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Twitches in the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid

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SUMMARY

Single muscle fibers continue to twitch for up to 20 min when immersed in ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) solutions containing less than 10^{-8} M free calcium. Failure of the twitch results from reversible depolarization, which occurs after 15–20 min in EGTA. The results make it clear that external calcium or calcium in the transverse tubules play no essential part in action potential propagation or excitation–contraction coupling.

Ca^{2+} has important effects on excitation–contraction coupling and on the action potential. Release of Ca^{2+} from the sarcoplasmic reticulum is known to be one of the final steps in coupling the action potential in a muscle fiber membrane to activation of the contractile filaments (see ref. 1 for review), but the link between depolarization of the muscle membrane and Ca^{2+} release is not known. There is now evidence that the release of Ca^{2+} from the sarcoplasmic reticulum is regenerative; *i.e.* Ca^{2+} itself leads to the release of more Ca^{2+} (ref. 2). This suggests the possibility that the initial triggering event may be the small influx of Ca^{2+} that is known to occur following membrane excitation[3]. Twitching continues for many minutes despite the removal of external Ca^{2+} (refs 4–6), but it seems possible that Ca^{2+} in a partially sequestered compartment like the transverse tubule system might remain high enough to allow Ca^{2+} to serve a trigger function. We have tested both this possibility and the possibility that Ca^{2+} plays a crucial role in action potential generation by immersing single muscle fibers in ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) containing Ringer solutions

Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

with extremely low Ca^{2+} concentrations. The results make it extremely unlikely that external Ca^{2+} or transverse tubule Ca^{2+} is crucially involved in action potential generation or in causing Ca^{2+} release from the sarcoplasmic reticulum.

Single fibers or small bundles of fibers were isolated from the semitendinosus muscle of the frog *Rana pipiens*. One end of the fiber was fixed and the other was connected to a mechano-electric transducer (RCA 5734) which measured tension. The fibers were stretched to 110 to 130% of their slack length, and all contractions were isometric. Stimulation was by means of two external electrodes. The compositions of the solutions used are given in Table I. pH of the EGTA solutions was adjusted with NaOH.

TABLE I

SOLUTION CONTENT

Designation	*mmoles/l*						
	K^+	Cl^-	Na^+	Ca^{2+}	HPO_4^{2-}	$H_2PO_4^-$	*EGTA*
Ringer solution	2.5	121	120	1.8	2.15	0.85	0
Low Ca^{2+} Ringer solution	2.5	117.4	120	$<4\cdot10^{-3}$	2.15	0.85	0
Low Ca^{2+} Ringer solution	2.5	117.4	124	$<2\cdot10^{-6}$	2.15	0.85	1

When changing from normal Ringer solution to a low Ca^{2+} EGTA solution, the fiber was first rinsed with low Ca^{2+} Ringer solution and after a few seconds with low Ca^{2+} EGTA Ringer solution. All experiments were performed at room temperature (about 20°C).

The free Ca^{2+} concentration in low Ca^{2+} EGTA Ringer solution was calculated from the equations given in ref. 9 to be $2\cdot10^{-9}$ M, assuming that the total concentration of contaminant calcium was 10^{-5} M. Actual measurements of the contaminant calcium concentration were kindly performed for us by Dr Y.S. Reddy, using a fluorimetric method, and the concentration was less than $4\cdot10^{-6}$ M. Free Ca^{2+} in our solutions may thus have been slightly less than the value given above.

Fig. 1 illustrates the result of immersing a fiber in low Ca^{2+} EGTA Ringer solution. The fiber was stimulated once every 10 s. The first twitches in the record were recorded in normal Ringer solution, and at the arrow this was changed first to low Ca^{2+} Ringer solution, and then to low Ca^{2+} EGTA Ringer solution. Immediately after the

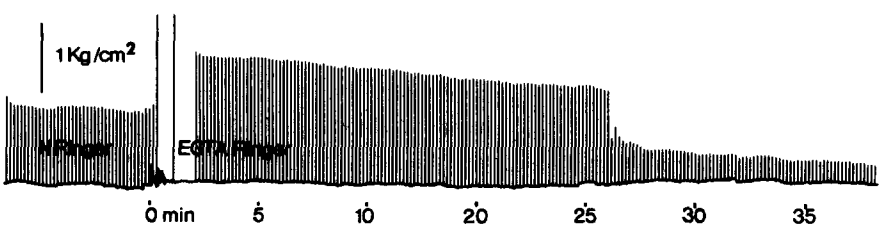

Fig. 1. Twitches in a single fiber on changing from normal Ringer's fluid to one containing 1 mM EGTA and no added calcium. Stimulation frequency, 0.1 Hz.

