Some Characteristics of Ca\(^{2+}\)-regulated Force Production in EGTA-treated Muscles from Rat Heart

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ABSTRACT McClellan and Winegrad (1980, J. Gen. Physiol., 75:283-295) have reported that in rat ventricular muscles that have reportedly been made "hyperpermeable" to small ions such as Ca\(^{2+}\), CaEGTA\(^{2-}\), and MgATP\(^{2-}\) by a soak in EGTA, the maximum Ca\(^{2+}\)-regulated force can be permanently increased by a short exposure to positively inotropic drugs, such as epinephrine or cAMP plus theophylline, in the presence of the detergent Triton X-100. The experiments reported here were begun as an attempt to repeat and extend this important observation. However, no evidence could be found for a potentiation of force that was not merely produced by Triton alone. In addition, the thickest muscles used (250-440 \(\mu\)m diameter) exhibited very low values for force per unit cross-sectional area, which suggested that either Ca\(^{2+}\) reached only a fraction of the myofibrils or the myofibrils were in a state of low contractility. The results of further experiments that were designed to test the permeability characteristics of these EGTA-treated muscles indicated that the movement of certain ions into these preparations was restricted, even in thin muscles (80-200 \(\mu\)m diameter). The rate of development of Ca\(^{2+}\)-regulated force was slow (\(t_{1/2} \approx 1-3\) min), but was greatly accelerated after the muscles had been superfused with Triton X-100 (\(t_{1/2} \approx 10-20\) s). Removal of creatine phosphate (CP) in the presence of MgATP produced a partial rigor contracture in the EGTA-treated muscles. The results were consistent with the suggestion that the EGTA-treated muscles were permeable to some extent to Ca\(^{2+}\) and HCP\(^{2-}\) ions but not to CaEGTA\(^{2-}\) and MgATP\(^{2-}\). Thus, it would seem unlikely that the [Ca\(^{2+}\)], [MgATP\(^{2-}\)], and [Mg\(^{2+}\)] in the immediate vicinity of the myofibrils in these preparations can be adequately controlled by the solution bathing the muscles.

INTRODUCTION

Winegrad and his collaborators (McClellan and Winegrad, 1978, 1980; Mope et al., 1980; Horowits and Winegrad, 1983; Weisberg et al., 1983; Winegrad et al., 1983) have reported that rat cardiac muscle can be made permeable to small ions.
ions such as Ca\(^{2+}\) and MgATP\(^{2-}\) by a prolonged soak in the Ca\(^{2+}\)-chelator EGTA. In these "hyperpermeable" muscles, as in skinned fibers (in which the sarcolemma has been completely disrupted), the ionic environment of the myofibrils can be directly controlled by the solution bathing the muscle; this allows the contractile properties of the myofibrils to be studied at constant and predetermined concentrations of Ca\(^{2+}\) and other ions. An advantage of the EGTA-treated muscles over skinned muscles is that they may retain the sarcolemmal and cytosolic enzymes and thus allow investigation of cellular processes that can produce enzyme-catalyzed modification of the myofibrils.

It is for these reasons that EGTA-treated cardiac muscles have proved useful in studies on the role of \(\beta\)-adrenergic-mediated phosphorylation of cardiac myofibrils. Once it became known that the positive inotropic action of epinephrine in intact isolated hearts was accompanied by the phosphorylation of troponin-I (England, 1976; Solaro et al., 1976), it seemed an attractive possibility that this phosphorylation could account for some of the increase in contractile force. However, this proved not to be the case, for the phosphorylation of troponin-I in isolated cardiac myofibrils and in EGTA-treated cardiac muscles in vitro was found to shift the sigmoidal \([\text{Ca}^{2+}]\)-ATPase and \([\text{Ca}^{2+}]\)-force relationships to higher Ca\(^{2+}\) concentrations, with little or no change in the maximum ATPase activity or maximum force (Ray and England, 1976; Mope et al., 1980). Thus, after phosphorylation of the myofibrils, a greater \([\text{Ca}^{2+}]\) was required to produce the same ATPase activity or force production as before. From this evidence, it would appear that catecholamines potentiate myocardial contraction solely by increasing the Ca\(^{2+}\) supply to the myofibrils during systole, and do so to such an extent that the inhibitory effects of troponin-I phosphorylation are overcome. There is now evidence that catecholamines do indeed produce large increases in the Ca\(^{2+}\) transients in intact cardiac cells that have been injected with the Ca\(^{2+}\)-sensitive photoprotein aequorin (Allen and Kurihara, 1980; Fabiato, 1981).

However, the direct actions of catecholamines on cardiac myofibrils may not be solely inhibitory, for McClellan and Winegrad (1980) have observed that the maximum Ca\(^{2+}\)-regulated force developed by EGTA-treated rat cardiac muscles can be permanently increased by up to 170\% by a brief exposure to positively inotropic agents such as epinephrine or cAMP plus theophylline if these drugs are applied to the muscle in a solution containing 1\% (vol/vol) of the detergent Triton X-100. This is an important observation because it implies that, in contradiction to the evidence cited above, a part of the positive inotropic effect of catecholamines may be due to a direct modification of the contractile machinery. In the experiments reported in the present paper, we set out to confirm and, if possible, extend the observations of McClellan and Winegrad (1980). Although we followed their experimental protocol closely, we were unable to confirm their results. However, we did find that the EGTA-treated cardiac muscles exhibited characteristics that were not consistent with a high sarcolemmal permeability to CaEGTA\(^{2+}\), MgATP\(^{2-}\), and possibly Ca\(^{2+}\).

