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Voltage dependence of inositol 1,4,5-trisphosphate-induced Ca^{2+} release in peeled skeletal muscle fibers

(skinned muscle fibers/sarcoplasmic reticulum/transverse tubule/excitation–contraction coupling)

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ABSTRACT **Excitation–contraction coupling in skeletal muscle is known to be under absolute control of plasmalemma voltage, but the steps from transverse (T)-tubule depolarization to Ca^{2+} release from the sarcoplasmic reticulum have not been elucidated. The effect of changing T-tubule membrane potential on inositol 1,4,5-trisphosphate ($InsP_3$) stimulation of Ca^{2+} release from the sarcoplasmic reticulum was studied to explore a possible role for $InsP_3$ as a chemical signal in excitation–contraction coupling. $InsP_3$ was microinjected into peeled rabbit skeletal muscle fibers at a pipette concentration of 0.5 μM; Ca^{2+} release from the sarcoplasmic reticulum was monitored as an isometric tension transient. The response to 0.5 μM $InsP_3$ was significantly larger when T-tubules were in a depolarized state than when they were in a polarized state, and this difference in response was independent of the ionic composition of the bathing solutions or the method for depolarizing the T-tubules. Thus, T-tubule depolarization may sensitize the sarcoplasmic reticulum to a preexisting low concentration of $InsP_3$ and greatly reduce the need for $InsP_3$ production. Plasmalemma voltage control of the stimulatory effects of $InsP_3$ may have relevance for mechanisms in excitable nonmuscle cells.**

Recent studies suggest inositol 1,4,5-trisphosphate ($InsP_3$) as a possible chemical signal in skeletal muscle excitation–contraction (EC) coupling (1–3). If $InsP_3$ is a transverse (T)-tubule to sarcoplasmic reticulum (SR) signal, steps in EC coupling would include (*i*) hydrolysis of phosphatidylinositol 4,5-bisphosphate in the T-tubule, (*ii*) $InsP_3$ diffusion to the SR, and (*iii*) $InsP_3$ action upon the SR. A major reservation is that these steps may take longer than the millisecond EC-coupling time course characteristic of skeletal muscle (4). In cell types that exhibit $InsP_3$ stimulation of Ca^{2+} release from endoplasmic reticulum, $InsP_3$ generation occurs in response to binding of agonist to receptors on the plasma membrane (5–7). The counterpart in skeletal muscle would be stimulation of plasmalemma in the form of depolarization to initiate, and repolarization to terminate, $InsP_3$-induced Ca^{2+} release from the SR (8–11). If $InsP_3$ plays a role in skeletal muscle EC coupling, then either $InsP_3$ production or its effectiveness as a stimulus for Ca^{2+} release must be tightly coupled to T-tubule membrane potential, as is contraction.

To our knowledge, a direct influence of membrane potential on inositol polyphosphate effects has not been reported previously. Here we show that T-tubule membrane potential governs sensitivity of the SR to $InsP_3$. T-tubule depolarization greatly enhances, and T-tubule polarization reduces, the $InsP_3$-induced, Ca^{2+}-activated tension transient in peeled rabbit skeletal muscle fibers. With T-tubule depolarization, an $InsP_3$ concentration as low as 0.5 μM induces maximal $InsP_3$-induced Ca^{2+} release. If T-tubule voltage controls the effectiveness of $InsP_3$ in EC coupling, the resting, or prestimulation, $InsP_3$ level could become a sufficient stimulus upon T-tubule depolarization. Then the concerns regarding time delays for phosphatidylinositol 4,5-bisphosphate hydrolysis and $InsP_3$ diffusion would be minimized.

METHODS

Preparation. Single fibers of rabbit adductor magnus skeletal muscle were skinned by mechanically peeling the sarcolemma in bathing solutions of ionic composition that maximizes T-tubule sealing and electrical polarization (12). Details regarding the animal preparation and methods for muscle excision and single-fiber isolation and peeling have been published (12).

Bathing Solutions and Ionic T-Tubule Polarization/Depolarization Protocol. Unless otherwise noted, single fibers were mechanically peeled in a "relaxing" bathing solution composed of 70 mM Na^+, 2 mM $MgATP^{2-}$, 15 mM creatine phosphate, 15 units of creatine kinase per ml, 1 mM Mg^{2+}, 50 μM EGTA (pCa = 7), propionate as major anion, and imidazole (pH = 7.0) concentration adjusted for μ = 0.15 ionic strength at 25 ± 1°C. Details for bathing-solution composition, preparation, and analyses were as published (12).

