Ca\(^{2+}\) Dependence of Transverse Tubule-mediated Calcium Release in Skinned Skeletal Muscle Fibers

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ABSTRACT Isometric force and \(^{45}\)Ca efflux from the sarcoplasmic reticulum were measured at 19°C in frog skeletal muscle fibers skinned by microdissection. After Ca\(^{2+}\) loading, application of the ionophores monensin, an Na\(^+(K^+)/H^+\) exchanger, or gramicidin D, an H\(^+\) > K\(^+\) > Na\(^+\) channel-former, evoked rapid force development and stimulated release of \(\sim 30\%\) of the accumulated \(^{45}\)Ca within 1 min, whereas CCCP (carbonyl cyanide pyruvate p-trichloromethoxyphenylhydrazone), a protonophore, and valinomycin, a neutral, K\(^+\)-specific ionophore, did not. When monensin was present in all bathing solutions, i.e., before and during Ca\(^{2+}\) loading, subsequent application failed to elicit force development and to stimulate \(^{45}\)Ca efflux. 5 min pretreatment of the skinned fibers with 50 \(\mu\)M digitoxin, a permeant glycoside that specifically inhibits the Na\(^+\),K\(^+\) pump, inhibited monensin and gramicidin D stimulation of \(^{45}\)Ca efflux; similar pretreatment with 100 \(\mu\)M ouabain, an impermeant glycoside, was ineffective. Monensin stimulation of \(^{45}\)Ca efflux was abolished by brief pretreatment with 5 mM EGTA, which chelates myofilament-space calcium. These results suggest that: (a) monensin and gramicidin D stimulate Ca\(^{2+}\) release from the sarcoplasmic reticulum that is mediated by depolarization of the transverse tubules, which seal off after sarcolemma removal and form closed compartments; (b) a transverse tubule membrane potential (myofilament space-negative) is maintained and/or established by the operation of the Na\(^+\),K\(^+\) pump in the transverse tubule membranes and is sensitive to the permeant inhibitor digitoxin; (c) the transverse tubule–mediated stimulation of \(^{45}\)Ca efflux appears to be entirely Ca\(^{2+}\) dependent.

INTRODUCTION Skinned skeletal muscle fibers have been used to study several aspects of Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) (for a review, see Stephenson, Address reprint requests to Dr. Elizabeth W. Stephenson, Dept. of Physiology, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, 100 Bergen St., Newark, NJ 07103. Dr. Volpe’s permanent address is Centro di Studio per la Biologia e la Fisiopatologia Muscolare del CNR, Istituto di Patologia Generale dell’Università di Padova, via Loredan 16, 35131 Padua, Italy.

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Electron microscope investigations (Costantin et al., 1965; Franzini-Armstrong, 1971) showed that the junction between transverse tubule (T-tubule) and the SR is preserved in mechanically skinned (Natori-type) fibers, so that T-tubule-SR coupling might be still functional. Csapo (1959), Natori and Isojima (1962), and Costantin and Podolsky (1967) found that mechanically skinned fibers could be activated by electrical stimulation, and Costantin and Podolsky (1967) suggested that current flow causing depolarization of membranes of the sarcotubular system (SR and/or T-tubule) could account for that phenomenon. More recent evidence shows that the T-tubule system has longitudinal elements (Peachey and Eisenberg, 1978), and the SR appears to have no appreciable resting membrane potential (for a review, see Oetliker, 1982). Therefore, in mechanically skinned fibers, T-tubules could seal off at the fiber surface and form the closed compartments that can be depolarized and initiate activation.

The experiments described in this paper were devised to evoke Ca\(^{2+}\) release from the SR of mechanically skinned fibers by manipulating T-tubule membrane potential with ionophores, on the assumption that the T-tubules are sealed and polarized, and T-tubule-SR junctional structures are functional. Ionophores that dissipate K\(^+\) and Na\(^+\) gradients did stimulate tension and \(^{45}\)Ca efflux from skinned fibers, and pretreatment with digoxin, a permeant inhibitor of the Na\(^+\),K\(^+\) pump, prevented the stimulation. These results imply that the T-tubules are the target of ionophore action, that ion gradients and a transmembrane potential are established across closed T-tubule compartments, and that the entire excitation-contraction (E-C) coupling pathway is functional. Moreover, ionophore-induced \(^{45}\)Ca release was inhibited in the presence of EGTA. This result indicates that Ca\(^{2+}\) plays a critical role in T-tubule-SR coupling and/or Ca\(^{2+}\) release from the SR of skeletal muscle (see Stephenson, 1981b; Fabiato, 1983).

**METHODS**

**Materials**

Monensin, gramicidin D, valinomycin, CCCP (carbonyl cyanide \(p\)-trichloromethoxyphenylhydrazone), phosphoenolpyruvate, pyruvate kinase, ouabain, and digitoxin were obtained from Sigma Chemical Co. (St. Louis, MO), caffeine from Aldrich Chemical Co. (Milwaukee, WI), and Triton X-100 from Rohm and Haas (Philadelphia, PA). \(^{45}\)CaCl\(_2\) was purchased from New England Nuclear (Boston, MA). Monensin, gramicidin D, valinomycin, CCCP (all at 2 mg/ml), and digitoxin were prepared as concentrated ethanolic solutions and diluted 1:200.

