed with glutaraldehyde and 2% tannic acid before en bloc staining with uranyl acetate. The sarcolemma is indented at each Z band (arrows). 100-Å filament profiles in transverse section occur at the Z bands at the edges of the fibrils (circles). A typical T-tubule (T) with junctional SR is illustrated. e.c.s., extracellular space. The mitochondrion in the lower left region appears to be within a surface bleb of the lower cell. B, Higher magnification view of the same preparation as A, illustrating the 100-Å filaments (small arrows) running transversely at the Z line where the sarcolemma is invaginated (large arrows). The section is cut at the surface of a fibril that the 100-Å filaments appear to encircle. α, actin, m, myosin.
ganic constituents (e.g., Na, K, Cl) of cytoplasm and organelles in dissociated cells which were exposed to ATP and free Ca$^{2+}$ concentrations sufficiently high ($10^{-7} - 10^{-6}$ M) to sustain Ca transport and phasic contractile activities, either in the presence or in the absence of oxalate. The results of elemental analyses of cytoplasm and mitochondria are summarized in Table I and expressed in mmol/kg dry weight. It is readily apparent from these measurements that cytoplasmic Na is much higher, and K much lower than expected in normal cells, clearly indicating electrochemical shunting across the cell membrane. It is noteworthy that such a shunting is a common feature of all cells probed.

An evaluation of the intracellular electrolyte concentrations may be obtained by estimating values for water content. Based on previous studies of mitochondrial water content (92) and comparing continuum x-ray counts obtained from cytoplasm and mitochondria, the cytoplasmic water is estimated to be 85% and 89% in the cells incubated in the absence (Table I A) and in the presence of oxalate (Table I B), respectively. These estimates are obtained by assuming a mitochondrial water content of 68%, compared to normal values of 63–66% (78, 92), consistent with the lack of apparent mitochondrial swelling in cryosections. A relatively high hydration of the cytoplasm in the dissociated cells is consistent with cellular swelling, which was observed by transmission electron microscopy. Based on these assumptions, and within the wide statistical variations indicated in Table I, the cytoplasmic Na$^+$ concentration was estimated to be 140 mM for both the A and the B series, which is quite close to the 158 mM concentration of the medium, and demonstrates a nearly complete equilibration of medium and cytoplasmic Na$^+$. Cytoplasmic K$^+$ was estimated to be 18.2 mM and 40.7 mM in the A and B series, respectively as
compared to 5.9 mM and 15.9 mM in the media used for the two series.

Thus, the normally high K⁺ concentration in the cytoplasm is not maintained after dissociation. On the other hand, the estimated cytoplasmic Cl concentration (80.6 mM and 72.9 mM in the two series) was lower than that of the medium (122.7 mM), probably due to Donnan equilibration with non-diffusible anions present in the cytoplasm.

The cytoplasmic P, Mg, and Ca contents of the dissociated cells were not significantly different than those of intact tissue. The high S content reflects the presence of MOPS buffer, in addition to the protein SH-residues. A comparison of the cytoplasmic and mitochondrial Ca content reveals no evidence of mitochondrial Ca uptake in spontaneously beating cells, under conditions permitting active transport by other organelles (Fig. 4A and B).

**Ca²⁺ Transport**

It was previously shown that myocytes obtained from rabbit heart accumulate Ca in the presence of low (10⁻⁷-10⁻⁶ M) concentrations of the divalent cations, which also causes the dissociated myocytes to undergo phasic contractile activity (13). The observed Ca accumulation was attributed to SR, although metabolic activity was required for this phenomenon, and a role of mitochondria appeared necessary.

We find that if mitochondrial ATPase is inhibited with oligomycin and the electron chain uncoupled with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), Ca accumulation is markedly reduced, and the myocytes remain relaxed. However, full transport and contractile activities are restored when the medium is supplemented with ATP and phosphocreatine (Figs. 5 and 6). Therefore, ATP is the substrate directly used for Ca transport, and mitochondria are only required to regenerate ATP when this nucleotide and/or phosphocreatine are not supplemented exogenously. These experiments indicate that Ca accumulation occurring in the presence of mitochondrial inhibition can be attributed exclusively to SR. In fact, loading of SR is demonstrated unambiguously by electron microscope visualization and electron probe analysis of calcium oxalate crystals in lateral cysternae (Fig. 7). On the other hand, even in conditions permitting full metabolic activity of mitochondria, no Ca accumulation by mitochondria is observed by electron probe analysis, when free Ca²⁺ is maintained at concentrations (10⁻⁷ – 10⁻⁶ M) sufficient to sustain