After peeling and mounting in the force transducer, each fiber was exposed to a 1-ml "load" bathing solution for 2 min to allow Ca^{2+} sequestration into the SR; the load solution was the same as the relaxing solution except that (*i*) 4 mM Na^+ plus 66 mM K^+ replaced 70 mM Na^+, (*ii*) 4 mM Cl^- replaced equimolar propionate, and (*iii*) EGTA was increased to 4 mM with Ca^{2+} added for pCa = 5.6. For all peeled-fiber bathing solutions, oligomycin (1 mg/ml) and ouabain (10 μM) were added to inhibit mitochondrial Ca^{2+} uptake and incompletely sealed T-tubules, respectively. The Ca^{2+}-loaded fiber was equilibrated in rinse solutions (two 1-ml aliquots for 1 min each), which were identical to the load solution except for reduction of EGTA to 20 μM and pCa to 6, with addition of 10 mM procaine to block caffeine contractures and Ca^{2+}-induced Ca^{2+} release (12). Load and rinse solutions established a polarized resting, or prestimulation, T-tubule state. Stimulation in the form of ionic depolarization was accomplished by switching the fiber from rinse solution to one in which 62 mM choline replaced equimolar K^+ and 62 mM Cl^- replaced equimolar propionate to a constant $[K^+] \times [Cl^-] = 4 \times 66$ mM^2 with all other variables the same. This ionic depolarization of T-tubules elicited SR Ca^{2+} release and a tension transient, referred to as "Cl^--induced" (12).

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Abbreviations: EC, excitation–contraction; T-tubule, transverse tubule; SR, sarcoplasmic reticulum; $InsP_3$, inositol 1,4,5-trisphosphate.

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$InsP_3$ was microinjected into the peeled fibers with T-tubules in polarized and depolarized resting states in the presence of 10 mM procaine to inhibit Ca^{2+}-induced Ca^{2+} release (12). On a sample of eight peeled fibers, paired data were collected consisting of three tension transients, in the following order, for each fiber: (*i*) $InsP_3$-induced with polarized T-tubules, (*ii*) Cl^--induced, and (*iii*) $InsP_3$-induced with depolarized T-tubules (Fig. 1).

Measurement of Ca^{2+} Release. Cl^--induced and $InsP_3$-induced SR Ca^{2+} releases, in the presence of procaine, were monitored as isometric tension transients, as shown in Figs. 1 and 2. In the absence of procaine, the Cl^--induced tension transient has primary (voltage-sensitive) and secondary (Ca^{2+}-induced) components (12). The secondary component was intentionally minimized in this study, by the presence of 1 mM free Mg^{2+} and 10 mM procaine in the bathing solutions for peeled fibers (3, 12, 13), because the mechanism of the primary component of SR Ca^{2+} release is most likely to reflect the voltage-dependent step of EC coupling (12). Cl^-- and $InsP_3$-induced tension transients were normalized by dividing their peak magnitudes by the magnitude of a maximum caffeine contracture (10 mM caffeine/100 μM EGTA/0.1 mM Mg^{2+}/66 mM Cl^- "depolarizing" solution) for the same peeled fiber (3). At the end of a protocol, each peeled fiber was maximally contracted in a bathing solution with saturating Ca^{2+} (pCa 3.6, 7 mM EGTA, otherwise matched to relaxing solution) to verify the magnitude of maximum force.

Bathing Solutions and Protocol for Zero Na^+ T-Tubule Depolarization. As an alternative to ionic depolarization of T-tubules, a sample of five fibers was tested by elimination of Na^+ from all bathing solutions. This was intended to minimize Na^+/K^+-ATPase polarization of sealed T-tubules in the peeled fiber preparation (12). The relaxing solution (70 mM Na^+) was not used in these experiments; all other bathing solutions had 4 mM Na^+ replaced with equimolar choline but were otherwise identical to the Na^+-containing solutions. Isolated fibers (cut ends) were soaked in a zero Na^+ rinse solution (containing 4 mM Cl^- and 20 μM EGTA) for at least 2.5 hr at 0°C and then peeled in the same solution. Experiments were conducted with only the 4 mM Cl^-, zero Na^+ load solution and the 4 mM Cl^-, zero Na^+ rinse solution with 10 mM procaine at 25 ± 1°C. The latter solution was the solvent for the 0.5 μM $InsP_3$ solution in the pipette.