**Preparation of Mechanically Skinned Fibers**

Fiber preparation and mounting have been described in detail previously (Stephenson, 1978a). In brief, the semitendinosus muscle of the southern frog (*Rana berlandieri*) was isolated and suspended for 90 min at 4-6°C in a low-Cl Ringer's solution containing (mM): 217 sucrose, 2.5 KCl, 1.8 CaCl\(_2\), and 3.1 NaH\(_2\)PO\(_4\) plus Na\(_2\)HPO\(_4\). From a bundle of fibers transferred to cold paraffin oil, single fibers were isolated from tendon to tendon and cut into segments that were skinned by microdissection just before use. Typically three to five fibers were used from each bundle.
Bathing Solutions

The bathing solutions contained (mM): 120 K propionate, 10 imidazole, 5 Na$_2$ATP, 5 MgSO$_4$, and other constituents as specified below (see Table I). pH was adjusted to 7.00. Free Mg$^{2+}$ was estimated at ~0.7–0.8 mM. Fiber and bath concentrations can differ because of factors such as Donnan effects and diffusional delays (Stephenson, 1981a), and stated concentrations refer to bulk solution values.

Isometric Force Measurements

The skinned segments, usually 2–2.5 mm long and 75–125 μm in diameter, were tied with monofilament thread to small stainless steel rods. One was attached to a leaf-spring photodiode transducer, which permitted continuous measurement of isometric force and transfer of the segment between bathing solutions maintained at 19°C in the wells of a spring-mounted thermoregulated chamber, as described previously (Stephenson, 1978a). Skinned segments were kept for several minutes in 0.1 mM EGTA bathing solution (solution B) to remove residual paraffin oil, and then loaded for 40 s in CaEGTA buffer solution, 0.375 mM CaEGTA with 0.5 mM total EGTA (solution C). Segments were then rinsed in 0.1 mM EGTA solution for 80 s and 0.01 mM EGTA solution (solution D) for 10 s, and challenged in solution A containing 10 μg/ml of either monensin, gramicidin D, valinomycin, or CCCP. If the segments did not develop tension, e.g., with valinomycin and CCCP, they were stimulated with 10 mM caffeine in bathing solution D. Finally, all segments were placed in 0.05% Triton X-100, 5 mM CaEGTA bathing solution (solution E) to measure maximal isometric force ($P_o$).

$^{45}$Ca Efflux Measurements

Procedures were essentially similar to those described in the previous section. Skinned segments were loaded for 40 s in $^{45}$CaEGTA buffer solution (final activity, ~20 μCi/ml). The segments were then rinsed three times in 0.1 mM EGTA bathing solutions for 2, 20, and 80 s, respectively, and once in 0.01 mM EGTA bathing solution for 10 s, transferred through a series of washout solutions, and finally extracted for 9–10 min in 0.05% Triton X-100, 5 mM CaEGTA, which removes the remaining $^{45}$Ca (Stephenson, 1978a, 1981a). Two types of responses were elicited: interrupted and pretreated responses (Stephenson, 1981a).
In the interrupted responses, skinned segments were challenged in solution A, containing the specific ionophore, for \( \sim 2 \) s and then transferred to washout solutions supplemented with 5 mM EGTA. In the pretreated responses, skinned segments were placed in 5 mM EGTA solution (solution F) for 10 s before the application of the ionophore in solution F, which contained 5 mM EGTA.

The amount of \(^{45}\text{Ca}\) lost into each solution was expressed as a fraction of the total initial \(^{45}\text{Ca}\) in the segment after rinsing, which is the sum of the \(^{45}\text{Ca}\) lost to the washout and extraction solutions (see Stephenson, 1981a). The fraction lost in each wash was expressed as a flux by dividing by the time actually spent in the wash (i.e., transfer times were not included). The flux divided by the mean fraction in the fiber during that interval gives an apparent first-order constant for \(^{45}\text{Ca}\) efflux (\( k_0 \)).

**TABLE II**

*Selectivities of Ionophores Used in this Study*

<table>
<thead>
<tr>
<th>Ionophore</th>
<th>Selectivity sequence</th>
<th>( \text{K}^+/\text{Na}^+ ) ratio*</th>
<th>( \text{K}^+/\text{Na}^+ ) (in ( \rightarrow ) out)</th>
<th>( \text{Na}^+/\text{K}^+ ) (out ( \rightarrow ) in)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin</td>
<td>( \text{Na}^+ &gt; \text{K}^+ )</td>
<td>0.1</td>
<td>1.2</td>
<td>480</td>
</tr>
<tr>
<td>Gramicidin D</td>
<td>( \text{H}^+ &gt; \text{K}^+ &gt; \text{Na}^+ )</td>
<td>1.8</td>
<td>21.6</td>
<td>26.6</td>
</tr>
<tr>
<td>CCCP</td>
<td>( \text{H}^+ )</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Valinomycin</td>
<td>( \text{K}^+ \gg \text{Na}^+ )</td>
<td>17,000</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

* Data are derived from Pressman (1976). Monensin, a carboxylic ionophore, forms a cationic complex in its deprotonated ionic form and carries ions as electrically neutral zwitterions, so the equilibrium catalyzed contains no electrical terms and is pH sensitive and independent of membrane potential. Gramicidin D introduces ion conductance channels into the membrane, and its action is not voltage dependent. CCCP transports protons only, and dissipates proton gradients. Valinomycin forms a specific, positively charged complex with \( \text{K}^+ \) and catalyzes an equilibrium defined by both the transmembrane concentration gradient and membrane potential.

