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Is a guanine nucleotide-binding protein involved in excitation–contraction coupling in skeletal muscle?

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Plasma membrane depolarization causes skeletal muscle contraction by triggering Ca^{2+} release from an intracellular membrane network, the sarcoplasmic reticulum. A specialized portion of the sarcoplasmic reticulum, the terminal cisternae, is junctionally associated with sarcolemmal invaginations called the transverse tubules, but the mechanism by which the action potential at the level of the transverse tubules is coupled to Ca^{2+} release from the terminal cisternae is still mysterious. Here we show that: (i) $GTP_\gamma S$, a non-hydrolyzable analog of GTP, elicits isometric force development in skinned muscle fibre; (ii) $GTP_\gamma S$ is unable to release CA^{2+} from isolated sarcoplasmic reticulum fractions; (iii) the threshold for tension development is shifted to higher $GTP_\gamma S$ concentrations by pre-incubation with pertussis toxin. These results suggest that a GTP-binding protein is involved in coupling the action potential of transverse tubules to Ca^{2+} release from the terminal cisternae.

Key words: muscle/excitation–contraction coupling/N protein

Introduction

Skeletal muscle contraction is initiated by sarcolemmal depolarization which triggers the release of Ca^{2+} from intracellular depots (Huxley and Taylor, 1958; Winegrad, 1970). The anatomical site at which the coupling of electrical events to Ca^{2+} release occurs is the triadic junction which is formed by sarcolemmal invaginations called the transverse tubules together with a specialized component of the sarcoplasmic reticulum called the terminal cisternae and bridging structures called 'feet' (Franzini-Armstrong, 1970). Although the anatomy of this coupling unit is well defined (Ferguson *et al.*, 1984), its physiology is a matter of dispute. In particular the molecular mechanism by which the action potential flowing along transverse tubules triggers Ca^{2+} release from terminal cisternae, i.e. the mechanism of excitation–contraction (EC) coupling, is still unknown (Endo, 1977). We have recently suggested that in muscle, as in other cell types (Berridge and Irvine, 1984), a chemical mediator, inositol trisphosphate ($InsP_3$) is responsible for EC coupling (Volpe *et al.*, 1985). In other cell types it has been proposed that the polyphosphoinositide (PPI) phosphodiesterase which generates $InsP_3$ from phosphatidylinositol(4,5)bisphosphate [PtdIns(4,5)P_2] is coupled to plasma membrane receptors via a GTP-binding protein (Np,p for 'phosphodiesterase') analogous to those involved in the control of adenylate cyclase activity (Cockroft and Gomperts, 1985). Furthermore, it has also been shown recently that pertussis toxin, also called islet activating protein (IAP), selectively inhibits several cell functions dependent on the activation of receptors linked to PPI turnover (Okajima and Ui, 1984).

This study was therefore aimed at investigating the effect of $GTP_\gamma S$ on Ca^{2+} release in isolated sarcoplasmic reticulum fractions and in chemically and mechanically skinned skeletal fibres.

Results and Discussion

$GTP_\gamma S$ elicits tension rise in chemically skinned fibres

Ca^{2+} release from the sarcoplasmic reticulum of chemically skinned muscle fibres of the rabbit (Salviati *et al.*, 1982) was followed indirectly as isometric force development. A segment of a single fibre was exposed to Ca^{2+} loading solution ($[Ca^{2+}]$= 0.25 μM) for 30 s, then rinsed with an EGTA-free solution and challenged with $GTP_\gamma S$ (Figure 1a). The addition of the guanine nucleotide elicited a tension rise which levelled off rather slowly. Similar results were obtained with another non-hydrolyzable GTP analog, GMP-PNP. The rise of tension was slower and sometimes preceded by a short lag, as compared with that elicited by caffeine, a drug known to cause Ca^{2+} release from terminal cisternae of isolated fractions and of intact and skinned fibres (Weber and Herz, 1968; Luttgau and Oetlicher, 1968; Stephenson, 1981). The latency disappeared, however, at high $GTP_\gamma S$ concentrations and also the rise of tension was faster (not shown in the figures). We think, therefore, that the slow response could be due, at least in part, to the time needed for $GTP_\gamma S$ to diffuse into the fibre. Seventeen fibres were challenged under identical experimental conditions with sequential $GTP_\gamma S$ additions: eight exhibited a threshold at 100 μM $GTP_\gamma S$, six at 200 μM, two at 250 μM and one at 500 μM. Despite this slight variability not a single fibre failed to respond to $GTP_\gamma S$. Moreover, $GTP_\gamma S$-