Bathing Solutions and Protocol for T-Tubule Depolarization by Ouabain. Ouabain was applied to the Na^+/K^+-ATPase sites on the inside of T-tubules prior to peeling by soaking isolated fibers (cut ends) in a 1 mM ouabain relaxing solution (see above) for at least 5 hr at 0°C; the fibers were peeled in this solution (12). This treatment selectively eliminates Cl^--induced tension transients, presumably by minimizing T-tubule polarization by the T-tubule Na^+/K^+-ATPase (12). Of eight fibers treated with ouabain, none responded to an increase in Cl^- concentration from 4 to 66 mM; all fibers responded to caffeine, demonstrating SR function and releasable Ca^{2+}. Only five were included in the study because three broke or deteriorated during initial caffeine contracture. Thus, in this protocol, $InsP_3$ was injected in the 4 mM Cl^- rinse solution under conditions for continuous T-tubule depolarization.

Criteria for Inclusion of Peeled Fibers. All peeled fibers included in the ionic T-tubule polarization/depolarization protocol responded, in the presence of procaine, to an abrupt increase in bathing solution Cl^- concentration with Ca^{2+} release from the SR; thus, they all demonstrated responsiveness to a depolarization-induced T-tubule-to-SR communication step (12, 14, 15). Fibers that were depolarized either by removal of Na^+ from the bathing solution or by pretreatment with 1 mM ouabain were included if, after the fibers were peeled and loaded with Ca^{2+}, they responded to 10 mM caffeine with a tension transient of peak magnitude equal to maximum force generation for the fiber at saturating Ca^{2+} concentration.

Microinjection of $InsP_3$. $InsP_3$ was prepared from crude brain phosphoinositides (Sigma P6023), purified, and assayed as reported (3). The method for microinjection of $InsP_3$ was as published (3). In brief, patch pipettes were filled with a solution containing 0.5 μM $InsP_3$ and 10 mM procaine, with either the 4 mM Cl^- rinse solution or the 66 mM Cl^- depolarizing solution as solvent. Peeled fibers were equilibrated in 1 ml of either the 4 mM Cl^- rinse solution or the 66 mM Cl^- depolarizing bathing solution (10 mM procaine in each) for at least 2 min to set resting, or prestimulation, T-tubule conditions. The fibers were then moved into silicone oil and microinjected with ≈1 nl of the 0.5 μM $InsP_3$ pipette solution. After the peak of the $InsP_3$-induced tension transient was reached, the peeled fiber was again immersed in 1 ml of the bathing solution used to set the resting T-tubule condition.

RESULTS

Ionic T-Tubule Polarization/Depolarization Protocol. Resting, or prestimulation, T-tubule depolarization significantly enhanced Ca^{2+} release stimulated by microinjected 0.5 μM $InsP_3$. In paired-protocol studies of a sample of eight fibers, seven responded with a larger $InsP_3$-induced tension transient when $InsP_3$ was microinjected with T-tubules in a depolarized as compared to polarized state. As shown in Fig. 1, after equilibration of the peeled fiber in the 4 mM Cl^- solutions to establish a polarized resting T-tubule state, microinjection of 0.5 μM $InsP_3$ (in 4 mM Cl^-) resulted in a tension transient (trace 1) that was smaller than that elicited by either ionic depolarization of T-tubules (Cl^--induced, trace 2) or microinjection of 0.5 μM $InsP_3$ using depolarizing bathing solutions (66 mM Cl^-) in the pipette and fiber (trace 3).

The increased fiber sensitivity to $InsP_3$ with resting T-tubule depolarization cannot be explained by fiber differences, since all data are paired. Nor can it be explained by cumulative effects of the microinjections, since each fiber was immersed in two 1-ml baths of bathing solution for at least 3 min between microinjections. Although the Ca^{2+}-induced Ca^{2+}-release mechanism of the SR might be enhanced by depolarizing bathing-solution conditions (16), $InsP_3$ appears to stimulate in the presence of procaine and 1 mM Mg^{2+}, which inhibit Ca^{2+} as a stimulus (3, 12).