Some of these results have been published in preliminary form (Jewell and Kentish, 1982).
METHODS

Experimental Preparation

EGTA-treated muscles from rat ventricles were prepared by the method of McClellan and Winegrad (1978). In brief, male rats (50–150 g, Wistar strain) were killed by cervical dislocation and their hearts were dissected in an oxygenated saline containing (mM): 140 NaCl, 4 KCl, 2.5 MgCl₂, 2.5 CaCl₂, and 10 imidazole, pH 7.2, at 4°C. Loops of fine silk were tied around the ends of selected right ventricular trabeculae, which were then cut out and pinned in a dish at approximately their in situ length. These muscles were then soaked for 24 h at 2–4°C in 20 ml of a disruption solution containing (mM): 140 K propionate, 2 Mg acetate, 10 H₂EGTA, 5 Na₂H₂ATP, 5 imidazole, brought to pH 7.2 at 4°C with KOH. This procedure is reported to make the ventricular cells "hyperpermeable" to small ions such as Ca²⁺, CaEGTA²⁻, and MgATP²⁻ (McClellan and Winegrad, 1978).

After being treated with EGTA, the trabeculae were mounted in a Lucite flow-through muscle bath (volume = 0.5 ml). One end of each muscle was tied to the carbon-fiber extension arm of an isometric force transducer (based on an AE801 strain gauge, Akjeselskapet Mikro-Elektronikk, Horten, Norway), and the other end was tied to a hook that was fixed to a micropositioner. The force transducer was connected to a bridge preamplifier and force and its first derivative \( \frac{dF}{dt} \) were displayed on a Gould (Cleveland, OH) chart recorder. Experimental solutions at 20°C were pumped through the muscle bath at 1 ml/min and were sucked away by a vacuum line.

The muscles could be made to contract and relax by superfusions with activating solutions of \([Ca^{2+}] = 2.4–190 \mu M\) and relaxing solutions of \([Ca^{2+}] \approx 1 \mu M\) (see below for details of solutions). The resting force was defined as the steady force obtained with the muscle in a relaxing solution, and the Ca²⁺-regulated force was defined as the difference between the steady total force obtained with the muscle in an activating solution and the mean of the resting forces before and after the perfusion with activating solution. To ensure that the sarcomere length was approximately constant from muscle to muscle, the muscles were stretched progressively until the Ca²⁺-regulated force measured at the optimal \([Ca^{2+}] = 190 \mu M\) was maximal. (It was not possible to set the sarcomere length to a known value by laser diffraction because the EGTA-treated muscles gave no distinct diffraction patterns from a 632.8-nm laser beam.) Once the muscles had been stretched to approximately \(L_{max}\) in this way, muscle length, width, and depth were measured with an eyepiece graticule fitted in the dissecting microscope. Muscles were usually round or slightly ellipsoid in cross section. Cross-sectional area was calculated on the assumption that the cross sections were perfectly circular or ellipsoid. In this paper the “diameter” of each muscle is defined as the mean of the width and depth measurements.

These experiments were originally designed to study the Ca²⁺-regulated force production of EGTA-treated rat trabeculae before and after treatment of the muscles with relaxing solution containing 1% (vol/vol) Triton plus 0.5 \mu M epinephrine or plus 1 \mu M cAMP plus 5 mM theophylline. These were the drugs that were reported by McClellan and Winegrad (1980) to produce the largest potentiation of force. Each muscle was superfused according to one of the following protocols (McClellan and Winegrad, 1980): (a) relaxing solution, relaxing solution plus 0.5 \mu M epinephrine (5 min), relaxing solution plus 0.5 \mu M epinephrine plus 1% Triton X-100 (30 min), relaxing solution (30 min); (b) relaxing solution, relaxing solution plus 5 mM theophylline (5 min), relaxing solution plus 5 mM theophylline plus 1 \mu M cAMP (10 min), relaxing solution plus 5 mM theophylline plus 1 \mu M cAMP plus 1% Triton X-100 (30 min), relaxing solution (30 min). Other
changes in solution composition (described in detail in the Results) were made to assess the permeability of the muscles before and after Triton treatment.

**Solutions**

The relaxing solution contained 140 mM KCl, 7 mM MgCl₂, 5 mM Na₂H₂ATP, 15 mM Na₂HCP, 0.1 mg/ml creatine kinase, 10 mM H₄EGTA (nominal concentration, see below), and 25 mM imidazole, pH 7.0, at 20°C. Activating solutions had the same composition, except that some or all of the EGTA was replaced by 4–10 mM CaEGTA. The free ionic concentrations in relaxing and activating solutions were calculated using a computer program that solved the set of equations which described the multiple equilibria in the solutions. The values for the true affinity constants were taken from Martell and Smith (1974), except for the following (in log,u form): 10.90 EGTA⁺⁻/Ca²⁺, 5.33 HEGTA⁺⁻/Ca²⁺ (Kentish, 1984); 1.15 HCP⁺⁻/Ca⁺⁺, 1.50 HCP⁺⁻/Mg⁺⁺ (Smith and Alberty, 1956). The constants for equilibria involving protons were converted from concentration constants to mixed constants by the addition of 0.11 log unit (Martell and Smith, 1974). The calculated free concentrations of some ionic species in the solutions were: 140 mM K⁺, 40 mM Na⁺, 1.72–2.13 mM Mg²⁺, 1 nM (relaxing solution) and 2.4–190 μM (activating solutions) Ca⁺⁺, 4.2–4.3 mM MgATP⁻⁻, 140 mM Cl⁻. The total ionic strength, which was calculated taking into account all ionic species, was 0.23 M. In some experiments, Na₂H₂ATP and Na₂HCP were replaced by NaCl, with [MgCl₂] adjusted to keep [Mg⁴⁺] constant and [KCl] raised to keep the ionic strength constant.