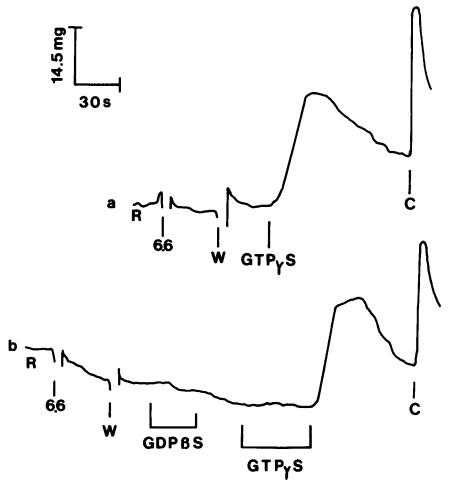

Fig. 1. $GTP_\gamma S$ elicits isometric force development in chemically skinned muscle fibres. Trace **a** shows rise of tension induced by 200 μM $GTP_\gamma S$. Caffeine (C) was 10 mM. Trace **b** demonstrates that GDPβS (500 μM) was completely ineffective and that nevertheless the fibre was responsive to subsequent additions of $GTP_\gamma S$ (500 μM). Single fibres were placed in solution R, then in Ca^{2+} uptake solution (pCa 6.6) and finally in solution W.

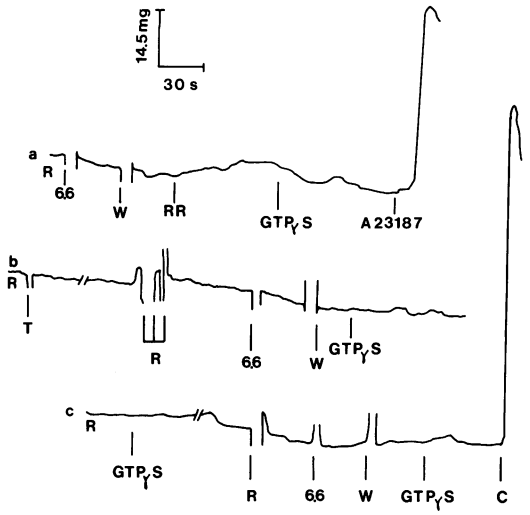

Fig. 2. $GTP_\gamma S$-induced rise of tension requires Ca^{2+} mobilization from sarcoplasmic reticulum. Trace **a** shows that in the presence of ruthenium red (RR) (20 μM), the effect of $GTP_\gamma S$ (250 μM) was abolished. A23187 (10 μg/ml), however, elicited a tension rise. Ruthenium red prevented a tension rise over the whole range of $GTP_\gamma S$ concentrations tested, i.e. from 100 to 500 μM, both at low or high $[Mg^{2+}]$. In trace **b** the fibre was first exposed to 3% Triton X-100 (T), then washed in solution R, loaded with Ca^{2+} and finally placed in solution W. $GTP_\gamma S$ was 250 μM. Caffeine (C) 10 mM. In trace **c** the fibre, before being loaded with Ca^{2+} and exposed to $GTP_\gamma S$ (500 μM) in solution W, was pre-treated with 500 μM $GTP_\gamma S$ in solution R for 8 min.

Table I. Effect of $GTP_\gamma S$ on isolated sarcoplasmic reticulum fractions

	Longitudinal sarcoplasmic reticulum	Terminal cisternae
Ca^{2+}-dependent ATPase (% of control activity)	98	98
Ca^{2+} loading (% of control rate)	102	95
Ca^{2+} uptake (% of control rate)	94	96
Extent of Ca^{2+} release (% of accumulated Ca^{2+})	1	2

$GTP_\gamma S$ was 500 μM. For experimental details see Materials and methods. Data are duplicate determinations from two different sarcoplasmic reticulum preparations.