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**TABLE 1**

Comparison of Mitochondrial and Cytoplasmic Concentrations in Freeze-dried Cryosections of Isolated Cardiac Cells

|                | Na (mmol/kg dry wt) | Mg (mmol/kg dry wt) | P (mmol/kg dry wt) | S (mmol/kg dry wt) | Cl (mmol/kg dry wt) | K (mmol/kg dry wt) | Ca (mmol/kg dry wt) |
|----------------|---------------------|---------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| **A. pCa > 6** |                     |                     |                   |                   |                   |                   |                   |
| Mitochondria   | 24                  | 579                 | 35                | 251               | 454               | 405               | 82                | 4                 | 1,247             |
| SEM            | ±5.3                | ±0.2                | ±1.6              | ±1                | ±0.9              | ±0.7              | ±9.1              |
| SD             | ±144                | ±19                 | ±47               | ±91               | ±163              | ±31               | ±6                | ±361              |
| Cytoplasm      | 20                  | 795                 | 50                | 158               | 502               | 457               | 103               | 7                 | 613               |
| SEM            | ±0.9                | ±3.3                | ±2.2              | ±3.0              | ±2.7              | ±1.5              | ±1.4              | ±6.6              |
| SD             | ±159                | ±21                 | ±39               | ±111              | ±162              | ±30               | ±13               | ±298              |
| **B. pCa > 6 + oxalate** |         |                     |                   |                   |                   |                   |                   |
| Mitochondria   | 19                  | 488                 | 24                | 289               | 280               | 269               | 169               | 5                 | 2,212             |
| SEM            | ±4.9                | ±1.7                | ±2.0              | ±2.0              | ±1.9              | ±1.3              | ±0.6              | ±13.0             |
| SD             | ±174                | ±12                 | ±28               | ±44               | ±109              | ±39               | ±5                | ±754              |
| Cytoplasm      | 16                  | 1,138               | 44                | 94                | 478               | 590               | 329               | 14                | 858               |
| SEM            | ±14.6               | ±3.3                | ±2.3              | ±5.5              | ±6.4              | ±3.7              | ±1.3              | ±8.5              |
| SD             | ±377                | ±24                 | ±64               | ±134              | ±184              | ±89               | ±8                | ±298              |

Grand weighted X ± SE ± SD. SEM, scanning electron microscope.
FIGURE 7  A, Calcium oxalate loaded isolated cardiac cell, frozen, processed by freeze substitution, and sectioned on glycerol. Large Ca signals were obtained in the regions of SR indicated by the arrows. The majority of the deposits occurred in the SR in the Z band region. The only stain present is the osmium included in the acetone. B, Similar to Fig. 1 but at higher magnification to illustrate the typical calcium oxalate deposits. C, Spectra from analysis of SR containing a calcium oxalate deposit and of a mitochondrion in the same region of a cryosection. Note the presence of a large Ca peak in the former and the absence of detectable Ca in the mitochondrion. The Ca peak in the SR is not associated with P which is compatible with the deposit being calcium oxalate.
Ca uptake by SR and phasic contractile activity.

In preliminary experiments with myocytes obtained from rabbit heart, an interdependence of Ca transport and phasic contractile activity was demonstrated (13), implying that Ca accumulated by the SR is phasically released to induce contractile activation, as observed by Fabiato and Fabiato (21-28) in skinned cardiac fibers. Owing to the large number of cells obtained by enzymatic dissociation, it is possible in these preparations to measure Ca transport by isotopic tracer and filtration methods, thereby obtaining time resolution and rates of transport. These measurements were carried out in myocytes obtained from rabbit heart, rat heart, and rat skeletal muscle. The purpose of these experiments was to demonstrate whether transport activity is proportional to the amount of SR in various muscles, and whether phasic contractile activity always follows the ability of the myocytes to accumulate Ca.

The dependence of the transport rates on free Ca\(^2+\) concentration is shown in Fig. 8. Half maximal activation of transport activity is obtained at approximately pCa values of 6.5, 6.8, and 7.0 with myocytes obtained from rabbit heart, rat heart, and rat diaphragm, respectively. It is noteworthy that the velocity of net accumulation is four- to fivefold higher in the diaphragm myocytes.