The effective total concentration of EGTA in relaxing solution and the ratio [total EGTA]/[total Ca] were measured by the pH-metric method of Moisescu and Pusch (1975), but imidazole was used instead of TES [N-tris(hydroxymethyl)-methyl-2-aminoethane sulfonic acid]. The 10 mM EGTA (nominal concentration, based on the weight of dried EGTA) was found to be effectively 9.60 mM. Most of the discrepancy was due to the impurity of EGTA (measured purity = 96.2%); the remainder was due to the binding of contaminant Ca, which was found to be ≈20 μM by atomic absorption spectroscopy in 1% LaCl₃. The computation of the free ionic concentrations took into account both the impurity of EGTA and the contaminant Ca.

Solution pH was checked with a three-place digital pH meter (model 130; Corning Medical and Scientific, Medfield, MA) in combination with separate glass pH and calomel reference electrodes. The electrodes were calibrated with phthalate and phosphate standards and were tested for sensitivity to the ionic strength of the solutions (cf. Illingworth, 1981; see Kentish, 1984).

**Chemicals**

Analytical reagent-grade chemicals were obtained from BDH Chemicals Ltd. (Poole, Dorset, England) except for imidazole, Triton X-100, Na₂HCP, caffeine, CCCP (carbonyl cyanide m-chlorophenylhydrazone), dithiothreitol (DTT), creatine kinase, cAMP, and theophylline, which were supplied by Sigma Chemical Co. (Poole, Dorset, England).

**RESULTS**

Fig. 1 shows a chart record of the forces developed by a typical EGTA-treated muscle at various concentrations of Ca²⁺ before and after treatment with relaxing solution plus 1% (vol/vol) Triton X-100 plus 1 μM cAMP plus 5 mM theophylline. The development of Ca²⁺-regulated force in the EGTA-treated muscles before exposure to drugs plus detergent was slow: the time taken to reach half the final force in a contracture (the half-time) was usually 1–3 min at the optimal [Ca²⁺]
of 190 μM and was even greater at a lower [Ca\(^{2+}\)], e.g., the half-time was >5 min at [Ca\(^{2+}\)] = 3.6 μM (Fig. 1a). However, after superfusion of the muscles with Triton X-100 (with or without inotropic drugs), force development was much faster, with the half-time sometimes being as little as 10 s (Fig. 1b). In 20 muscles the half-time for the development of a contracture in 190 μM Ca\(^{2+}\) was significantly decreased (paired t test, P < 0.01) from 103.3 ± 18.7 s (mean ± SE) to 16.6 ± 3.7 s by treatment of the muscles with Triton X-100. Thus, a plateau of

![Figure 1](image-url)

**FIGURE 1.** Record of force in an EGTA-treated rat trabecula before, during, and after superfusion of the muscle with 1 μM cAMP plus 5 mM theophylline plus 1% Triton X-100 in relaxing solution. In both panels the upper trace shows the force developed by the muscle and the lower trace shows a schematic representation of the solution changes. The muscle was first superfused with relaxing solution containing ≈1 nM Ca\(^{2+}\) and activating solutions containing 2.4–190 μM Ca\(^{2+}\). Then the muscle was bathed in the following substances dissolved in relaxing solution: 5 mM theophylline (5 min), 5 mM theophylline plus 1 μM cAMP (10 min), 5 mM theophylline plus 1 μM cAMP plus 1% Triton X-100 (30 min). The previous relaxing and activating solutions were then reapplied to the trabecula. The muscle was also superfused for 3 min with 5 mM dithiothreitol (DTT) in relaxing solution, and was stretched (L\(_r\)) to return resting force to its pre-Triton level. Note that the chart speed is the same in the two panels. Muscle diameter, 100 μm.
force was attained much more quickly after Triton X-100 and so the perfusion time of each solution could be reduced, as is shown in Fig. 1. Triton decreased the \([\text{Ca}^{2+}]\) required to produce 50% of maximum force from 5.55 ± 0.38 \(\mu\text{M}\) (mean ± SE, \(n = 15\)) to 4.85 ± 0.30 \(\mu\text{M}\). This small decrease, which was equivalent to an increase of 0.07 pCa unit, was statistically significant (paired \(t\) test, \(P < 0.01\)).