$^d$ Effective selectivity values are the product of the selectivity ratios and the initial relative concentrations in the source solutions; the concentrations assumed for illustrative purposes are 120 mM [\( \text{K}^+ \)] and 10 mM [\( \text{Na}^+ \)] (the bath composition) and 2.5 mM [\( \text{K}^+ \)] and 120 mM [\( \text{Na}^+ \)] (extracellular composition in the T-tubule lumen after sealing and active Na/K transport).

**RESULTS**

*Monensin Elicits Isometric Force Development in Skinned Fibers*

Monensin, an \( \text{Na}^+/(\text{K}^+) /\text{H}^+ \) exchanger (Pressman, 1976; see Table II) elicited isometric force development in skinned fibers (Fig. 1A). The response occurred without delay. In this fiber, half-peak tension was reached in 0.5 s and the relative peak force \( P/P_o \) (see legend to Table III) was \( \sim 0.8 \) (Fig. 1A). The spontaneous, slow decline in force was greatly accelerated in 5 mM EGTA solution. In two experiments (not shown), where the bathing solutions were supplemented with an ATP-regenerating system (6 mM phosphoenolpyruvate and 50 U pyruvate kinase), the response to monensin was not modified. The spontaneous force decay was not accelerated, which indicates that ATP depletion was not involved. The mean values in 15 skinned segments stimulated by monensin are summarized in Table III (line a). However, when monensin was present before, during, and after \( \text{Ca}^{2+} \) loading, no force developed with application at the usual stimulus
FIGURE 1. Effect of monensin on isometric force development of skinned fibers. Skinned segments were loaded in Ca-EGTA buffer solution for 40 s and rinsed in 0.1 mM EGTA solution for 80 s (not shown) and in 0.01 mM EGTA solution for 10 s. (A) 10 μg/ml monensin evoked force development. In this experiment, half-peak tension was reached in 0.5 s, and $P/P_0$ was 0.82 (see also Table III). (B) 10 μg/ml monensin was present in Ca+ loading and rinsing solutions. The application of 10 μg/ml monensin at the usual stimulus time failed to evoke force development, whereas the subsequent addition of 10 mM caffeine induced force development.

TABLE III
Effect of Ionophores and Glycosides on Isometric Force Development of Skinned Fibers

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>With 10 mM caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time to half-peak</td>
</tr>
<tr>
<td></td>
<td>tension $n$</td>
</tr>
<tr>
<td>(a) Monensin (10 μg/ml)</td>
<td>15 0.65±0.03 0.86±0.05</td>
</tr>
<tr>
<td>(b) Monensin in all solutions</td>
<td>7    — — —</td>
</tr>
<tr>
<td>(c) Gramicidin D (10 μg/ml)</td>
<td>7    0.60±0.03 0.82±0.06</td>
</tr>
<tr>
<td>(d) Valinomycin (10 μg/ml)</td>
<td>5    — —</td>
</tr>
<tr>
<td>(e) CCCP (10 μg/ml)</td>
<td>6    — —</td>
</tr>
<tr>
<td>(f) Monensin + digitoxin* (50 μM)</td>
<td>8  — —</td>
</tr>
<tr>
<td>(g) Monensin + ouabain (100 μM)</td>
<td>7  0.63±0.04 0.80±0.03</td>
</tr>
<tr>
<td>(h) Gramicidin D + digitoxin (50 μM)</td>
<td>4 — —</td>
</tr>
</tbody>
</table>

Skinned segments were exposed to 10 mM caffeine only when they did not develop tension in the presence of ionophores. Digitoxin or ouabain was present in all bathing solutions (lines f, g, and h) and skinned segments were exposed to glycosides for ~5 min before monensin (or gramicidin D) application. $P/P_0$ is the ratio between $P$, the force developed in the presence of either ionophore or caffeine, and $P_0$, the maximal force developed in 0.05 Triton X-100, 5 mM Ca EGTA (free Ca$^{2+}$ = 24.6 μM). Results are expressed as means ± SEM for the number of experiments in column $n$.

* In one additional experiment, digitoxin incompletely inhibited monensin-induced force development ($P/ P_0 = 0.08$).
time (upward arrow in Fig. 1B). The subsequent application of 10 mM caffeine (double arrow in Fig. 1B) rapidly evoked large force development, which indicates that the SR was loaded with calcium available for release. The mean values in seven segments are summarized in Table III. Caffeine induces Ca\textsuperscript{2+} release from the SR of intact (Lüttgau and Oetliker, 1968; Howell, 1969; J. S. Frank and Winegrad, 1976) and skinned fibers (Endo, 1977; Stephenson, 1981a) and from purified SR vesicles (Miyamoto and Racker, 1982; Su and Hasselbach, 1984), and is believed to act directly on the SR.