With regards to the relevance of Ca uptake to phasic contractile activity, it is shown in Fig. 9 that most rabbit and rat heart myocytes acquire phasic contractile activity already at the minimal Ca\(^2+\) concentration sustaining transport by SR. On the other hand, the Ca\(^2+\) concentration dependence of the beating rates is more graded, exhibiting a behavior which is parallel to that of the transport rates (Fig. 10). If Ca accumulation is prevented by the addition of Ca ionophores, no phasic contractile activity is observed.

Contrary to cardiac myocytes, we never saw skeletal muscle fibers undergoing phasic contraction, even though displaying high Ca transport activity (Fig. 8). When the Ca\(^2+\) concentration was raised to levels higher than those required for phasic contractile activity, both cardiac and skeletal myocytes developed permanent contractures. In these conditions, the regular sarcomere pattern was disrupted, myofilaments were disorganized, and large cell surface blebs appeared which were filled with mitochondria. The majority of mitochondria contained small dense deposits consisting of Mg, Ca, and P. In supercontracted cells, freeze-substituted and cut on glycerol, the range of elemental concentrations in the mitochondrial granules were (mmol/kg): Mg, 81-947; P, 128-2,582; Ca, 61-3,054 (the large spread in concentration arises from variations in granule size and whether the microvolume irradiated by the electron beam included only a granule or surrounding matrix.
as well). The Ca:Mg ratio was 1.4 ± 0.23 S.E.M. (Standard Error of Measurement), n = 9 (one extreme value of 10 was deleted), and the P:Ca + Mg ratio was 0.93 ± 0.05 S.E.M.

The range of Ca\(^{2+}\) concentrations producing permanent contractures of the dissociated myocytes was best determined when a divalent cation ionophore was used to prevent Ca accumulation by SR, and the occurrence of phasic contractions. In these conditions, Ca binding to activating sites on the myofibrils was only dependent on equilibration of medium and cytoplasmic Ca\(^{2+}\), and not on release from SR. It is shown in Fig. 12 that Ca\(^{2+}\) concentrations producing contractures are not identical in myocytes obtained from various muscles, although in the range known to produce direct activation of the myofilaments (60). Owing to a wider divergence between Ca\(^{2+}\) concentrations triggering phasic contractions and those producing permanent contracture, rat heart myocytes provide the most favorable experimental system.

**Modulation of Transport Activity**

A most interesting question is whether Ca transport by the SR can be modulated by physiological control mechanisms. In this regard, it was previously shown that Ca uptake by vesicles isolated from cardiac muscle, is increased by cyclic-AMP (cAMP) (11, 54, 74, 95, 96). Demonstration of this effect with isolated vesicles requires medium supplementation with protein kinase and sometimes is rendered difficult by a disturbing lability of the isolated vesicles of cardiac SR.

We now find that the effect of cAMP on Ca transport can be unambiguously demonstrated in cardiac myocytes, without addition of exogenous protein kinase. In fact, the velocity of uptake can be doubled with cAMP concentrations as low as 1 \(\mu\)M (Fig. 13 A). Maximal stimulation is obtained with cAMP concentrations ranging between 1 and 5 \(\mu\)M.

The functional relevance of the cAMP effect on Ca transport
is indicated by the studies of Fabiato and Fabiato (23) on mechanically skinned fibers, demonstrating an increase in phasic contractile tension following addition of analogous concentrations of cAMP. In both the above and our studies, the beating frequency of the dissociated myocytes was increased by cAMP concentrations, which also stimulate the rates of Ca accumulation (Table II).

Studies on isolated cardiac SR vesicles have suggested that protein kinase mediated phosphorylation of a low molecular weight regulatory protein is involved in the cyclic nucleotide stimulated increase in Ca$^{2+}$ transport (83, 84, 95). It is of interest that the stimulation of Ca$^{2+}$ transport by the cyclic nucleotide can be demonstrated in the dissociated myocytes without the addition of exogenous enzyme (i.e., protein kinase), such as is necessary in experiments with isolated SR. In contrast to the cardiac myocytes, no effect of cAMP on Ca sequestration was observed in skeletal myocytes (Fig. 13 B).

Epinephrine, a physiological neurotransmitter which increases cAMP production and contractile tension in intact cardiac tissue, was only slightly and irreproducibly effective in stimulating Ca sequestration in the dissociated myocytes (not shown). This may be attributed to denaturation of the neurotransmitter receptors on the outer surface of the cell membrane, as a consequence of enzymatic dissociation, or to interference of the electrochemical shunt with the stimulus transduction across the external membrane.