It is clear that in the muscle shown in Fig. 1, the maximum \(\text{Ca}^{2+}\)-regulated force (achieved at \([\text{Ca}^{2+}] = 190 \mu\text{M}\)) was smaller after treatment with Triton X-100 plus drugs than before. However, a comparison of the forces before and after Triton was complicated by two factors. First, non-ionic detergents such as Triton X-100 can contain sulfhydryl-oxidizing agents (Chang and Bock, 1980), which would decrease the maximum force production of the cardiac myofibrils (Jewell and Kentish, 1981). To regenerate any oxidized sulfhydryl groups, 5 mM DTT was added to the relaxing solution (Jewell and Kentish, 1981). Maximum \(\text{Ca}^{2+}\)-regulated force was usually potentiated, but only slightly, by this addition (Fig. 1b).

The second problem was that the resting force often decreased during superfusion of the muscles with solutions containing Triton X-100. Thus, it was possible that sarcomere length had altered during this period, with the consequence that the muscle was no longer at \(L_{\text{max}}\). Accordingly, the Triton-treated muscles were stretched to return their resting force to the pre-Triton value. Usually this procedure increased the \(\text{Ca}^{2+}\)-regulated force, but even after the muscles had been treated with DTT and had been stretched, the maximum \(\text{Ca}^{2+}\)-regulated force was only approximately the same after treatment with Triton X-100 plus drugs as before treatment (Fig. 1). Thus, muscle force was not markedly potentiated after superfusion of the muscle with Triton X-100 plus drugs.

It seemed possible that the differences between these results and those of McClellan and Winegrad (1980), who observed substantial increases in force in muscles treated with Triton plus drugs, was due to the thickness of trabeculae used. The muscles routinely used in this laboratory are <200 \(\mu\text{m}\) in diameter (e.g., that in Fig. 1 = 100 \(\mu\text{m}\)), whereas McClellan and Winegrad (1980) probably used muscles of diameter 200–400 \(\mu\text{m}\) (estimated from data in Mope et al., 1980). The experiments were therefore repeated with thicker muscles.

The collected results from 16 muscles of diameter 60–440 \(\mu\text{m}\) are given in Fig. 2. This figure shows how the muscle diameter influenced the degree of potentiation of \(\text{Ca}^{2+}\)-regulated force by Triton, with or without drugs (Fig. 2a), and the maximum force per unit cross-sectional area in the EGTA-treated muscles prior to Triton treatment (Fig. 2b). It should be emphasized that for the comparison of forces before and after Triton X-100 (Fig. 2a), the “force before Triton” was taken as the \(\text{Ca}^{2+}\)-regulated force at \([\text{Ca}^{2+}] = 190 \mu\text{M}\) in the last contracture before the muscle was superfused with Triton, and the “force after Triton” was taken as the greatest \(\text{Ca}^{2+}\)-regulated force at \([\text{Ca}^{2+}] = 190 \mu\text{M}\) that was obtained either before or after treating the muscle with DTT or stretching the muscle. In general, superfusion of the EGTA-treated muscles with Triton plus cAMP or epinephrine inhibited maximum \(\text{Ca}^{2+}\)-regulated force if the muscle diameter was below \(~250 \mu\text{m}\), but in muscles thicker than this, the force was
potentiated by up to 50% (Fig. 2a). However, the same relationship between muscle diameter and the potentiation of force was also seen with Triton X-100 alone; thus, the increase of force in the thicker muscles after superfusion with detergent plus drugs appeared to be an effect of the detergent rather than of the drugs.

Fig. 2b shows the maximum force per unit cross-sectional area in the EGTA-

![Graph](image)

**Figure 2.** Dependence of the contractile properties of the EGTA-treated muscles upon muscle diameter. (a) Ratio between the greatest force obtained in 190 μM Ca²⁺ after the muscle had been treated with Triton and the force obtained in 190 μM Ca²⁺ in the last contracture before Triton. The muscles were bathed in relaxing solution containing 1% Triton X-100 alone (●), Triton X-100 plus 0.1 μM adrenaline (□), or Triton X-100 plus 5 mM theophylline plus 1 μM cAMP (△). (b) Force per unit cross-sectional area in the EGTA-treated muscles in 190 μM Ca²⁺ in the last contracture before Triton. Although the muscles had not been superfused with Triton, with or without drugs, at this stage, the symbols used in a have been used in b so that the two properties can be seen for each muscle.

treated muscles before superfusion with Triton X-100. The thinnest preparations (diameter 60–150 μm) developed a force of ~20–50 mN/mm². However, the force per unit area decreased with an increase in muscle diameter, so that at a muscle diameter of ~300 μm, the force per unit area was only ~10% of its value at a diameter of 100 μm. Thus, the contractile performance of the EGTA-treated trabeculae depended greatly upon muscle diameter.
The characteristics of the EGTA-treated muscles before treatment with Triton X-100 (Figs. 1 and 2b) were not as would be expected if all the myofibrils in these muscles could be rapidly and completely activated by Ca\(^{2+}\) from the bathing solution. Further experiments were therefore undertaken to investigate whether the EGTA-treated muscles were freely permeable to Ca\(^{2+}\), CaEGTA\(^{2-}\), Mg-ATP\(^{2-}\), and other small ions. To aid comparisons between different types of contractures, all the following chart records are from the same muscle (diameter 90 \(\mu\)m); the responses were similar in five other muscles.