Effect of Glycosides (Ouabain and Digitoxin) on Monensin-induced Force Development

If monensin acts on polarized T-tubules, its effect may be influenced by digitoxin and ouabain, glycosides that are specific inhibitors of the plasma membrane Na\textsuperscript{+},K\textsuperscript{+} pump (Shuurmans Stekhoven and Bonting, 1981). They are identical in potency (Jorgensen, 1982) but differ in that digitoxin is permeant to the T-tubule membrane, whereas ouabain is not (Lau et al., 1979). The binding site for glycoside is extracellular and the Na\textsuperscript{+},K\textsuperscript{+} pump is present in T-tubule as well as sarcolemma membranes of frog skeletal muscle (Venosa and Horowicz, 1981), but is absent from SR (Lau et al., 1979).

In the experiment shown in Fig. 2A, the skinned segment was exposed to 50 \(\mu\)M digitoxin, in all bathing solutions, for a total of \(\sim 5\) min before the application of monensin. After this treatment, monensin (upward arrow) failed to evoke force development. However, the subsequent application of 10 mM caffeine (double arrow) induced a rapid and large force development, which indicates Ca\textsuperscript{2+} release. The results of eight such experiments, summarized in Table III, suggested that the T-tubule was the target of monensin action.

In contrast, when a skinned segment was exposed to 100 \(\mu\)M ouabain for \(\sim 5\) min (Fig. 2B), monensin action (upward arrow) was unaffected. The differential effects of digitoxin and ouabain (also summarized in Table III) were consistent with the expectation that glycosides had to cross the T-tubule membrane barrier to bind and act at the external (luminal) face, i.e., the T-tubules are sealed off and the lumen is inaccessible to impermeant solutes. We also made several attempts to block monensin-induced force development by exposing muscle bundles for 90 min to 0.1 mM ouabain before skinning, when the luminal binding site should be accessible (not shown). A similar treatment inhibits chloride responses in rabbit peeled skeletal muscle fibers (Donaldson, 1982, 1983), but our attempts were unsuccessful. Plausible explanations for this discrepancy are: (a) the ouabain entry into the T-tubule lumen might be less effective under our experimental conditions, because we exposed the whole muscle bundle and not the single fiber as Donaldson did, and (b) different animal species were used (frog vs. rabbit). In this respect, it is worth mentioning that Yamakawa and Mobley (1984) failed to confirm Donaldson's results using an almost identical experimental protocol but a different preparation (frog fibers).

Effect of Gramicidin D, CCCP, and Valinomycin on Isometric Force Development

In order to investigate further the ionic mechanism underlying monensin stimulation of Ca\textsuperscript{2+} release, other ionophores were tested. Gramicidin D, an H\textsuperscript{+} >
K⁺ > Na⁺ channel-forming ionophore (Pressman, 1976; see Table II), also elicited rapid force development (Table III). The force pattern evoked by gramicidin D was similar to that evoked by monensin (lines a and c in Table III). Gramicidin D, like monensin, failed to evoke force development when the skinned segments had been exposed to 50 μM digitoxin for ~5 min (line h in Table III). In the same segments, the subsequent application of 10 mM caffeine produced a large and rapid force development, which indicates that Ca²⁺ was available for release.

On the other hand, CCCP, a protonophore, and valinomycin, a neutral, K⁺-

specific ionophore (Pressman, 1976; see Table II), were ineffective. The skinned segments that were unresponsive to valinomycin or CCCP were stimulated subsequently by 10 mM caffeine, which induced rapid force development (lines d and e in Table III).

Taken together, these results suggest that Ca²⁺ release occurs when both Na⁺ and K⁺ gradients are dissipated, but not when dissipation of only a K⁺ or an H⁺ gradient is facilitated.

**Effect of Monensin, Gramicidin D, and Glycosides on ⁴⁰Ca Efflux from Skinned Fibers (Interrupted Responses)**

Development of force (Fig. 1A and Table III) is indirect evidence of net Ca²⁺ release from the SR. Consequently, Ca²⁺ efflux was measured directly by means of isotope techniques (Stephenson, 1981b). Skinned segments were loaded in a ⁴⁰CaEGTA buffer solution, rinsed in low-EGTA bathing solutions (solutions B
and D), and then challenged with 10 μg/ml monensin in solution A for ~2 s. The segments were then transferred through a series of monensin washout solutions containing 5 mM EGTA (see Methods for details), in order to minimize 45Ca reaccumulation by the SR.

Fig. 3A and Table IV show that monensin stimulated 45Ca efflux and that, on the average, 30.6% of the accumulated 45Ca was released within 55 s. Under these washout conditions, the apparent first-order rate constant of efflux into the bathing solution during the first 2-s wash, in the absence of EGTA, was 2.67 ± 0.20 × 10⁻² s⁻¹ (n = 10). The actual rate of 45Ca efflux from the SR was higher and a minimal estimate is given in the last section of the Results.

When monensin was present in all bathing solutions, no force development was recorded (cf. Fig. 1B), and the small 45Ca efflux (Fig. 3A and line b in Table IV) did not exceed the control in 5 mM EGTA (cf. Table V).

