Calcium Ion-Insensitive Contraction of Glycerinated Porcine Cardiac Muscle Fibers by Mg-Inosine Triphosphate

ITP as a Tool to Dissociate the Contraction Mechanism from the Regulatory Mechanism

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SUMMARY The contraction of cardiac muscle that has been treated with glycerol requires Ca\(^{2+}\) (pCa 8–5), when MgATP is used as a substrate. In contrast, this preparation contracts, even in the absence of Ca\(^{2+}\) (pCa 8–10), when ATP is replaced by ITP. Ca\(^{2+}\) dependency was not observed after increasing free Ca\(^{2+}\) concentrations from pCa 8.0 to 5.0, or after increasing MgTTP concentration from 5 to 80 mM. On the other hand, rabbit skeletal muscle fiber treated by the same method as cardiac muscle demonstrates Ca\(^{2+}\) dependency in the presence of both MgTTP and MgATP, although this Ca\(^{2+}\) regulation is less in the presence of MgTTP. Loss of Ca\(^{2+}\) dependency was confirmed by the finding that, in contrast to ATPase, the ITPase activity of cardiac myofibrils was not dependent on Ca\(^{2+}\) concentrations. Furthermore, the very fast tension responses (quick phases) following quick stretch and quick release were missing in MgTTP, and the contractions were similar to rigor. These were not rigor however, because phosphate liberation from FTP continued, and muscle shortening occurred in MgTTP. These findings suggest that MgTTP dissociates the contraction mechanism from the regulatory mechanism, modulating the regulatory properties of cardiac muscle fiber. Circ Res 49: 1360–1365, 1981

FOR muscle contraction, Mg-adenosine triphosphate (MgATP) is used as a substrate (Engelhardt and Ljubimowa, 1939), and small amounts of Ca\(^{2+}\) regulate the contraction of all muscle tissues (Ebashi, 1980). In the absence of Ca\(^{2+}\) (pCa > 7), both cardiac and skeletal muscle contraction are repressed by troponin and troponyosin, which are situated on thin filaments. The inhibitory action of troponin is prevented in the presence of Ca\(^{2+}\) (pCa < 6), resulting in muscle contraction (Ebashi et al., 1968, 1975).

The physiological role of Ca\(^{2+}\) in the regulation of cardiac muscle contraction is modified in some pathological states, such as intramyocardial acidosis (Schäider, 1967; Katz and Hecht, 1969; Ebashi et al., 1975; Fabiato and Fabiato, 1978), or myocardial ischemia (Toyo-oka and Ross, 1979; Toyo-oka and Ross, 1981) and by the effect of catecholamine (England, 1975). The role of Ca\(^{2+}\) is important for an exact understanding of the mechanism of muscle contraction and for the clarification of pathological changes in cardiac muscle contractility at the myofibrillar level.

In this paper, we report that contractions of cardiac muscle fibers made permeable to Ca\(^{2+}\) buffers and substrate by glycerol treatment are independent of free Ca\(^{2+}\) concentrations when MgTTP is used as a substrate. Possible explanations of these findings are discussed.

Methods

Preparation of Glycerinated Muscle Fibers

The septomarginal fibers of porcine right ventricle were freshly obtained from the slaughter house, separated from connective tissue, and treated as described by Heinl et al. (1974).

Psoas muscle fibers from male adult rabbits were used for the skeletal muscle study and were treated as were cardiac muscle fibers. The muscle fibers were then stored at −20°C for up to 1 week.

Measurement of Isometric Tension of Glycerinated Fibers

After mounting on the force transducer (RCA 5734), muscle fibers were washed with a relaxing solution containing 90 mM KCl, 10 mM MgCl\(_2\), 4 mM ethyleneglycol-bis-(β-aminoether)-N,N'-tetraacetic acid (EGTA), 10 mM ATP, and 20 mM imidazole buffer (pH 6.7).
In this condition, the length of the fibers was around 3 mm, cross-sectional area was 0.3 mm², and the sarcomere length was between 2.0 and 2.2 μm as determined by laser diffraction.

After stabilization for 10 minutes at 25°C, isometric tension was induced by changing the incubation medium from the relaxing solution to another solution, as described in each experiment. In the course of the tension measurement study, the incubation mixture was stirred gently, and the cell jacket was circulated in water at 25°C.