To eliminate the possibility that contaminants inherent in the 66 mM Cl^- solution were causing apparent increased sensitivity to $InsP_3$, fibers were microinjected exclusively with the 4 mM Cl^- $InsP_3$ solution after equilibration in either T-tubule-polarizing or T-tubule-depolarizing bathing solutions. The risk was that the 4 mM Cl^- $InsP_3$ injectate would repolarize fibers, making all $InsP_3$ responses characteristic of the resting polarized state, since the 1 nl microinjected represents approximately half of the peeled fiber volume in oil (3). Indeed, two of five fibers tested appeared to be repolarized by $InsP_3$ in the 4 mM Cl^- solution in that they showed no difference in response to $InsP_3$ regardless of resting, or prestimulation, polarization state of the T-tubules; the $InsP_3$-induced tension transients in these two fibers were close to the mean value of all fibers for $InsP_3$ (in 4 mM Cl^-) microinjected with T-tubules in a polarized state. However, three fibers exhibited a larger $InsP_3$-induced tension transient under conditions for resting T-tubule depolarization than they did under polarization conditions despite the fact that $InsP_3$ was microinjected in the 4 mM Cl^- solution. The enhanced effectiveness of $InsP_3$ under conditions for T-tubule depolarization indicates that, in some fibers, the $InsP_3$ effect was more rapid than the polarizing effect of the pipette solution.

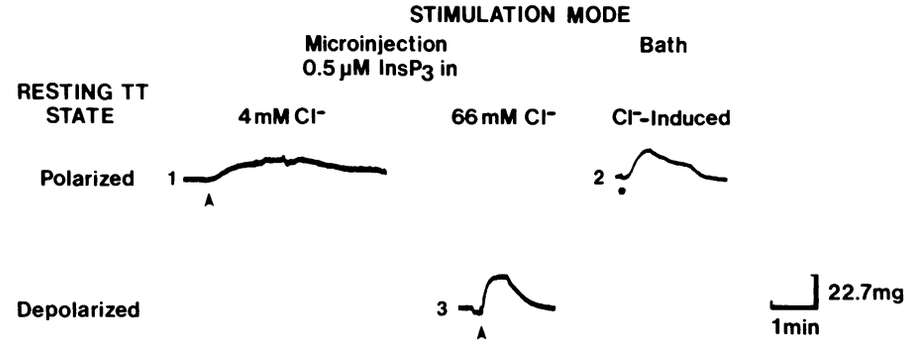

FIG. 1. Ca^{2+}-activated tension transients of peeled fibers stimulated by microinjections of $InsP_3$ and ionic depolarization. The tension traces are all from the same rabbit adductor magnus fiber (outer diameter, 60 μm) and are numbered according to order of occurrence. Trace 1: after loading of Ca^{2+} into the SR and equilibration in rinse solution, the fiber was placed in silicone oil and 1 nl of 0.5 μM $InsP_3$ (dissolved in 4 mM Cl^- rinse solution) was microinjected into the myofilament lattice (arrowhead); at the completion of the tension transient, the fiber was returned to the 4 mM Cl^- rinse solution (1-ml bath) to wash away exogenous $InsP_3$. Trace 2: the fiber was transferred from 4 mM Cl^- rinse solution to 66 mM Cl^- ionic depolarization solution (dot). Trace 3: the fiber was moved from the 66 mM Cl^- bath to silicone oil and microinjected (arrowhead) with 0.5 μM $InsP_3$ (in 66 mM Cl^-). TT, T-tubule.

Fig. 2 shows the effect of altered resting T-tubule conditions on the responses of one fiber to stimulation with $InsP_3$ in 4 mM Cl^-. Responses shown as traces 1 and 4–7 appear relatively small and similar and were elicited after exposure of the fiber to the T-tubule-polarizing (4 mM Cl^-) bathing solution. The larger tension transients (traces 3, 8, and 9) were elicited by the same $InsP_3$ (in 4 mM Cl^-) stimulus but under conditions for depolarized resting T-tubules established by equilibration for 2 min in 1 ml of the 66 mM Cl^- bathing solution before transfer of the fiber to oil and microinjection.