Digitoxin (50 μM) and ouabain (100 μM) had different effects on monensin-
stimulated $^{45}$Ca efflux (Fig. 3A), which confirmed the force results shown in Fig. 2 and Table III. After a 5-min exposure before stimulation, digitoxin inhibited monensin-stimulated $^{45}$Ca efflux, whereas ouabain did not (Fig. 3A and Table IV). In the presence of digitoxin, the $^{45}$Ca lost was reduced to control values and the apparent first-order rate constant of the initial wash was decreased by a factor of 20. In contrast, ouabain did not affect the extent and rate of monensin-stimulated $^{45}$Ca efflux (lines g and a in Table IV).

Fig. 3B shows that the skinned segments pretreated with digitoxin or exposed to monensin from the beginning of the experiments (cf. Figs. 2A and 1B) did release $^{45}$Ca when 10 mM caffeine was applied subsequently. In both cases, ~40% of the $^{45}$Ca present in the skinned fibers was released by caffeine (see also Table IV). There was no evidence that monensin and digitoxin decreased $^{45}$Ca loading by the SR.

### Table IV

**Effect of Ionophores and Glycosides on $^{45}$Ca Efflux from Skinned Fibers (Interrupted Responses)**

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>n</th>
<th>$k_o$ (s$^{-1}$)</th>
<th>$^{45}$Ca lost</th>
<th>$k_o$ (s$^{-1}$)</th>
<th>$^{45}$Ca lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Monensin (10 µg/ml)</td>
<td>10</td>
<td>2.67±0.20</td>
<td>30.54±1.20</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(b) Monensin in all solutions</td>
<td>5</td>
<td>0.14±0.03</td>
<td>7.38±0.37</td>
<td>2.57±0.22</td>
<td>40.68±2.34</td>
</tr>
<tr>
<td>(c) Gramicidin D (10 µg/ml)</td>
<td>5</td>
<td>2.62±0.17</td>
<td>31.08±1.61</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(d) CCCP (10 µg/ml)</td>
<td>4</td>
<td>0.21±0.04</td>
<td>6.96±0.69</td>
<td>2.65±0.39</td>
<td>39.12±3.11</td>
</tr>
<tr>
<td>(e) CCCP (10 µg/ml)</td>
<td>4</td>
<td>0.19±0.02</td>
<td>6.48±0.58</td>
<td>2.69±0.25</td>
<td>40.16±1.97</td>
</tr>
<tr>
<td>(f) Gramicidin + digitoxin (50 µM)</td>
<td>5</td>
<td>0.13±0.02</td>
<td>6.98±0.53</td>
<td>2.86±0.21</td>
<td>41.30±2.51</td>
</tr>
<tr>
<td>(g) Monensin + ouabain (100 µM)</td>
<td>5</td>
<td>2.66±0.40</td>
<td>29.88±1.58</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(h) Gramicidin D + digitoxin (50 µM)</td>
<td>4</td>
<td>0.17±0.04</td>
<td>6.77±0.78</td>
<td>2.78±0.21</td>
<td>40.80±2.05</td>
</tr>
</tbody>
</table>

Procedures are described in the Methods and in the legends to Figs. 3 and 4. $k_o$ is the apparent first-order rate constant of the first 2-s wash containing ionophore or caffeine. The amount of $^{45}$Ca lost after 50–60 s is expressed as the percentage of the total $^{45}$Ca in the skinned segments after rinsing. Caffeine was applied only when ionophores did not elicit force development. The skinned segments stimulated with caffeine had lost, on the average, 10% of the accumulated $^{45}$Ca at the time of caffeine application. Results are expressed as means ± SEM for the number of experiments in column n.

In extension of the force results shown in Table III, gramicidin D also stimulated $^{45}$Ca efflux (Fig. 4A). The initial rate and total extent of $^{45}$Ca loss were indistinguishable from those obtained with monensin (Fig. 3A and Table IV). In addition, when skinned segments were exposed to 50 µM digitoxin, gramicidin D stimulation of $^{45}$Ca efflux was inhibited (line h in Table IV). The pattern of inhibition was similar to that of monensin-stimulated $^{45}$Ca efflux (line f in Table IV) and consistent with the isometric force responses (line h in Table III).

**Effect of CCCP and Valinomycin on $^{45}$Ca Efflux from Skinned Fibers (Interrupted Responses)**

Consistent with the force results, CCCP and valinomycin did not stimulate $^{45}$Ca efflux (Fig. 4A and Table IV). The same segments rapidly released $^{45}$Ca when subsequently stimulated with 10 mM caffeine (Fig. 4B and Table IV).
Monensin-stimulated \(^{45}\text{Ca}\) Efflux is \(\text{Ca}^{2+}\) Dependent

In order to assess the \(\text{Ca}^{2+}\) dependence of the monensin-stimulated \(^{45}\text{Ca}\) efflux, three experimental protocols were employed in skinned segments from the same fiber. One segment was challenged with monensin in solution A for 2 s (interrupted response) and then transferred to 5 mM EGTA washout solutions containing monensin, as in the preceding experiments. A second segment was exposed to 5 mM EGTA for 10 s before applying monensin in 5 mM EGTA solution (pretreated response). A third segment was pretreated with 5 mM EGTA but was not stimulated with monensin (control).