One salient feature of every EGTA-treated muscle was that the development of Ca\(^{2+}\)-regulated force was very slow before the muscle had been treated with Triton X-100 and was much faster afterward (e.g., Fig. 1). This observation was consistent with the idea that Triton removed a functional barrier to Ca\(^{2+}\) or CaEGTA\(^{2-}\) diffusion into the muscle. To establish whether this barrier was specific for Ca\(^{2+}\) (or CaEGTA\(^{2-}\)) or extended to other ions, such as MgATP\(^{2-}\) and HCP\(^{2-}\), the rate of force development in a Ca\(^{2+}\)-regulated contracture was compared with the rate of relaxation of a rigor contracture after MgATP\(^{2-}\) and HCP\(^{2-}\) had been added back to the solutions. This was done both before and after superfusion of the muscle with Triton X-100. Theoretically, in these multicellular preparations the development of force in a Ca\(^{2+}\)-regulated contracture should reflect the rate of entry of Ca\(^{2+}\) (or CaEGTA\(^{2-}\)) into the muscle, while the rate of relaxation of a rigor contracture should reflect the rate of entry of MgATP\(^{2-}\) or HCP\(^{2-}\). (In fact, evidence is presented below which suggests that at least some of the EGTA-treated cells were not freely permeable to MgATP\(^{2-}\), so it is likely that the rate of relaxation of a rigor contracture reflects the rate of entry of HCP\(^{2-}\) alone.) Fig. 3a shows results from a typical muscle prior to treatment with Triton X-100. A Ca\(^{2+}\)-regulated contracture at the optimal \([\text{Ca}^{2+}]\) of 190 \(\mu\)M reached half-maximal force in \(-1\) min, but \(-10\) min was required for steady force to be reached. The muscle was subsequently bathed in relaxing solution for 15 min and was then put into rigor by removal of ATP and CP from the solution. Rigor contractures exhibited a characteristic biphasic shape, with a peak force about half of that in a maximal Ca\(^{2+}\)-regulated contracture. When the ATP and CP were resupplied, the muscle relaxed fairly rapidly. The absolute magnitude of the peak \(dF/dt\) during relaxation from rigor was approximately four times as great as the peak \(dF/dt\) during development of Ca\(^{2+}\)-regulated force.

After the same muscle had been superfused for 30 min with 1% Triton X-100 in relaxing solution, the peak rate of development of Ca\(^{2+}\)-regulated force was increased fivefold (Fig. 3b), even though in this case the maximum Ca\(^{2+}\)-regulated force was in fact 20% less than before. By contrast, the peak rate of relaxation of a rigor contracture was slightly less than that before Triton. However, it should be noted that the absolute magnitude of force at the end of the rigor contracture in the Triton-treated muscle was only 60% of that before Triton; if this decrease in force was taken into account, the normalized peak \(dF/dt\) during relaxation of the rigor contracture was in fact 25% greater than before Triton. This faster relaxation after Triton X-100 was also apparent from the shortening of the time course of the negative \(dF/dt\) transient. However, this small increase
Figure 3. Ca\textsuperscript{2+}-regulated contractures and rigor contractures before (a) and after (b) superfusion of an EGTA-treated muscle with 1% Triton X-100 in relaxing solution. In each panel, the top trace shows the time differential of force, the middle trace shows force, and the bottom trace illustrates schematically the changes in solution composition. Ca\textsuperscript{2+}-regulated contractures were induced by a change from the relaxing solution of \([\text{Ca}\textsuperscript{2+}] = 1 \text{ nM}\) to an activating solution of \([\text{Ca}\textsuperscript{2+}] = 190 \mu\text{M}\). Rigor contractures were induced by replacement of the ATP and CP in the relaxing solution by NaCl and KCl. To assess muscle stiffness, the muscles were occasionally given transient releases (\(\downarrow\)) or stretches (\(\uparrow\)) of 10 \(\mu\text{m}\) (small arrows) or 50 \(\mu\text{m}\) (large arrows). Muscle diameter, 90 \(\mu\text{m}\).
in the rate of relaxation from a rigor contracture was very much less than the fivefold increase in the rate of development of a Ca\(^{2+}\)-regulated contracture.

To investigate whether the very slow development of Ca\(^{2+}\)-regulated force in the EGTA-treated muscles before Triton was the result of active sequestration of Ca\(^{2+}\) within the muscles, 10 mM caffeine or 10 \(\mu\)M CCCP was added to the bathing solutions to block Ca\(^{2+}\) uptake by the sarcoplasmic reticulum (Weber, 1971) or mitochondria (Heydt and Prichard, 1962), respectively, or 5 mM NaN\(_3\) was added to block mitochondrial ATPase activity (Fambourg, 1964). Typical results are shown in Fig. 4. Like skinned muscle preparations, the EGTA-treated muscles exhibited a gradual loss of contractile performance in repeated contractures. To allow for this, "control" Ca\(^{2+}\)-regulated contractures with no added inhibitors were produced before (trace 1 in Fig. 4) and after (trace 5) the contractures with inhibitors present (traces 2-4). Caffeine had no effect on the
rate of development of Ca\(^{2+}\)-regulated force, although it did appear to decrease slightly the steady force attained. Both NaN\(_3\) and CCCP accelerated force development to a small extent, but the contractures were still very slow compared with those in the same muscles after treatment with Triton X-100 (e.g., Fig. 3).