For a given Ca$^{2+}$ concentration, increasing the free Mg$^{2+}$ concentration from 2 to 10 mM or lowering the temperature, produced simultaneous inhibition of both Ca transport and contractile activation. Similar effects of Mg$^{2+}$ on mechanically skinned cardiac cells have been reported by Fabiato and Fabiato (25).

Since caffeine has been used extensively as a Ca releasing agent in skinned fibers, we also studied the effects of methyloxanthines on dissociated heart cells. We found that theophylline (Fig. 14) and caffeine at concentrations higher than 0.2–0.3 mM, produced a strong inhibition of net Ca uptake and phasic contractile activity (Table II). In suitable conditions, cAMP could reverse the methyloxanthines inhibition of both Ca transport (Fig. 14) and phasic contractions (Table II).

Lower concentrations (50–100 μM) of methyloxanthines which did not affect significantly the levels of net Ca accumulation, nevertheless increased the beating frequency of the myocytes. This effect seems more in line with the actual pharmacological action of methyloxanthines, and is likely produced by lowering the threshold (18) for the occurrence of phasic release, without direct interference with Ca transport.

It is probable that the effects of methyloxanthines are due to stimulation of Ca release (18, 88, 90), causing (phasic) Ca release at low concentrations and depletion of the SR at high concentrations. Our data do not permit any conclusion on whether the effects of methyloxanthines are mediated by phosphodiesterase inhibition.

**DISCUSSION**

Dissociation of cardiac myocytes retaining electrochemical competence of the surface membrane has been reported (6, 42, 68), providing an experimental system for the study of electrophysiological and contractile parameters. Alternatively, a hyperpermeable state can be induced by the procedure of enzymatic dissociation (13, 71) and the cells so obtained can be used for studies of the cellular events underlying the phasic contractile activity that occurs when the external membrane is electrochemically shunted. The latter method of preparation,
used in this study, yields a population of relatively homogeneous single cells permitting experimental studies that require large numbers of cells in suspension. To obtain a homogeneous population of relaxed myocytes that retain cylindrical shape (Fig. 1A), it is important to perfuse with solutions containing no added Ca and to maintain the Ca²⁺ concentration of the suspension media below 10⁻⁷. Single myocytes prepared by this method provide complementary information to that obtained with cardiac fibers that are skinned mechanically (21-28). They can be compared also to multicellular preparations treated with EDTA (93) or exposed to saponin (19), which in some cases may not be completely permeable (21).

The plasma membrane of the enzymatically isolated myocytes retains its structural continuity at the level of resolution of scanning and transmission electron microscopy (40), although freeze-fractured preparations show some abnormalities of protein particle distribution within the external membrane and open gap junctions. The ultrastructure of dissociated myocytes is generally well preserved, although some swelling is usually apparent. The dilution of other cytoplasmic components by swelling and the use of tannic acid as a mordant agent (14, 53, 62, 77) are probably responsible for the excellent visualization of the 100-Å filaments (Figs. 3A and B). These filaments occur and are structurally indistinguishable in a variety of muscle and nonmuscle cells, even though some differences in their protein subunit composition is apparent (57, 71). In smooth muscle the filaments are frequently, though not invariably, associated with dense bodies that are analogous to the Z lines of striated muscle (79). Their numbers are increased in hypertrophied smooth (3, 41) and cardiac (1) muscle. Intermediate (100-Å) filaments are relatively scarce in adult skeletal muscle, although they occur in large numbers and are longitudinally oriented in normal 5-d myocytes; they decrease in number during development and become localized to the Z lines (2, 73, 85). In adult skeletal and cardiac muscle 100-Å filaments in various numbers are located transversely at the Z lines (36, 52, 66), and have been called anchor filaments as they are thought to stabilize the sarcotubular structures (81).

In Z discs isolated from striated muscle, antibodies to desmin (the major protein of the 100-Å filaments) are localized encircling the Z disc (57, 58). The presence of the 100-Å filaments at the junction between Z line and surface plasmalemma in isolated cardiac myocytes (Fig. 3A) also suggests that they are attachment sites and may be responsible for the scalloped appearance of cardiac muscle cells.