After pretreatment of a segment with 5 mM EGTA, monensin application resulted in no detectable force development and a loss of only 6.2% of the accumulated \(^{45}\text{Ca}\) to the bath (Table V). This small release did not differ from that of control experiments, in which segments exposed to 5 mM EGTA but not stimulated lost 6.4% of the accumulated \(^{45}\text{Ca}\). The initial apparent first-order rate constant (Table V) and mean \(^{45}\text{Ca}\) efflux to the bath (Fig. 5) under pretreated
and control conditions were indistinguishable. These results indicated that stimulation of \(^{45}\text{Ca}\) efflux by monensin was entirely \(\text{Ca}^{2+}\) dependent, within the accuracy of the measurements.

The results of the interrupted response can be used to estimate the minimum efflux rate of \(^{45}\text{Ca}\) from the SR in the absence of EGTA. Since monensin is not stimulatory in the presence of EGTA (Table V), the cumulative \(^{45}\text{Ca}\) loss to the

![Image](https://via.placeholder.com/150)

**Figure 5.** Effect of 5 mM EGTA pretreatment on the mean monensin-stimulated \(^{45}\text{Ca}\) efflux plotted as in Fig. 3. Procedures are described in the Methods and in the legend to Table V. Interrupted responses (■); pretreated responses (○); control responses (△). Data are expressed as means ± SEM for five different experiments.
bath during the interrupted response, corrected for the $^{45}$Ca loss in the pretreated response after the first wash, presumably had been released from the SR to the myofilament space (MFS) before EGTA application (Stephenson, 1978a). By this calculation, at least 24% of the fiber tracer was released from the SR during the first 3.1 s of monensin stimulation (1.5 s wash plus 1.6 s transfer time): $\sim 5\%$ diffused into the bath during the first wash and 19% was still in the MFS when EGTA was applied. A minimal estimate of the initial $^{45}$Ca efflux rate from the SR to the MFS is $8\%$ s$^{-1}$, which is equivalent to $\sim 120$ $\mu$M Ca liter fiber$^{-1}$ s$^{-1}$. This does not take into account factors such as $^{45}$Ca reaccumulation by the SR during the first wash, transfer time, and EGTA diffusion time into the fiber (Stephenson, 1978a).

**DISCUSSION**

This study shows that monensin and gramicidin D, ionophores that can dissipate Na$^{+}$ and K$^{+}$ gradients, stimulate Ca$^{2+}$ release from the SR of mechanically skinned fibers. Furthermore, pretreatment with digitoxin, a permeant glycoside inhibitor of the Na$^{+}$,K$^{+}$ pump, prevents stimulation of Ca$^{2+}$ release. These results imply that the T-tubules of Natori-type skinned fibers can seal off and redevelop transmembrane ion gradients and potential of normal polarity, forming the only plausible target for such glycoside-sensitive stimulation. Therefore, the T-tubule–SR coupling pathway appears to be functional in mechanically skinned fibers, and the observed Ca$^{2+}$ dependence of ionophore stimulation suggests that Ca$^{2+}$ plays a crucial role in the T-tubule-mediated Ca$^{2+}$ release from the SR of intact fibers.

*Previous Evidence of Stimulation of Mechanically Skinned Muscle Fibers through the T-Tubules*

In skeletal muscle, the depolarization of the sarcolemma and its radial propagation along the T-tubules initiate Ca$^{2+}$ release from the SR by unknown mechanisms. If electrical stimulation (Costantin and Podolsky, 1967) and chloride substitution (Stephenson and Podolsky, 1977; Stephenson, 1981b; Donaldson, 1982, 1983) activate skinned muscle fibers through the T-tubules, depolarization of the T-tubules would mimic the ionic depolarization of intact fibers, and contraction of the skinned fibers would involve T-tubule–SR coupling.

Costantin and Podolsky (1967) showed that short skinned regions of muscle fibers could be activated reversibly by electrical stimulation and that fibers obtained from a muscle exposed to 10–20 $\mu$M strophanthidin, a cardiac glycoside, were not electrically responsive. These authors concluded that “removal of the surface membrane permits the restoration of excitability” and discussed the possibility that “the transverse tubules (T-system) can reseal following their disruption from the surface membrane, and that operation of a metabolically supported sodium pump within the T-system is then capable of decreasing the potassium concentration within the tubular lumen”; the potassium diffusion gradient results in a “resting potential across the internal membranes.” These authors also showed that skinned fibers contracted reversibly when droplets of
140 mM KCl were applied, and that electrical activation and Cl− stimulation were mutually exclusive, which suggested that they acted through a common mechanism. Later, Stephenson and Podolsky (1977) showed that stimulation of skinned fibers by KCl replacement of K propionate was increased by pretreatment of the intact fiber with low-Cl− solution, which would increase the Cl− gradient across sealed-off T-tubules membranes of the skinned fiber. Similar stimulation of 45Ca efflux was blocked in fibers stretched to twice slack length, whereas stimulation by caffeine or Mg²⁺ reduction was unaffected (Stephenson, 1978b). Recently, Donaldson (1982, 1983) has reported that force development stimulated by substitution of choline Cl for K propionate could be blocked when rabbit muscle fibers had been exposed to 50 μM ouabain for 2–3 h before mechanical skinning. These results strongly suggest that the Cl− stimulus acts at the level of the sealed-off T-tubules.