In a preliminary communication, Elder et al. (1981) reported that the removal of HCP\(_2^-\) from a relaxing solution bathing an EGTA-treated rat trabecula resulted in a rigor-like contracture, even if the [MgATP\(^{2-}\)] in the solution was maintained. If this was so, the muscles cannot have been fully permeable to MgATP\(^{2-}\). In a similar experiment, shown in Fig. 5a, the 15 mM Na\(_2\)HCP in the relaxing solution was replaced by NaCl and KCl at a constant concentration (4.3 mM) of MgATP\(^{2-}\). This change induced a contracture, which relaxed again when the Na\(_2\)HCP was resupplied. Transient 10-\(\mu\)m stretches of the muscle (arrows, Fig. 5) showed that the long-term stiffness of the muscle during this "zero CP" contracture was much greater than during a Ca\(^{2+}\)-regulated contracture (small arrows, Fig. 5a), but was somewhat less than during a rigor contracture induced by removal of both ATP and CP (arrows, Fig. 3b). After the muscle had been superfused with 1% Triton X-100 in relaxing solution for 30 min, removal of the Na\(_2\)HCP had no effect on resting force (Fig. 5b), as would be expected for a muscle that was permeable to MgATP\(^{2-}\).

**DISCUSSION**

A consistent finding in these experiments was that the development of force in a Ca\(^{2+}\)-regulated contracture was very slow in the EGTA-treated trabeculae, but was accelerated greatly after superfusion of the muscles with 1% Triton X-100. This implies that the detergent disrupted or removed a functional obstacle to the diffusion of Ca\(^{2+}\) or CaEGTA\(^{2-}\) into the EGTA-treated muscles. This barrier could have been a physical structure such as the sarcolemma, which in EGTA-treated muscles appears abnormal but intact (McClellan and Winegrad, 1978) and which is impermeable to extracellular La\(^{3+}\) (Elder et al., 1981). By contrast, if living cardiac muscle is skinned with 1% Triton X-100, the sarcolemma is disrupted to such an extent that it becomes permeable to large molecules such as ferritin (Kentish, 1982). Alternatively, a functional barrier to Ca\(^{2+}\) or Ca-EGTA\(^{2-}\) diffusion would have been the result if Ca\(^{2+}\) were bound or taken up by intracellular organelles before reaching the myofibrils, i.e., if there were a powerful Ca\(^{2+}\) "sink" in the muscle cells. Both the sarcoplasmic reticulum (SR) and mitochondria have Ca\(^{2+}\)-sequestering activity and both may be active under certain conditions in the EGTA-treated muscles (McClellan and Winegrad, 1978; Weisberg et al., 1983). The action of Triton to accelerate the development of Ca\(^{2+}\)-regulated force could then be explained by the destruction of these organelles by this detergent (though even this hypothesis assumes some restriction of Ca\(^{2+}\) entry into the EGTA-treated cells; otherwise, the Ca\(^{2+}\)-sequestering processes would quickly become saturated with Ca\(^{2+}\) and force would rapidly attain a steady level). However, CCCP and caffeine, inhibitors of mitochondrial and SR Ca\(^{2+}\) uptake, respectively (Heytler and Prichard, 1962; Weber, 1971), failed to reproduce the pronounced acceleration of Ca\(^{2+}\)-regulated force that was seen after treatment of the muscles with Triton. It would therefore appear that either
the putative Ca\(^{2+}\) sink did not involve the SR and mitochondria, or, as seems more likely, the access of Ca\(^{2+}\) to the myofibrils in the EGTA-treated muscles was limited not by the intracellular sequestration of Ca\(^{2+}\) but by a restriction of Ca\(^{2+}\) entry through the sarcolemma. The observation that superfusion of the muscles with Triton accelerated the rate of development of Ca\(^{2+}\)-regulated force (which probably reflects the rate of entry of Ca\(^{2+}\) or CaEGTA\(^{2-}\) into the muscle) to a much greater extent than the rate of relaxation of a rigor contracture (which in these muscles probably reflects the rate of entry of HCP\(^{2-}\)) suggests that Triton removed a functional barrier that restricted the movement of Ca\(^{2+}\) or CaEGTA\(^{2-}\) but did not greatly impede the movement of HCP\(^{2-}\).

In spite of this evidence for a restriction on the diffusion of Ca\(^{2+}\) or CaEGTA\(^{2-}\) into the EGTA-treated trabeculae, the muscles must have been permeable to some extent to Ca\(^{2+}\) because the steady force produced at the optimal bathing [Ca\(^{2+}\)] of 190 \(\mu\)M was little altered after treatment of the muscles with Triton X-100 alone (Figs. 1 and 2a). Thus, it would seem likely that when a steady force had been attained, the [Ca\(^{2+}\)] surrounding the myofibrils was the same before and after Triton, provided that the other ionic conditions were constant (though this may not have been so; see below). One way in which these observations may be assimilated is by assuming that the EGTA-treated muscles were permeable to Ca\(^{2+}\) but not to CaEGTA\(^{2-}\); in this case, the [Ca\(^{2+}\)] in the immediate vicinity of the myofibrils would have equilibrated with the bathing [Ca\(^{2+}\)], but only slowly because of the high Ca\(^{2+}\)-buffering capacity of the myofibrils and possibly other intracellular binding sites. In contrast, the Triton-treated muscles would have been permeable to CaEGTA\(^{2-}\) because Triton essentially destroys the sarcolemma (Kentish, 1982), and so equilibration of the [Ca\(^{2+}\)] between the inside and the outside of the muscles would have been much faster.