**Force Transients after Quick Changes of Muscle Length**

In the steady state of tension development, the muscle length was suddenly (within 0.3 msec.) changed by 0.65% of the initial length, as described earlier (Heinl et al., 1974). The reaction mixture was the same as for the tension measurement study, but was changed 5 seconds before the quick change, to avoid nucleotide triphosphate starvation. The force transducer had a resonance frequency of 10 kHz. The duration of the length change step was 5 seconds. All results were recorded on x-y chart recorder.

**Assay of ATPase and ITPase Activity of Cardiac Myofibrils**

Cardiac myofibrils were prepared from porcine ventricular muscle, as described previously (Solaro et al., 1971). The reaction mixture included 2.75 mg/ml myofibrils, 30 mM KCl, 10 mM MgCl₂, 1 mM Na₃EGTA, 20 mM imidazole buffer (pH 6.7), 4 mM Ca-EGTA buffer (pCa from 8.0 to 5.0). The enzyme reaction was initiated by the addition of either ATP or ITP (10 mM in final concentration), and stopped with the addition of the same volume of ice-cold 15% trichloroacetic acid (TCA). The control was prepared by adding TCA before addition of ATP or ITP.

The amount of inorganic phosphate released was determined by the Rockstein and Heron method (1951). Both ATPase and ITPase activity were assayed in the steady state of inorganic phosphate liberation.

**Miscellaneous**

Protein concentration was determined by the biuret method (Gornall et al., 1949), using bovine serum albumin (Sigma) as a standard.

ATP and ITP, which were obtained from Sigma or Boehringer, were purified further with Dowex-1 column chromatography (Hurlbert, 1957) and passed through chelating resin (Chelex, Sigma) to remove contaminant divalent cations, before mixing with other reagents. The purity of ATP or ITP were checked by thin layer chromatography of polyethyleneimine cellulose (Polygram CEL 300, Macherey-Nagel). Samples were developed in 0.75 mM KH₂PO₄, 10 mM ethylenediamine tetraacetic acid, adjusting the pH to 3.4 with H₃PO₄ (Greene and Eisenberg, 1980).

The ionic strength of the reaction mixtures was adjusted with 3 mM KCl. The final concentrations of ATP or ITP were measured by UV spectrophotometry. The contamination of ATP or ITP by Ca²⁺ was less than 1 μM as analyzed by the atomic absorption method (Sandell, 1959).

Free Ca²⁺ concentrations were calculated, using the apparent binding constants determined by Porzehl et al. (1964). The binding constants of Mg⁺ and Ca²⁺ to ITP were assumed to be of the same order as those to ATP (Sillén and Martell, 1970). The binding constants used here are different from those used by others (Ogawa, 1968). To compare the present results to other data (Toyo-oaka and Ross, 1981), present pCa values should be shifted to the right by 0.7 pCa unit.

**Results**

**Isometric Tension Development with MgATP or MgITP**

After glycerine treatment, cardiac muscle fibers responded to MgATP and Ca²⁺ added in the reaction mixtures. In the presence of 4 mM EGTA (pCa 8.0) and 10 mM ATP, tension did not develop. With stepwise increase of free Ca²⁺ concentrations from pCa 8.0 to 5.0, tension developed (Fig. 1). After changing to pCa 8.0, the muscle relaxed again (Fig. 1).

When MgATP was replaced by MgITP, without changing other conditions, the same muscle fiber developed tension at pCa 8.0. Stepwise increase of free Ca²⁺ concentrations from pCa 8.0 to 5.0 did not change the tension level (Fig. 1). On incubation in 10 mM MgATP at pCa 8.0, the same muscle fiber relaxed again. That fiber developed tension after changing to MgITP, even in the presence of 20 mM EGTA (pCa 10.0). The rigid tension development was induced by adding a nucleotide free solution. It was quite different from the contraction produced by either MgATP or MgITP in both time course and final tension level (Fig. 1).

Using another fiber, clear Ca²⁺ dependency of the force development in MgATP was shown, while no dependency was revealed in MgITP (Fig. 2). Compared with skeletal muscle, cardiac muscle demonstrated a less steep force pCa curve, as we have reported earlier (Ebashi et al., 1975).