**Increased Permeability of the External Membrane**

Evidence accumulated in this and previous studies indicates that the surface membrane of myocytes prepared in the manner described here is hyperpermeable and would not support cyclic electrical activity that could account for the beating of such cells. The results of electron probe analysis, demonstrating dissipation of the transmembrane gradients of Na and K (Table I), show that hyperpermeability is a general property of the entire cell population. These observations are consistent with the absence of significant electrical potential across the surface membrane of these cells while undergoing phasic contractile activity (71). The electron micrographs of the cells treated with tannic acid as a mordant (Fig. 2) also show free communication of the T tubules with the extracellular space, excluding the possibility that sealing of the tubular openings may have created a compartment that would support electrical potentials and contractile activation. Therefore, the beating observed in the isolated myocytes prepared by our method, like that in mechanically skinned fibers (21-28), can be attributed to primary release of Ca from SR. Indeed, perhaps the most interesting feature of the beating activity in myocytes isolated by our method and in mechanically skinned fibers is that phasic contractile activation occurs independently of sarcolemmal excitation.

**Ca Transport in SR**

An experimental advantage of the dissociated cells is that they permit direct measurements of Ca transport by isotopic tracer and filtration methods. In the presence of 10⁻⁷-10⁻⁴ M free Ca²⁺, Ca accumulation is an energy dependent process that can be supported by endogenous mitochondrial ATP synthesis, but can also proceed in the presence of mitochondrial inhibitors such as FCCP and oligomycin, if supported by exogenous ATP and phosphocreatine (Fig. 5). This feature and the enhancement of Ca accumulation by oxalate are characteristic of active Ca transport by SR (46). The "swiss cheese appearance" of calcium oxalate deposits exposed to the electron beam has previously been observed in cardiac (10, 91) SR; the latter study (91) also localized Ca in the terminal cisternae of cat papillary muscle. In our preparation, localization of accumulated Ca in SR was unambiguously proven by electron probe analysis (Fig. 7).

Both the maximal loading capacity and the rate of Ca²⁺ transport is greater in skeletal than in the cardiac myocytes (Fig. 8). These findings are of interest, because the maximal Ca loading capacity of the SR in the presence of oxalate is thought to be limited largely by its volume. However, the respective volumes of total SR in rat ventricular (3.5%, reference 67) and in rat skeletal (6%, reference 14) muscle cannot account for the observed elevenfold difference in Ca loading. Considering that a larger proportion of the skeletal muscle SR consists of saclike expanses (terminal cisternae) compared to the tubular structure of cardiac SR (for review, see reference 81) it is possible that a greater distention of the terminal cisternae is responsible for the larger calcium oxalate loading capacity of skeletal muscle. The differences in calcium oxalate loading could be better accounted for by volume differences (sixfold) between the terminal cisternae (junctional SR) of cardiac (0.35%) and mammalian fast skeletal (2%; see references 17, 67) muscle, as it is likely that the terminal cisternae were the only part of the SR loaded with calcium oxalate crystals.
Lower rates of Ca transport in cardiac than in skeletal muscle were previously observed in fragmented SR preparations obtained from the two tissues (9, 32, 35, 50, 74). Our findings indicate that the differences in rates can be observed also in cellular preparations and, therefore, are not due to damage to SR during isolation procedures, but rather to inherently lower rates of net uptake. A lower density (about half) of ATPase particles in freeze-fracture preparations of cardiac (81) as compared to skeletal muscle SR, has been noted.

Lack of Mitochondrial Role in Physiological Ca Transport

Mitochondria in rabbit cardiac myocytes do not accumulate Ca at Ca\(^{2+}\)-concentrations (10^{-7}--10^{-6} M) supporting phasic contractile activity, but only when the free Ca\(^{2+}\) concentration is raised to levels (>10^{-6} M) that cause contractures. Under these conditions, mitochondrial granules containing Ca, P, and also Mg are observed (Figs. 11). The absence of mitochondrial Ca loading at physiological, as contrasted to higher Ca\(^{2+}\) concentrations, is consistent with the high apparent \(K_m\) of isolated cardiac mitochondria (55, 72). In fact, in a variety of cell systems such as smooth muscle (78), cardiac muscle (7), and epithelial cells (43), mitochondrial Ca loading in the form of granules is observed only in the presence of abnormally high cytoplasmic free Ca\(^{2+}\) concentrations due to cell damage. It is clear that mitochondria do not play a significant role in the regulation of free cytoplasmic Ca\(^{2+}\) under physiological conditions.