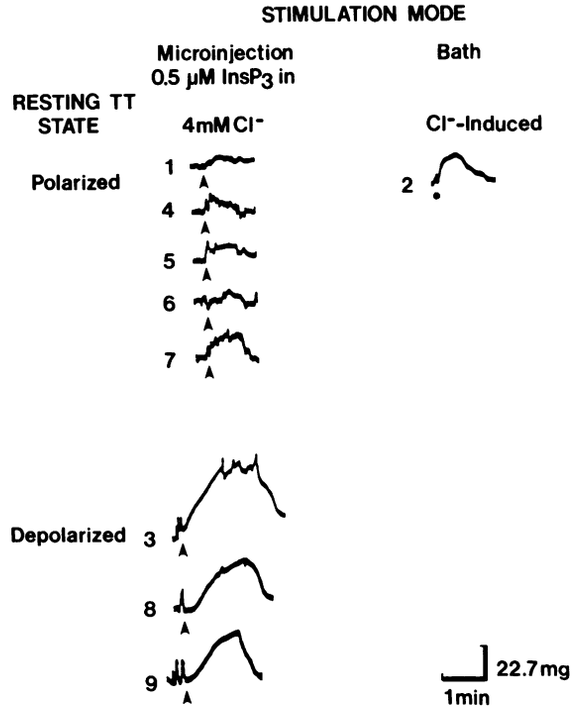

FIG. 2. Peeled-fiber tension transients stimulated by $InsP_3$ under various conditions of T-tubule (TT) polarization and by bath Cl^-. The peeled fiber was from rabbit adductor magnus (outer diameter, 70 μm). See *Methods* for Ca^{2+} loading protocol. All microinjections were made into the peeled fiber while it was immersed in silicone oil, and the pipette solution was always 0.5 μM $InsP_3$ in the polarizing rinse solution (4 mM Cl^-). Tension traces are numbered in order of occurrence. Trace 1: tension transient elicited in response to $InsP_3$ microinjection (arrowhead) after equilibration of the peeled fiber in 1 ml of polarizing rinse (4 mM Cl^-) solution. Trace 2: the Cl^--induced tension transient, in response to an abrupt increase (dot) in the Cl^- concentration of the bathing solution. Trace 3: tension transient stimulated by microinjection of $InsP_3$ but with the fiber fluid equilibrated with 66 mM Cl^- depolarizing solution. Traces 4–7: $InsP_3$-induced tension transients due to multiple microinjections with the fiber continuously in oil. The resting T-tubule polarization was accomplished by prior microinjection(s) of 1 nl of 4 mM Cl^- rinse solution (as solvent for $InsP_3$). Traces 8 and 9: tension transients elicited in the same manner as for trace 3.

For trace 1 in Fig. 2, the peeled fiber was equilibrated (3 min) in 1 ml of the 4 mM Cl^- bathing solution before it was transferred to oil and microinjected with $InsP_3$ (in 4 mM Cl^-). In contrast, traces 4–7 were elicited by successive microinjections of $InsP_3$ (in 4 mM Cl^-) without removing the fiber from oil. After the microinjection of $InsP_3$ (in 4 mM Cl^-) for trace 3, the fluid in the peeled fiber was polarizing for T-tubules for traces 4–7. For analysis, only data from traces 1, 2, and 3 from this fiber were included in the mean values of Table 1. The responses of this fiber (Fig. 2) demonstrate that, as observed previously (3), multiple $InsP_3$-induced tension transients can be elicited by successive microinjections and that, in addition, the magnitude of the $InsP_3$-induced tension transient correlates with fluid composition in the peeled fiber rather than in the pipette.

$InsP_3$ in the 66 mM Cl^- solution was not similarly tested with the fiber in the polarized resting T-tubule state, because the increase in Cl^- concentration would constitute a concurrent, additional Ca^{2+}-release stimulus as shown in traces 2 of Figs. 1 and 2. In previous work, when relatively high bath concentrations of $InsP_3$ were used to trigger Ca^{2+} release (3), we observed that equilibration of a peeled fiber in 100–300 μM $InsP_3$ did not always trigger Ca^{2+} release when the bathing solution was 4 mM Cl^-. However, subsequent ionic depolarization with 66 mM Cl^- often yielded a greatly enhanced tension transient compared to the Cl^--induced one in the absence of $InsP_3$ (unpublished data). These observations provided the impetus for this study.