Skeletal muscle fibers were prepared from rabbit psoas muscle by the same method as cardiac muscle fibers. ATP at pCa 8.0 did not produce tension. After changing to ITP at pCa 8.0, a partial muscle contraction was induced, and additional tension was demonstrated at pCa 5.0. The developed tension level by MgITP was less than that by MgATP, but was still Ca²⁺ sensitive (Fig. 3). These data from skeletal muscle are in agreement with the ATPase and ITPase activity reported by Weber (1969). Using skeletal muscle myofibrils, she showed a higher...
ITPase activity than ATPase in the absence of Ca$^{2+}$, and lower ITPase activity than ATPase activity in the presence of Ca$^{2+}$. Clear Ca$^{2+}$ dependency was still demonstrated in MgITP, as was the case in the tension level in the present study (Fig. 3).

**Ca$^{2+}$ Sensitivity at Different MgITP Concentrations**

When MgATP concentration is decreased to 10$^{-5}$ M, ATPase activity, or tension development, of mechanically skinned muscle has been shown to exhibit less Ca$^{2+}$ sensitivity (Weber, 1969; Fabiato and Fabiato, 1975b).

To examine this effect with MgITP, we studied the Ca$^{2+}$ sensitivity under different MgITP concentrations. Cardiac muscle fibers, which show normal Ca$^{2+}$ sensitivity in the presence of 10 mM MgATP, did not show Ca$^{2+}$ sensitivity in the presence of MgITP, 5-80 mM (data not shown). The ionic strength of more concentrated MgITP solutions exceeded physiological conditions. Fibers remained relaxed both in the presence and absence of Ca$^{2+}$.

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**Force Transients after Quick Length Changes**

Figure 4A shows the quick (within 0.3 msec) length changes (0.65% of the initial length). In MgATP (pCa 5.0), after quick stretch of the fiber, an elastic phase, a quick phase, and an activation phase were evident. The subsequent quick release to the initial muscle length induced an elastic phase, a quick phase, a plateau phase, and a reactivation phase (Fig. 4B).

These data confirmed the previous results using glycerine-treated skeletal muscle (Heinl et al., 1974) and cardiac muscle (Yamamoto and Herzig, 1978), and were similar to living skeletal muscle (Huxley and Simmons, 1971).

In MgITP (pCa 5.0), sudden stretch showed only an elastic phase without the quick and activation phases seen with MgATP (Fig. 4C). Subsequent quick release induced an elastic phase, but neither a quick phase nor a plateau phase were demonstrated (Fig. 4C).

It has been shown that in the rigor state, cardiac muscle has neither quick phases, an activation phase, a plateau phase, nor a reactivation phase (Yamamoto and Herzig, 1978). Therefore, we measured force transients in rigor. As shown in Figure 4D, the results obtained during rigor were very
FIGURE 4  Force transients after quick stretch and subsequent quick release. A: Length change (0.65% of the initial length) within 0.3 msec. B: Force transients in MgATP at pCa 5.0, C: in MgITP at pCa 5.0, and D: in rigor, which was induced by nucleotide free solution. Ionic strength was adjusted by KCl. Bar length denotes 1 second and 10 mg, respectively.

similar to those in MgITP, although the elastic phase was slightly smaller in rigor than in MgITP.

ATPase and ITPase Activity of Cardiac Myofibrils

As summarized in Figure 5, at pCa 8.0, the phosphate release from MgATP was inhibited, and that inhibition was removed at pCa 5.0. However, the amount of phosphate liberation from MgITP was nearly the same at both pCa 8.0 and 5.0. These data correspond very well with the tension measurements, i.e., Ca\(^{2+}\)-sensitive tension development in MgATP and insensitive development in MgITP (Figs. 1 and 2). These data also indicate that the tension development in MgITP was not rigor tension, because phosphate liberation was measured from MgITP.

Discussion

Previously, Szent-Györgyi's group reported the significance of inosine phosphate in living muscle (McLaughlin et al., 1955), and Hasselbach (1955) has shown the existence of high concentrations of ITP in skeletal muscle. However, the physiological function of ITP has not yet been clarified.

The regulatory mechanism of cardiac muscle and skeletal muscle has been believed to be the same, at least qualitatively (Ebashi, 1980). In skeletal muscle, the steric relationship among the head of myosin (S1), actin, tropomyosin, and troponin was presented by Huxley (1972), Haselgrove (1972), and Potter and Gergely (1974). Following this model, in the absence of Ca\(^{2+}\), tropomyosin is situated in the groove formed by two stranded F-actin filaments to inhibit the interaction between S1 and actin. In the presence of Ca\(^{2+}\), tropomyosin sinks into the groove, allowing S1 to interact with actin. After that, muscle contraction is induced (Potter and Gergely, 1974).