Involvement of SR in Phasic Contractile Activation

It was previously demonstrated that Ca loading of SR is required for the occurrence of rhythmic contractions in skinned cardiac fibers (21–28). It is interesting that spontaneous contractions apparently not due to electrical activity of the surface membrane, were also observed in frog ventricles that contain only a very small volume of SR (94), probably related to myofilament dependent oscillations (29). Furthermore, such rhythmic activity is not completely unique to cardiac SR, since slowly occurring rhythmic contractions have also been observed in mechanically skinned frog and crayfish skeletal muscle fibers (20, 38, 70). However, the rhythmic contractions occur at a higher rate and are easier to elicit in (mammalian) cardiac myocytes than in skeletal fibers (21–28). In fact, in the conditions of our experiments we never observe any contractile activation of the diaphragm fibers, even though they display a very high transport activity of the SR. On the other hand, all the dissociated myocytes obtained from rat and rabbit hearts undergo very obvious contractile activation producing beating rates varying between 20 and 60/min with an \(\sim\) 30% shortening per each beat. All the dissociated cardiac cells have similar energy (Figs. 5 and 6) and free Ca\(^{2+}\) (Figs. 8 and 10) requirements for Ca transport and for contractile activity. Furthermore, the sequential pattern of sarcomere activation indicates a self propagating mechanism of Ca release, and if Ca accumulation by SR is prevented by Ca\(^{2+}\) ionophores the myocytes remain in the relaxed state.

A most important advantage of our preparation is that we are able to measure accurately the rates of Ca uptake by SR, and to compare the Ca\(^{2+}\) concentration dependence of these rates to that of contractile activity (Figs. 9 and 10). It is then demonstrated clearly that phasic contractile activity is already present at the lowest Ca\(^{2+}\) concentrations that sustain Ca transport activity of SR: 10^{-7} M for the rat, and 5 \(\times\) 10^{-7} M for the rabbit. Our experiments also demonstrate that the beating rates increase in parallel with the velocities of transport. Therefore we conclude that Ca uptake by SR is a requirement and a rate limiting factor for the occurrence of phasic contractile activity in dissociated cardiac cells prepared by our method.

In previous studies carried out with skinned preparations it was found that to obtain rhythmic contractions it was necessary to increase the Ca\(^{2+}\) concentration in the medium to levels higher than those required for activation of SR (19). On the contrary, our observations are more in line with the low levels reported by Fabiato and Fabiato (27) for rat cardiac fibers. It should be pointed out that the high Ca\(^{2+}\) concentrations used in previous studies must have been only transient, since permanent contractures were not produced. In fact, slowly added Ca can be buffered by SR in some instances.

It is noteworthy that in previous studies on skinned cells, loading of SR was assessed by releasing the Ca load with caffeine. Even though some estimates of Ca loading velocities were previously obtained in fibers by radioisotope and optical methods (31, 38, 82), we report here the first comparison of directly measured transport rates with parameters of contractile activation, in the presence of constant Ca\(^{2+}\) concentrations in the medium.

Although Ca accumulation by SR is a requirement and a rate limiting factor for the occurrence of phasic contractions, a number of observations indicate that, even in the presence of loaded SR, Ca\(^{2+}\) must be added to the medium to obtain phasic contractions if the Ca\(^{2+}\) concentration is below a certain level. The requirement for such an addition led to the concept of "Ca-induced Ca release" (20, 21, 37), a term originally introduced by Bianchi (5). Our experimentation did not permit direct detection of such a requirement, although the self propagating patterns that we observed are consistent with it. The simplest mechanism consistent with our observations, would be one requiring occupancy of the high-affinity Ca sites of the SR pump to obtain release of accumulated Ca. In fact, the pump cycle includes two interconverting states of the Ca site: one state of high affinity and outward (cytoplasm) orientation, and an other state with low affinity and inward orientation with respect to the cysternal space (49). Evidence obtained with SR vesicles indicates that occupancy of the high-affinity Ca sites maintains the membrane in a state of high permeability to Ca\(^{2+}\) (12, 89), permitting exchange or net fluxes depending on the experimental conditions. It is clear that the presence of ATP sustains inward active transport. On the other hand cardiac SR must develop oscillations of transmembrane equilibrium to account for phasic release of accumulated Ca. We do not know whether the release is accompanied by electrical phenomena. In this regard, the self propagating pattern of sarcomere activation (71) and the optical signals detected with potential sensitive dyes (26) suggest but do not prove changes in electrical potential.

Independent of the mechanism producing changes of the (electro-) chemical equilibrium across the SR membrane, it is clear that the phasic Ca release sustaining contractile activity requires a Ca\(^{2+}\) gradient established by active transport. The rate of transport is rate limiting for the occurrence of phasic release and contractile activation, as these two phenomena are influenced in a parallel fashion by cAMP (Figs. 13 and 14), methylxanthines (Figs. 14 and Table II), Mg\(^{2+}\), and temperature, even when the Ca\(^{2+}\) concentration in the medium is maintained constant.
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