Table 1 shows mean magnitudes of the Ca^{2+}-release tension transients for each stimulation type and resting T-tubule condition for 4 mM Na^+ solutions. The mean magnitude of the tension transient for $InsP_3$ (in 4 mM Cl^-) microinjected into fibers with polarized resting T-tubules is significantly smaller than that obtained with $InsP_3$ (in 66 mM Cl^-) microinjected into the same fibers with depolarized T-tubules ($P < 0.01$). The mean of the initial tension transient for $InsP_3$ (in 4 mM Cl^-) injected into fibers with ionically depolarized resting T-tubules (0.62 ± 0.07) is not significantly different from that for $InsP_3$ (in 66 mM Cl^-) injected under the same conditions for depolarized resting T-tubules (0.71 ± 0.06). However, there was no significant difference ($P > 0.05$) in the means for $InsP_3$ (in 4 mM Cl^-) injected into

Table 1. Normalized peak magnitudes of tension transients in response to SR Ca^{2+} release stimulated by microinjection of 0.05 μM $InsP_3$ or by bath Cl^--induced depolarization of T-tubules

Resting T-tubule state	Normalized peak magnitude*		
	Microinjection		Cl^--induced depolarization
	$InsP_3$ in 4 mM Cl^-	$InsP_3$ in 66 mM Cl^-	
Polarized	0.30 ± 0.08 (n = 8)†¶		0.25 ± 0.08 (n = 8)‡§
Depolarized			
Ionically	0.62 ± 0.07 (n = 5)	0.71 ± 0.06 (n = 8)†‡	
Ouabain	0.67 ± 0.05 (n = 5)§¶		

*Peak magnitude of tension transient/maximum Ca^{2+}-activated tension. Values are means ± SEM (n = no. of fibers); $P > 0.05$, nonsignificant
†,‡Statistically significant difference ($P < 0.01$, t test for paired data).
§,¶Statistically significant difference ($P < 0.01$, Mann–Whitney U test).

fibers with polarized resting T-tubules (0.30 ± 0.08) and those with ionically depolarized resting T-tubules (0.62 ± 0.07). This nonsignificance is attributed to T-tubule-polarizing effects of the injectate in two of the five fibers tested in the paired-data protocol.

Zero Na^+ T-Tubule Depolarization Protocol. Experiments were conducted with bathing solutions devoid of Na^+ to determine whether the increase in Cl^- concentration and/or variations in other ionic constituents of the bathing solutions might have been increasing $InsP_3$ effectiveness independently of their effects on T-tubule potential. In this protocol, the bathing solutions for peeled fibers matched the composition of the load and rinse solutions of the ionic polarization/depolarization protocol, except that choline was substituted for Na^+ (4 mM). The zero Na^+, 4 mM Cl^- $InsP_3$-induced tension transients (0.51 ± 0.01, n = 5) were not significantly different (P = 0.19, Mann–Whitney U test) from those elicited by microinjection of $InsP_3$ in 4 mM Cl^- into fibers where T-tubules were ionically depolarized by 66 mM Cl^- in the bathing solutions (0.71 ± 0.06, n = 8, Table 1).

The pooled mean for 4 mM Cl^-, $InsP_3$-induced tension transients (4 mM Na^+ plus zero Na^+) under the resting depolarized T-tubule state (0.56 ± 0.04, n = 10) was significantly larger ($P < 0.05$, Mann–Whitney U test) than that for 4 mM Cl^-, $InsP_3$-induced tension transients under polarized resting T-tubule conditions (0.30 ± 0.08, n = 8, Table 1). Thus, significantly larger $InsP_3$-induced tension transients are elicited when T-tubules are depolarized, either ionically through a diffusion potential or by equilibration in zero Na^+ medium, independent of the concentrations of Cl^-, K^+, and counterions of the bathing solutions.

The requirement of SR Ca^{2+} loading for the $InsP_3$ effect in the absence of Na^+ was tested in three fibers to correspond with control experiments conducted previously with 4 mM Na^+ bathing solutions (3). After the initial $InsP_3$-induced tension transient in zero Na^+ solution, procaine was washed away in rinse solution (5 min) and the Ca^{2+} content of the SR was diminished by stimulation with 10 mM caffeine, which resulted in a large tension transient. After a 2-min wash in rinse solution to remove caffeine, each fiber was again microinjected with $InsP_3$ (zero Na^+, 4 mM Cl^-); none showed an increase in tension. However, after the SR was reloaded with Ca^{2+}, all three again responded with an $InsP_3$-induced tension transient (data not shown).