Ikemoto et al. (1984) came to a similar conclusion using an isolated SR fraction that includes triads, i.e., the junctional association of terminal cisternae and T-tubules. Replacement of K gluconate with choline Cl triggered rapid Ca²⁺ release, which was prevented when the T-tubule–SR junction had been disrupted by French press treatment.

**Mechanism of Stimulation by Monensin and Gramicidin D**

Monensin and gramicidin D, ionophores that transport monovalent cations by different mechanisms (see Table II), both stimulate ⁴⁵Ca efflux from the SR. Thus, dissipation of monovalent cation gradients appears to initiate activation.

The SR is an unlikely target for this ionophore action because (a) there is no evidence for ionic gradients or membrane potential across the SR (Somlyo et al., 1977; Oetliker, 1972) that could be dissipated by these ionophores, and (b) it is already highly permeable to monovalent cations (Miller, 1978) and protons (Meissner and Young, 1980). Furthermore, Ca²⁺ release from isolated SR vesicles is not affected by gramicidin D (Louis et al., 1980; MacLennan et al., 1982; Volpe et al., 1983), or by protonophores (Scarpa and Inesi, 1972; MacLennan et al., 1982) and valinomycin (Scarpa et al., 1972; Meissner, 1984). These observations argue against direct ionophore stimulation of Ca²⁺ release from the SR.

Our results are consistent with the interpretation that monensin and gramicidin D stimulate Ca²⁺ release from the SR by depolarizing sealed-off T-tubules in the mechanically skinned fiber. Polarized T-tubules seem the only plausible target for ionophore action.

When skinned segments are transferred from paraffin oil to aqueous solution (solution B), the operation of the Na⁺,K⁺ pump in sealed T-tubules would maintain or increase intraluminal Na⁺, [Na]o, and decrease [K]o, establishing cation gradients. Assuming that myoplasm concentrations, [Na]i, and [K]i, are 10 and 120 mM, respectively, as in the bathing solution, and that PNa/PK is very low, as in intact fibers (Hodgkin and Horowicz, 1959), the outward K⁺ gradient would result in a resting membrane potential (myofilament space negative), established and maintained by the Na⁺,K⁺ pump. Before skinning, the my-
The plasma membrane of the T-tubule is exposed to the normal myoplasm, which is high in K\(^+\) and low in permeant anions. Thus, if the T-tubules seal off soon after skinning, the operation of the Na\(^+\),K\(^+\) pump might establish a resting membrane potential in oil (Costantin and Podolsky, 1967) before exposure to aqueous solution.

Given this ion distribution, a possible mechanism of the ionophore action is as follows: monensin dissipates both K\(^+\) and Na\(^+\) gradients across T-tubule membranes, with net Na\(^+\)/K exchange down the respective chemical gradients. Both K\(^+\) and Na\(^+\) transport by monensin are coupled to proton exchange (Table II), but net proton movement need not occur if protons associate and redissociate at each membrane interface, and bulk electroneutrality is maintained by the Na\(^+\) and K\(^+\) fluxes. The probability of each cation moving unidirectionally would depend on the selectivity ratio (Table II) and the concentrations in the source compartment, giving the illustrative "effective selectivities" shown in Table II. The net fluxes would depend on the concentration gradients, so the net monensin-mediated Na\(^+\) flux from the lumen would be accompanied by net K\(^+\) flux into the lumen, with dissipation of the K\(^+\) gradient and consequent T-tubule depolarization. Additionally, as [K]\(_o\) increased and the membrane potential became less negative, P\(_{Na}\) might also increase and accelerate depolarization. Gramicidin D, a channel-forming ionophore, could depolarize the T-tubule membrane by simultaneously increasing Na conductance directly and changing [K]\(_o\)/[K].

When skinned segments are exposed to monensin continuously from the first transfer to aqueous solution (line 6 in Tables III and IV), ion gradients and a resting membrane potential should not be established. Consistent with this interpretation, the application of monensin after Ca\(^{2+}\) loading failed to evoke force development (Fig. 1B) or Ca\(^{2+}\) release (Fig. 3A). Since a T-tubule resting membrane potential may be re-established before transfer to aqueous solution (Costantin and Podolsky, 1967), one might expect Ca\(^{2+}\) release and contraction to take place upon the initial exposure to monensin. Since this was not the case, either repolarization was not yet adequate or insufficient endogenous Ca\(^{2+}\) remained in the SR to be released (Reuben, 1982).