induced force development was also observed when $[Mg^{2+}]$ was raised from 0.1 to 2.6 mM. A non-hydrolyzable analog of GDP, GDPβS, did not cause tension rise whereas subsequent additions of $GTP_\gamma S$ elicited force development (Figure 1b). Since myofibrils possess binding sites for nucleotides, $GTP_\gamma S$ could elicit force development simply by interfering with these sites, i.e. by interacting directly with the myofibrils. This possibility was ruled out because, as Figure 2a shows, $GTP_\gamma S$-triggered contraction, but not that triggered by the Ca^{2+} ionophore A23187, was completely abolished by 20 μM ruthenium red, a blocker of Ca^{2+} channels in terminal cisternae (Antoniu *et al.*, 1985). Furthermore, the blocking effect of ruthenium red could not be overcome by increasing $GTP_\gamma S$ up to the higher concentration tested, 500 μM, either in low or high Mg^{2+} (not shown). Secondly, pretreatment of skinned fibres with Triton X-100, which destroys the integrity of the sarcoplasmic reticulum compartment and of the sarcolemma without impairing the contractile apparatus, prevented the tension rise evoked by $GTP_\gamma S$ (Figure 2b). Finally, fibre exposure to $GTP_\gamma S$ for 8 min in an EGTA-containing medium, before Ca^{2+} loading, did not develop tension (Figure 2c). In this experiment the fibre was pre-incubated with 500 μM $GTP_\gamma S$ in solution R (see Materials and methods for description of solutions used) for 8 min. The fibre was then rinsed once again in solution R, loaded with Ca^{2+} (pCa 6.6), placed in solution W and stimulated with $GTP_\gamma S$ and later with caffeine. $GTP_\gamma S$-induced contraction was clearly dependent on the presence of intact terminal cisternae with Ca^{2+} stores. Furthermore Figure 2b also suggests that the pre-treatment of skinned fibres with $GTP_\gamma S$, under conditions which are non-permissive for contraction, somehow desensitized the fibre to a subsequent addition of the guanine nucleotide.

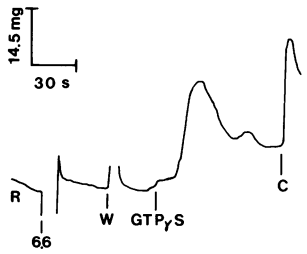

Fig. 3. $GTP_\gamma S$ triggers tension rise in mechanically skinned fibres. The fibre was challenged with $GTP_\gamma S$ (250 μM) under the same experimental conditions already detailed for chemically skinned fibres (see Figure 1). Caffeine was 10 mM.

$GTP_\gamma S$ does not release Ca^{2+} from isolated sarcoplasmic reticulum fractions

The results so far presented do not clarify whether $GTP_\gamma S$ mobilized Ca^{2+} directly from the sarcoplasmic reticulum of skinned fibres or possibly generates a mediator which is the direct agent of Ca^{2+} release. Although it was already known that GTP does not evoke Ca^{2+} efflux from isolated sarcoplasmic reticulum fractions (Meissner, 1984; Morii and Tonomura, 1983), we addressed this issue directly in the experiments summarized in Table I. $GTP_\gamma S$ did not influence Ca^{2+} fluxes either in longitudinal sarcoplasmic reticulum fractions (LSR) or in terminal cisternae, as judged by the lack of effect on Ca^{2+}-dependent ATPase activity, Ca^{2+} loading, Ca^{2+} uptake and Ca^{2+} release. Therefore $GTP_\gamma S$ has a compulsory requirement for the presence of the surface membrane in order to exert its Ca^{2+} releasing action. We suggest that in chemically skinned fibres, which retain a major portion of the plasma membrane (sarcolemma and transverse tubules) and of the associated enzymes (Eastwood *et al.*, 1979), $GTP_\gamma S$ promotes the generation of a mediator which in turn triggers Ca^{2+} release from the terminal cisternae. Pre-incubation with cyclic AMP phosphodiesterase (20 μg/ml) did not prevent the action of $GTP_\gamma S$ (375 μM) (not shown), thus excluding a possible involvement of the adenylate cyclase complex, which is known to be activated by $GTP_\gamma S$ (Ross and Gilman, 1980). Furthermore, it is known that cyclic AMP does not induce Ca^{2+} release from sarcoplasmic reticulum (Moroii and Tonomura, 1983).

$GTP_\gamma S$ elicits tension rise in mechanically skinned fibres

Although the experiments discussed so far suggest that the guanine nucleotide needs the presence of the sarcolemma to exert its Ca^{2+} releasing activity, they do not give us any hint as to which part of the plasma membrane is involved in $GTP_\gamma S$-induced Ca^{2+} release. Mechanical skinning of fibres completely removes

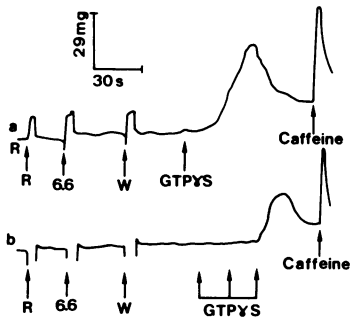

Fig. 4. Pertussis toxin shifts the threshold of GTP$_\gamma$S-induced tension. Trace **a**, control segment. Trace **b**, segment exposed to 10 μg/ml of IAP for 30 min. See Figure 1 and Materials and methods for further details.