Ouabain T-Tubule Depolarization Protocol. Pretreatment of fibers with ouabain, to inhibit Na^+/K^+-ATPase, was employed as another means of achieving sustained depolarization of T-tubules in the 4 mM Cl^- bathing solutions. In this protocol, the peeled fibers were shown to be insensitive to ionic depolarization with Cl^-, and the $InsP_3$ responses were tested with only 4 mM Cl^- bathing solutions in the peeled fiber and the pipette. Thus the bathing solution composition was identical to that for the ionic T-tubule polarization protocol where 0.5 μM $InsP_3$ elicited the smallest responses. With ouabain-induced resting T-tubule depolarization, $InsP_3$ elicited a significantly larger ($P < 0.01$) tension transient in the 4 mM Cl^- bathing/pipette solutions (0.67 ± 0.05) as compared to the effects of the same solutions and injectate under conditions for ionically polarized T-tubules (0.30 ± 0.08, Table 1). The effect of 0.5 μM $InsP_3$ on Ca^{2+} release is not significantly different for ionic versus ouabain-induced depolarization of T-tubules whether the $InsP_3$ is dissolved in solutions of high or low Cl^- concentration (P values > 0.05, Table 1).

Cl^--Induced Tension Transients. The mean Cl^--induced tension transient is similar in magnitude (Table 1) to that reported for peeled rabbit adductor fibers in the presence of procaine and 1 mM Mg^{2+} (12). Thus, the peeled fibers in the ionic T-tubule polarization/depolarization protocol of this study were typical of rabbit skeletal muscle and had functional T-tubule–SR voltage-dependent coupling mechanisms.

DISCUSSION

Stimulation of SR Ca^{2+} Release by Submicromolar $InsP_3$. The concentration of $InsP_3$ used in this study (0.5 μM) is, to our knowledge, the lowest published for stimulation of Ca^{2+} release in peeled muscle fibers. The mean magnitude of tension stimulated by 0.5 μM $InsP_3$ under conditions of T-tubule depolarization is nearly the same as those reported for peeled rabbit adductor fibers microinjected with 1.0 and 10 μM $InsP_3$, which were maximal responses (3).

Since oligomycin in the bathing solutions should have blocked mitochondrial Ca^{2+} cycling, the SR is the only major internal store of releasable Ca^{2+} in the peeled fibers. The finding that pretreatment with caffeine, an agent that greatly reduces the Ca^{2+} content of peeled-fiber SR (12, 13), eliminates the $InsP_3$-induced tension transient supports the view that $InsP_3$ stimulates Ca^{2+} release from the SR. $InsP_3$ also elicits internal Ca^{2+} release in saponin-treated fibers (1, 2) in which the SR is the only likely nonmitochondrial internal store of Ca^{2+} (12).

$InsP_3$ Acts on the SR. The site of action of $InsP_3$ cannot be directly demonstrated in the peeled fiber preparation. $InsP_3$ is unlikely to be stimulating through a mechanism dependent on T-tubule membrane potential, since $InsP_3$ is more effective under conditions that depress T-tubule-depolarization-induced Ca^{2+} release from the SR in this preparation (12) and in intact skeletal muscle fibers (9). It is also unlikely that $InsP_3$ effects are mediated by T-tubule Ca^{2+} channels and T-tubule Ca^{2+} release, since $InsP_3$ stimulates Ca^{2+} release from the SR in saponin-skinned fibers where T-tubule membrane integrity is compromised (1, 2). Furthermore, under the conditions of this study, Ca^{2+} is unlikely to stimulate Ca^{2+} release from the SR. The most likely site of $InsP_3$ action is the SR. A report of $InsP_3$-induced Ca^{2+} release from isolated SR vesicles of rabbit skeletal muscle has appeared (2).