Compelling evidence that T-tubules mediate the monensin and gramicidin D stimulation of \(^{45}\)Ca efflux is provided by the effect of glycosides, specific inhibitors of the Na\(^+\),K\(^+\) pump, which is located in T-tubules but is absent from the SR (Lau et al., 1979). Pretreatment of the skinned fiber with the permeant glycoside digitoxin fully prevents ionophore stimulation of \(^{45}\)Ca efflux from the SR, and this result clearly indicates that the operation of the Na\(^+\),K\(^+\) pump is required for the skinned fiber response. Digitoxin, by turning off the Na\(^+\),K\(^+\) pump, would lead to dissipation of a resting membrane potential or prevent its re-establishment across T-tubules. The T-tubules would remain depolarized so that ionophores could not stimulate them. The ineffectiveness of ouabain, an impermeant glycoside, is consistent with inaccessibility of the luminal glycoside binding site from the myofilament space in this preparation, i.e., T-tubule sealing.

In view of the evidence that the effective ionophores act on the polarized T-tubule, the stimulation of Ca\(^{2+}\) release from the SR implies that T-tubule-SR coupling is functional in these mechanically skinned fibers.
The ineffectiveness of Valinomycin and CCCP

The proposed mechanism of action for monensin and gramicidin D is strengthened by the lack of effect of valinomycin and CCCP. Our results show that valinomycin, a K⁺-specific ionophore that does not transport Na⁺, and CCCP, a protonophore, do not stimulate ⁴⁰Ca efflux from the SR of skinned fibers. The T-tubule already has a high Pₖ (Hodgkin and Horowicz, 1960; Eisenberg and Gage, 1969), and valinomycin might act only by changing [K]₀/[K]. If [K]₀ ≫ [K], because of the Na⁺,K⁺ pump activity, the resting membrane potential could be close to the K⁺ equilibrium potential. If, for example, the K⁺ equilibrium potential were 99 mV (based on a 120:2.5 [K] ratio) and Pₙₐ/Pₖ were 0.01, the resting membrane potential would be −97 mV. Increasing Pₖ with valinomycin would only clamp or hyperpolarize the membrane voltage.

The ineffectiveness of CCCP suggests that dissipation of proton gradients across SR and/or T-tubule membranes does not stimulate Ca²⁺ release from the SR. This also implies that gramicidin D does not act by dissipation of putative proton gradients. The present experimental result is in disagreement with previous studies on chemically (EGTA) skinned fibers of the rabbit by Shoshan et al. (1981), who suggested that both CCCP and gramicidin caused rapid transient tension by dissipating a proton gradient across the SR (Shoshan et al., 1981) or T-tubule membranes (MacLennan et al., 1982). The discrepancy may be due to different fiber preparations and experimental conditions; in particular, chemically skinned fibers have permeable T-tubule membranes (Eastwood et al., 1979), and their positive response to CCCP also differs from the absence of response in SR vesicles, as noted above.

T-tubule-mediated Ca²⁺ Release Is Ca²⁺ Dependent

One of the most interesting results of our study is that ionophore stimulation of ⁴⁰Ca efflux is entirely Ca²⁺ dependent; it should be noted that this occurs at physiological Mg²⁺ levels (Table 1). The stimulation of Ca²⁺ release was completely abolished after a 10-s pretreatment with 5 mM EGTA to chelate MFS Ca²⁺. Since monensin and gramicidin D appear to act by depolarizing sealed-off T-tubules, as discussed above, this result implies that T-tubule–SR coupling and/or the Ca²⁺ release process is inhibited if free Ca²⁺ is lowered at the T-tubule–SR junction or in the vicinity of SR Ca²⁺ release channels.

Among the models proposed to explain T-tubule–SR coupling and the Ca²⁺ release process from SR (see Schneider and Chandler, 1973; Endo, 1977; Mathias et al., 1980; Stephenson, 1981b; G. B. Frank, 1982; Eisenberg et al., 1983), a role for Ca²⁺ has recently gained support. Calcium might: (a) mediate transmission at the T-tubule–SR junction, i.e., Ca²⁺ is the messenger; (b) be involved in enzymatic reactions within the junction; or (c) interact with Ca²⁺-gated Ca²⁺ channels of SR responsible for massive Ca²⁺ release that activates contraction. The physiological significance of early observations on skinned fibers (Endo et al., 1970; Ford and Podolsky, 1970) was later questioned (Endo, 1977) because high levels of applied free Ca²⁺ appeared to be required to induce Ca²⁺ release at physiological Mg²⁺ levels. More recent findings have indicated that: (a) Cl⁻-induced ⁴⁰Ca efflux from the SR of mechanically skinned fibers is highly Ca²⁺
dependent (Stephenson, 1981a, 1983); (b) Ca$^{2+}$ release from the SR of mechanically skinned fibers takes place at higher Mg$^{2+}$ when bath free Ca$^{2+}$ is changed very rapidly (within 0.2 s) from resting values to 1 μM (Fabiato, 1982, 1983, 1985); and (c) isolated SR vesicles derived mainly from terminal cisternae also display Ca$^{2+}$-induced Ca$^{2+}$ release at micromolar free Ca$^{2+}$ (Miyamoto and Racker, 1982; Kim et al., 1983; Meissner, 1984) and contain Ca$^{2+}$-gated Ca$^{2+}$ channels (Meissner, 1984).

The present results are consistent with EGTA interference with any of the three steps on which Ca$^{2+}$ might act, and further experiments are needed to differentiate among them.

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