The idea that the EGTA-treated muscles were permeable to small ions such as Ca\(^{2+}\) (\(\approx 40\) mol wt) but not to larger ions such as CaEGTA\(^{2-}\) (\(\approx 418\) mol wt) is supported by experiments in which HCP\(^{2-}\) was removed from the relaxing solution. Like Elder et al. (1981), we found that removal of HCP\(^{2-}\) in the absence of added Ca\(^{2+}\) induced a contracture, even though 4.3 mM MgATP\(^{2-}\) was present (Fig. 5). The stiffness of the muscle during this contracture indicated that some of the myofibrils had gone into rigor. Thus, at least some of the EGTA-treated cells cannot have been permeable to MgATP\(^{2-}\) (\(\approx 572\) mol wt). However, the cells were permeable to the smaller ion HCP\(^{2-}\) (\(\approx 209\) mol wt), since the reapplication of HCP\(^{2-}\) relaxed the muscles once more. Consequently, the [MgATP\(^{2-}\)] inside these EGTA-treated muscle cells must have been maintained not by the MgATP\(^{2-}\) but by the HCP\(^{2-}\) in the bathing solution (in combination with the residual creatine kinase in these muscles; McClellan and Winegrad, 1978).

However, the concept that the EGTA-treated cells were leaky only to molecules below a certain size may be too simplistic. It is possible that the permeant molecules entered via specific carrier mechanisms rather than through nonspecific holes in the sarcolemma. For example, Ca\(^{2+}\) (but not CaEGTA\(^{2-}\)) could have entered via the Na\(^{+}\)/Ca\(^{2+}\) exchange, as it appears to be able to do in EGTA-treated muscle from frog heart (Miller, 1979).
It should also be pointed out that the cells of amphibian cardiac muscles can be loaded with photoproteins during a 30–40-min exposure to EGTA (Sutherland et al., 1980; Morgan and Morgan, 1982). Thus, we have the apparent paradox that mammalian cardiac muscles treated for 24 h with EGTA do not seem to be freely permeable to small ions such as CaEGTA$^{2-}$ or MgATP$^{2-}$, whereas amphibian cardiac muscle becomes permeable to molecules as large as proteins during a much shorter soak in EGTA. This is probably not due to a difference between animal classes, because amphibian cardiac muscle does not appear to be freely permeable to small ions after a 24-h treatment with EGTA (Miller, 1979).

One possible reason for this discrepancy is that the photoproteins entered the cells by a process such as pinocytosis, which does not depend upon a nonselective breakdown of the sarcolemma. Another possibility is that the cardiac sarcolemma is made highly permeable to proteins during the 24 h in the EGTA-containing disruption solution, but that it reseals afterwards. For example, this could arise if the high permeability to proteins was caused not by the presence of EGTA (or the absence of Ca$^{2+}$), but by an excess of ATP over Mg. In all these studies (McClellan and Winegrad, 1978; Sutherland et al., 1980; Morgan and Morgan, 1982) and in the present experiments, the disruption solution contained 5 mM total ATP and 2 mM total Mg. The amphibian cells, at least, could be resealed by increasing the [Mg] to 10 mM (and thereby decreasing the [free ATP]), even though EGTA was still present (Sutherland et al., 1980; Morgan and Morgan, 1982). In a similar way, mast cells can be made permeable with ATP (probably as ATP$^+$) and this is reversed by the addition of excess Mg (Gomperts, 1983).

Thus, it is possible that the EGTA-treated muscles became highly permeable to molecules as large as proteins during the exposure to the disruption solution, but that on exposure to relaxing and activating solutions (which contain a 2-mM excess of total Mg over total ATP), the sarcolemma resealed and the high permeability to proteins was lost. If this is so, it raises the possibility that cytosolic proteins could be washed out during the period in disruption solution.

Miller (1979) originally suggested that frog ventricular muscle was not made permeable to small ions by treatment with EDTA or EGTA, and in a preliminary report Elder et al. (1981) indicated that the same may be true for rat ventricular muscle. The observations reported in the present paper substantiate the results of Elder et al. (1981). For this reason, we have chosen to refer to these preparations as EGTA-treated muscles rather than as hyperpermeable muscles. The importance of this is that if the sarcolemma in the EGTA-treated cardiac cells is not highly permeable to MgATP$^{2-}$ and CaEGTA$^{2-}$, then the concentrations of MgATP$^{2-}$, Ca$^{2+}$, and Mg$^{2+}$ in the local environment of the myofibrils may not approximate the concentrations in the bathing solution and could vary in an unknown way throughout the experiment. Under these conditions, changes in the sarcolemmal permeability or in the activities of any non-myofibrillar ATPases (e.g., the SR Ca$^{2+}$-ATPase) could alter the ionic milieu of the myofibrils and could thereby indirectly alter force production. For example, stimulation of the SR Ca$^{2+}$-ATPase by phosphorylation could produce an increase in pH (caused by increased CP hydrolysis), which would lead to an increase in the force
production. It should be pointed out that this applies only to EGTA-treated multicellular preparations of cardiac muscle, for there is considerable evidence that EGTA-treated single fibers from skeletal muscle are adequately skinned (Katz et al., 1978).