Resting Membrane Potential Effects Specific for T-Tubules. Variations in K^+ and Cl^- concentrations in the bathing solution most likely affect T-tubule rather than SR membrane potential in this preparation, since the polarizing effects of these ions are eliminated by agents specific for T-tubules, such as ouabain (12). There is no evidence that SR membrane potential would be altered by the changes in ionic composition of the bathing solutions (12, 17, 18) employed in this study, and SR depolarization does not appear to trigger Ca^{2+} release in peeled skeletal fibers (12). Similarly, there is no evidence for SR depolarization as a stimulus for EC coupling *in vivo* (19). Further, in the ouabain depolarization protocol, only the T-tubules should have been affected by ouabain and no changes in ionic composition of the bathing solutions were employed. Thus, resting T-tubule membrane potential probably governs the sensitivity of the SR to $InsP_3$, perhaps through a structural or conformational change (20) involving the SR "feet" structures that span the gap between the T-tubule and the SR (21).

$InsP_3$ Effects and Functional Integrity of the T-Tubules and SR. Controversy as to the possible role of $InsP_3$ as a T-tubule–SR signal stems primarily from negative results (22) and the slow time course of $InsP_3$-induced SR Ca^{2+} release (4) reported by others using skinned fiber preparations.

In those studies the responsiveness of the skinned fibers to the physiological stimulus of T-tubule depolarization was not tested (4, 22), so that T-tubule–SR functional integrity was unknown, or $InsP_3$ was applied under conditions for polarized T-tubules (22). $InsP_3$ can be shown to stimulate a large SR Ca^{2+} release with polarized T-tubules, but relatively high concentrations must be microinjected. Positive results were obtained in other skinned fiber preparations where it is likely that depolarized T-tubules sensitized the SR to low concentrations of bath $InsP_3$ as a result of saponin treatment (1, 2) or glycerol-induced disengagement of T-tubules from sarcolemma (23). Rabbit skeletal fibers with cut ends and attached T-tubules contract in response to microinjected $InsP_3$ (unpublished data), suggesting that T-tubule detachment is not a requirement for $InsP_3$ stimulation of Ca^{2+} release from the SR.

$InsP_3$ in Skeletal Muscle EC Coupling. Voltage sensitivity of SR responsiveness to $InsP_3$ is of potential importance in defining the physiologic mechanism of skeletal muscle EC coupling. The influence of voltage observed in this study raises the possibilities that $InsP_3$ is a chemical stimulus for SR Ca^{2+} release and that T-tubule depolarization activates by sensitizing the SR to the preexisting $InsP_3$ concentration. Repolarization could terminate Ca^{2+} release by desensitizing the SR to $InsP_3$. The critical time factor would thus be for a physical step, T-tubule to SR, that could occur in a millisecond time frame. In this scheme, phosphatidylinositol bisphosphate hydrolysis and $InsP_3$ production could still be increased by electrical stimulation (1), but $InsP_3$ need not diffuse from T-tubule to SR to initiate Ca^{2+} release.

There is convincing evidence that voltage-dependent influx of extracellular Ca^{2+} is not the stimulus for SR Ca^{2+} release in skeletal muscle EC coupling (24, 25). Similarly in peeled fiber studies, Ca^{2+} release induced by T-tubule depolarization can occur when the Ca^{2+} stimulation of the Ca^{2+}-induced mechanism of Ca^{2+} release from the SR is specifically blocked (12).

$InsP_3$ could be a primary stimulus for SR Ca^{2+} release in skeletal muscle, since, as demonstrated here, it can stimulate SR Ca^{2+} release under conditions that inhibit Ca^{2+} stimulation. The slow time course of $InsP_3$-induced tension transients of skinned fibers remains problematic in that Ca^{2+} release is much faster *in vivo*. Uneven $InsP_3$ distribution cannot fully explain this, since tension transients in response to internally preequilibrated caged $InsP_3$ were also slow (4). However, measurement of the rate of skinned-fiber tension generation may not be an adequate assay for the rate of $InsP_3$-induced Ca^{2+} release, since the former also reflects the degree of longitudinal uniformity of Ca^{2+} release and activation in a preparation where T-tubule–SR functional integrity is likely to be uneven. A more direct measure of the rate and spatial distribution of $InsP_3$-induced Ca^{2+} efflux is needed, as well as further experiments involving manipulation of endogenous $InsP_3$, to determine whether $InsP_3$ is an essential cofactor or a modulator in skeletal muscle EC coupling.

T-tubule voltage regulation of $InsP_3$ effectiveness may be relevant for other aspects of voltage control of contraction in skeletal muscle. Voltage control of $InsP_3$ effects on Ca^{2+} release from endoplasmic reticulum in other cells has not been explored.

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