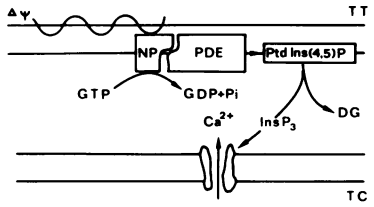

Fig. 5. Tentative description of the events responsible for excitation–contraction coupling at the transverse tubule–terminal cisternae junction. $\Delta\psi$, transverse tubule potential changes; Np, GTP-binding protein; PDE, phosphodiesterase. As soon as transverse tubule membrane depolarizes, Np exchanges bound GDP for GTP. This generates an activated conformation of Np which stimulates PDE. PtdIns(4,5)P_2 is split and InsP_3 diffuses to the terminal cisternae membrane where it triggers Ca^{2+} release.

the sarcolemma but not transverse tubules (Constantin *et al.*, 1965). Figure 3 shows also that in mechanically skinned fibres GTP$_\gamma$S elicits tension development thus indicating that the target of the guanine nucleotide is at the level of the transverse tubules.

Pertussis toxin inhibits GTP$_\gamma$S-induced tension development

One of the main tools for investigating the role of GTP and of GTP-binding proteins (N proteins) in the adenylate cyclase system has been pertussis toxin, also called islet activating protein (IAP). This toxin irreversibly ADP rybosylates and inactivates the inhibitory GTP-binding protein (N_i) of the adenylate cyclase complex (Katada and Ui, 1981; Katada, *et al.*, 1984). The pre-treatment of neutrophils with this toxin prevents secretion, intracellular Ca^{2+} changes and phosphoinositide breakdown induced by the chemotactic peptide fMet-Leu-Phe (Okajima and Ui, 1984; Molski *et al.*, 1984; Volpi *et al.*, 1984). It has therefore been proposed that an N protein, maybe stimulatory, is also involved in phosphoinositide turnover and its inactivation by IAP inhibits the cleavage of plasma membrane inositol lipids. In Figure 4 two segments of the same fibre were incubated for 30 min in solution R, containing also 20 mM dithiothreitol (DTT) and 2.5 mM NAD, pH 8.0, in the presence and absence of IAP. At the end of the incubation the fibres were rinsed in solution R, allowed to accumulate Ca^{2+}, rinsed in solution W and challenged with GTP$_\gamma$S. The pre-treatment with IAP significantly shifted the threshold to higher GTP$_\gamma$S concentrations. In fact the segment which had received IAP contracted only when exposed to 300 μM GTP$_\gamma$S, while the control had a threshold at 100 μM GTP$_\gamma$S. Furthermore the tension, normalized against that of caffeine, was 0.83 with 100 μM GTP$_\gamma$S in the absence of IAP and 0.48 with 300 μM GTP$_\gamma$S in the presence of IAP. If DTT was omitted from the incubation medium, no dose shift was observed, as one would expect if inhibition of force development was due to a specific action of the catalytic unit (Okajima and Ui, 1984). IAP was tested in three skinned fibres, and in all cases the threshold was shifted to higher (3- to 4-fold) GTP$_\gamma$S concentrations as compared with control segments (not treated with IAP). We took care always to use segments of the same fibre to study the effect of IAP because, as already pointed out, the threshold for GTP$_\gamma$S varied in different fibre preparations.

In a previous report (Volpe *et al.*, 1985) we showed that InsP_3 was able to release Ca^{2+} from terminal cisternae and to elicit force development in chemically skinned fibres. Other authors have recently confirmed this observation, also demonstrating the generation of InsP_3 following a 5 s tetanus in frog muscle (Vergara *et al.*, 1985). It seems legitimate to propose a role for InsP_3 in EC coupling. The gap between transverse tubules depolarization and InsP_3 formation is, however, still to be filled. We suggest that, as in non-muscle cells, PtdIns(4,5)P_2 is cleaved in muscle by a GTP-modulated phosphodiesterase. As depicted in Figure 5, transverse tubule depolarization triggers the exchange of bound GDP for GTP at the level of Np. The activated Np is thus allowed to interact with the catalytic unit, the PPI phosphodiesterase, which finally splits PtdIns(4,5)P_2 into InsP_3 and diacylglycerol. The physiological relevance of the model is supported by the observation that GTP$_\gamma$S triggers tension development also in the presence of physiological [Mg^{2+}], and in mechanically as well as in chemically skinned fibres. In fact one of the major criticisms raised against the InsP_3 hypothesis for EC coupling is that exogenous InsP_3 elicits contraction only at unphysiologically low concentrations of Mg^{2+} (Somlyo, 1985). Our findings with GTP$_\gamma$S suggest, however, that if InsP_3 is generated '*in situ*', i.e. at the transverse tubule–terminal cisternae junction, this limitation can be overcome. The experiments with mechanically skinned fibres further support this view. In these fibres the sarcolemma is completely removed, whereas the transverse tubule system remains junctionally associated with the terminal cisternae (Constantin *et al.*, 1965). GTP$_\gamma$S elicited tension rise in mechanically skinned fibres and this indicates a transverse tubule localization of the enzymatic complex putatively activated by GTP$_\gamma$S. This is what one would expect if this mechanism has to generate InsP_3 as close as possible to the terminal cisternae in order to reduce the latency between the transverse tubule depolarization and Ca^{2+} release.