The low effective permeability of the EGTA-treated muscles to CaEGTA\(^{2-}\) and MgATP\(^{2-}\) may have made it impossible for these ions to reach the inner myofibrils in the muscles of diameter exceeding \(\sim 200 \mu m\). This would explain why the force per unit cross-sectional area in the thicker muscles was much less than would have been expected if all the myofibrils in the muscles had been fully activated with Ca\(^{2+}\) (Fig. 2). Alternatively, some or most of the myofibrils in the thicker muscles may have been in a state of low contractility. This possibility is favored by the observation that Triton, although it greatly increased the membrane permeability to CaEGTA\(^{2-}\) and MgATP\(^{2-}\), did not restore normal contractile performance in the thicker muscles (Fig. 2). Whatever the reason for the poor contractile performance of the thicker EGTA-treated muscles, it would seem advisable to use muscles of diameter less than \(\sim 200 \mu m\) to ensure that all the myofibrils can be activated by Ca\(^{2+}\) and are capable of normal contractile performance.

The thinnest EGTA-treated rat trabeculae developed a force of 20-50 mN/mm\(^2\), which is comparable to the force developed by living trabeculae that had been skinned with detergents (49 ± 5 mN/mm\(^2\), mean ± SE, \(n = 7\), Hibberd and Jewell, 1982; 29 ± 4 mN/mm\(^2\), mean ± SE, \(n = 22\), J. C. Kentish, unpublished observations). These values are considerably smaller than those for mechanically skinned single cells from rat ventricle (\(\approx 110 \) mN/mm\(^2\), Fabiato, 1981), chiefly because the thinnest trabeculae often contain much connective tissue, which adds to muscle diameter without adding to the Ca\(^{2+}\)-regulated force (Kentish, 1982).

Superfusion of the EGTA-treated muscles for 30 min with relaxing solution plus 1% Triton X-100 by itself had little effect on maximum Ca\(^{2+}\)-regulated force in muscles of diameter less than \(\sim 200 \mu m\) (Fig. 2). In muscles thicker than this, the Ca\(^{2+}\)-regulated force seemed to be potentiated slightly after the detergent treatment, to an extent that increased with muscle diameter; this was probably the result of Triton X-100 skinnning a small percentage of the cells that were effectively impermeable to Ca\(^{2+}\) or CaEGTA\(^{2-}\) in the EGTA-treated muscle. These results confirm those of McClellan and Winegrad (1980), who found a small but statistically nonsignificant potentiation of force after Triton. On the other hand, we could find no evidence for a marked potentiation of Ca\(^{2+}\)-regulated force after the muscles had been superfused with 0.5 \(\mu M\) adrenaline or 1 \(\mu M\) cAMP plus 5 mM theophylline in the presence of 1% Triton X-100 (Figs. 1 and 2). McClellan and Winegrad (1980) reported increases of force of 173 and 159%, respectively, after treatment of the muscles with these concentrations of adrenaline and cAMP plus theophylline; these increases were observed without the muscles being stretched or treated with DTT after the superfusion with Triton. In the present experiments, there was some slight potentiation of force in the thicker muscles, but the increase in force was no different from that found after Triton X-100 without drugs. In the muscles of diameter <200 \(\mu m\), potentiation of force was never observed, whether or not drugs were applied.
with the detergent. The reason for the discrepancy between these results and those of McClellan and Winegrad (1980) is not clear. The only difference between the experimental protocols was that we used an EGTA concentration of 10 mM rather than 3 mM to provide better buffering of Ca^{2+}, but in preliminary experiments we used an EGTA concentration of 3 mM and the results were no different from those reported here. Although Winegrad and his collaborators (Winegrad and McClellan, 1979; Winegrad et al., 1983) have pointed out that the degree of force enhancement depends on the hormonal and isozymic status of the cardiac muscle immediately before it is treated with EGTA, all of the relevant factors in our experiments would tend to maximize the potentiation of force.

For example, Winegrad and McClellan (1979) have observed that the potentiation of force after cAMP plus theophylline plus detergent is somewhat diminished if there was considerable β-adrenergic stimulation in the living muscle. However, the initial degree of β-adrenergic stimulation, as measured by the increase in the pCa for 50% Ca^{2+} activation after detergent treatment (Winegrad and McClellan, 1979), was very small in our preparations. In any case, the force enhancement observed with epinephrine plus detergent is not affected by the β-adrenergic status of the living muscle (Winegrad and McClellan, 1979). Another important factor is the age of the rats. As the rats become older, the potentiation seen with cAMP plus theophylline plus Triton decreases, whereas that seen in Triton alone increases, apparently because of the shift in the relative concentrations of the V₁ and V₅ myosin isozymes (Winegrad et al., 1983). The rats used in the present study were ~3–6 wk old; at this age, the increase in force should have been very large (cf. Winegrad et al., 1983). Furthermore, the magnitude of the force potentiation was not correlated with the weight (and thus presumably with the age) of the rats (unpublished results).

Thus, it would seem that there is some other, as yet undefined detail of the procedure used by McClellan and Winegrad (1980) that is necessary for ensuring that the EGTA-treated muscles are responsive to treatment with Triton plus inotropic drugs.

Taken together, the results in Fig. 2, a and b, demonstrate that even after the potentiation by Triton (with or without inotropic drugs), the force produced in the thicker muscles was still much less than expected if all the myofibrils in the muscle were maximally activated. In fact, this would have been so even if, like McClellan and Winegrad (1980), we had observed a two- or threefold increase in force after Triton plus drugs.

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