change, the fiber began to respond tetanically to each stimulus (the recorder saturated, and these responses are clipped) and stimulation was interrupted. After 2 min, stimulation was resumed, and the apparent twitch tension was about 50% greater than in normal Ringer solution, probably because the fiber responded with two action potentials to each stimulus, as often happens in low calcium solutions. Twitch amplitude then slowly declined over the course of 19 min, and then abruptly dropped as one end of the fiber began failing. The older end continued to contract and stretch out the failing part until the fiber was returned to normal Ringer solution after 43 min in low Ca^{2+} EGTA Ringer solution. Five other fibers were almost equally resistant to low Ca^{2+} EGTA Ringer solution. In four of them twitching had not failed at the time of reimmersion in normal Ringer solution, after 8.5 to 14.6 min in low Ca^{2+} EGTA Ringer solution. Most of the fibers twitched well when returned to normal Ringer solution.

Failure of the twitch could result either from failure of the action potential or from defective coupling of the action potential to the contractile machinery. To determine which seemed more likely, we followed the resting potential in a bundle of three fibers after immersion in low Ca^{2+} EGTA Ringer solution, and the results are shown in Fig. 2. The open symbols show the resting potentials in normal Ringer solution before and after immersion in low Ca^{2+} EGTA Ringer solution. Twitching was followed visually, and

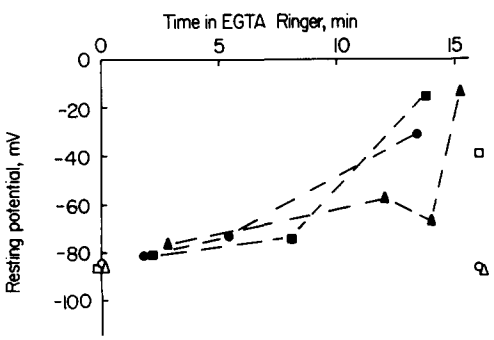

Fig. 2. Internal potential in three fibers exposed to low Ca^{2+}, 1 mM EGTA Ringer's fluid. Open symbols represent resting potentials in normal Ringer's fluid before and after recovery from exposure to the EGTA Ringer's fluid.

continued throughout the slow phase of depolarization. None of the fibers twitched when their resting potentials had fallen to the low values shown at the end of the period in low Ca^{2+} EGTA Ringer solution. Two of the three fibers recovered completely on re-immersion in normal Ringer solution. Failure of the twitch in low Ca^{2+} EGTA Ringer solution thus seems to be the result of electrical failure: when the resting potential falls low enough, the sodium conductance of the fiber membrane becomes inactivated, making action potentials impossible. These results are quite similar to those of Edman and Grieve[5] who studied fibers in low calcium solutions without EGTA.

From these results it is immediately clear that extracellular calcium plays no essential role in action potential generation. The calcium concentration at the fiber surface must have fallen within a second or two to less than 10^{-8} M, and action potentials continued to propagate normally for 10 min or more thereafter. In myelinated nerve, in contrast, at least 10 μM Ca^{2+} is necessary to sustain conduction[7]. The difference may be that muscle fibers have a large chloride permeability which helps to stabilize the resting potential at a negative value. Nerve fibers do not have such a large chloride permeability, and for this reason they may depolarize more rapidly than muscle fibers. Endo[8] found that the dye Lissamine Rhodamine B 200 (mol. wt. 558) diffused readily into the transverse tubule system, and washed out with a half time of 4 to 10 s. EGTA (mol. wt 380) in our experiments within no more than a minute must have reached a concentration of about 1 mM in the transverse tubule system, lowering the free Ca^{2+} concentration there to about $2 \cdot 10^{-9}$ M. The small Ca^{2+} efflux measured from muscle fibers could not have raised the concentration significantly above this level, and the fibers nonetheless continued to twitch for many minutes. These results strongly indicate that Ca^{2+} in the transverse tubules cannot be involved in triggering the release of Ca^{2+} from the sarcoplasmic reticulum. A simple calculation helps to make the point. If it is assumed that the Ca^{2+} influx caused by an action potential ($0.2 \cdot 10^{-12}$ M·cm^{-2}·impulse^{-1} in 1.8 mM Ca^{2+}) is proportional to the external Ca^{2+} concentration then at $2 \cdot 10^{-9}$ M Ca^{2+} the influx is only $0.2 \cdot 10^{-18}$ M·cm^{-2}·impulse^{-1}. This is roughly one calcium ion per sarcomere, surely too little to serve as a reliable trigger.

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