It is difficult to assume that a similar mechanism is working in cardiac muscle, because in MgITP the same tension level was developed at both pCa 8.0 and 5.0 (Figs. 1 and 2), and because the amount of phosphate liberation was not dependent on Ca\(^{2+}\) concentrations (Fig. 5).

Compared with the basal level of myosin ATPase activity at pCa 8.0, the ITPase activity was slightly higher at pCa 8.0 (Fig. 5). In contrast, the tension developed in MgITP at pCa 8.0 was much higher than that in MgATP. This gives the impression that the efficiency of force generation was better when MgITP was used as a substrate, than when MgATP was used. However, the rate of force generation was much slower in MgITP (data not shown). The force generation is regulated by the attachment-detachment cycling of S1 to actin (Solaro et al., 1980). The relationship between force generation and substrate consumption is of great importance (Herzig et al., 1981), although the interpretation of the result is difficult.

The force transients after the quick changes of muscle length suggest that the attachment of S1 to actin in MgITP was similar to that in rigor (Fig. 4). We want to stress that the absence of quick phases does not always denote that the muscle is in rigor, since ITP hydrolysis continues. Furthermore, cardiac fibers can shorten after a 10% release from the initial muscle length (data not shown). This is further evidence that MgITP can be used as a substrate for cardiac muscle contraction and shortening.

In skeletal muscle, Moos has reported that S1 has a higher actin affinity in MgITP than in MgATP (1972). Therefore, S1 in MgITP might cause tropomyosin to move and allow the interaction between S1 and actin even in the absence of Ca\(^{2+}\), as might be the case in the low MgATP concentrations (Bremel and Weber, 1972). Once this linkage is formed, another S1 might bind with actin, because seven actin molecules are governed by one tropomyosin molecule (Bremel and Weber, 1972).

Cardiac muscle troponin may be less potent than skeletal muscle troponin (Toyo-oka and Ross,
1980). This may be another reason why cardiac muscle fibers showed a loss of Ca\(^{2+}\) sensitivity in MgITP, whereas skeletal muscle fibers demonstrated a low but distinct Ca\(^{2+}\) sensitivity in the same solutions (Figs. 1 and 3).

The slopes of force vs. pCa and ATPase activity vs. pCa were less steep in cardiac muscle than in skeletal muscle (Figs. 2 and 5), confirming our previous results (Ebashi et al., 1975). This is not due to long-term glycerin treatment, since cardiac muscle fibers demonstrated similar results after chemical skinnning for only 30 minutes (Endo and Kitazawa, 1978). These data are in contrast with force vs. pCa curves, obtained from mechanically skinned rat heart muscle fibers (Fabiano and Fabio, 1975a). Therefore, the fact that the slope of the force vs. pCa reported in the present study is much lower than that observed in single, mechanically skinned cardiac cells seems to be related to the multicellular nature of the preparation rather than to a deterioration caused by long-term glycerin treatment.

The loss of Ca\(^{2+}\) sensitivity is not thought to be induced by the selective destruction of troponin by the endogenous protease (Toyobo-oka et al., 1978; Toyobo-oka and Masaki, 1979), as was shown in actomyosin prepared from ischemic myocardium (Toyobo-oka and Rose, 1981), because the loss of Ca\(^{2+}\) sensitivity in MgITP is shown to be completely reversible (Fig. 1).

In the present study, we used purified ITP, because contamination by ATP induced some, but not full, Ca\(^{2+}\)-dependent tension development. Most commercially available ITP contains a small amount of ATP.

This paper clearly demonstrates that Ca\(^{2+}\)-dependent regulation of cardiac muscle contraction is drastically changed in MgITP, and that cardiac muscle contractile mechanisms can be dissociated from the regulatory mechanisms in MgITP.

**Acknowledgments**


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Calcium ion-insensitive contraction of glycerinated porcine cardiac muscle fibers by Mg-inosine triphosphate. ITP as a tool to dissociate the contraction mechanism from the regulatory mechanism.

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doi: 10.1161/01.RES.49.6.1350

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1981 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

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