In addition to the classical adenylate cyclase complex, a role for N proteins is being recognized in a variety of events: secretory exocytosis in granulocyte mast cells and platelets, phototransduction in retinal cells, activation of protein kinase and cyclic AMP phosphodiesterase by insulin (Okajima and Ui, 1984; Molski *et al.*, 1984; Volpi *et al.*, 1985; Gomperts, 1983; Haslam and Davidson, 1984; Stryer, 1984; Heyworth *et al.*, 1983a, 1983b). The plausibility of such a device for stimulus–activation coupling is strengthened by the demonstration of the occurrence of receptor- (Vicentini *et al.*, 1985; Di Virgilio *et al.*, 1985) or GTP$_\gamma$S-activated (Cockroft and Gomperts, 1985) PPI splitting at physiological resting cystolic Ca^{2+} concentrations (i.e. 100 nM). This mechanism can, therefore, operate in the absence of previous elevations of cytoplasmic Ca^{2+}.

We are aware of the difficulty in accepting for a fast process such as EC coupling a model which postulates several enzymatic steps and a diffusible chemical mediator. On the other hand, the latency of myoplasmic Ca^{2+} rise after transverse tubule depolarization is calculated to be of ~2.5 ms at 10°C (Vergara *et al.*, 1985), a lag even longer than that occurring between the arrival of the pre-synaptic impulse and the observation of the first post-synaptic electrical changes in neuromuscular junction (Hill,

1982). Yet neuromuscular transmission is generally believed to involve a complex sequence of intracellular biochemical reactions and the release of a chemical mediator (acetylcholine). Therefore we believe that diffusible chemical mediators should be seriously considered in the search for the missing link in excitation–contraction coupling.

Materials and methods

Reagents

Caffeine, antipyrylazo III, disodium phosphocreatine, creatine phosphokinase and ruthenium red were obtained from Sigma, St. Louis, MO. Ionophore A23187 was from Calbiochem-Behring, San Diego, CA. GTP$_\gamma$S, GMP-PNP and GDPβS were purchased from Boehringer Mannheim GmbH, FRG. The GTP analogs were used without further purification, therefore a small contamination by GDP is to be expected, which is, however, ineffective or inhibitory (Cockroft and Gomperts, 1985). IAP was a kind gift of Dr Rino Rappuoli, Sclavo Research Laboratories, Siena, Italy. All other reagents were analytical grade.

Preparation of chemically skinned fibres

Rabbit adductor muscle bundles were chemically skinned (Salviati *et al.*, 1982) and stored at −20°C in a solution containing 50% glycerol, 5 mM K_2-EGTA, 0.17 M K-propionate, pH 7.0, 2.5 mM K_2-Na_2-ATP, 2.5 mM Mg-propionate and 10 mM imidazole-propionate.

Preparation of mechanically skinned fibres

Rabbit extensor digitorum longus was the source of fibres for the mechanical skinning. The muscle was isolated and kept in mammalian Ringer's solution at 0°C for 30 min. From a bundle of fibres transferred to cold paraffin oil, single fibres were isolated from tendon to tendon and cut into segments that were skinned by microdissection just before use (Stephenson, 1981).

Isometric force measurements

In order to measure isometric force development, segments of single fibres were inserted between two clamps, one of them attached to a strain gauge (800 series, Akers Electronics, Horton, Norway), and stretched to 130% of the slack length (Salviati *et al.*, 1982). Fibres were kept for 30–45 s in solution R (5 mM K_2-EGTA, 0.17 M K-propionate, 2.5 mM Mg-propionate, 5 mM K_2-Na_2-ATP and 10 mM imidazole-propionate pH 7.0), exposed for 30 s to a Ca^{2+}-containing solution (pCa 6.6, 0.17 M K-propionate, 2.5 mM Mg-propionate, 5 mM K_2-Na_2-ATP, 1.6 mM $CaCl_2$, 5 mM K_2-EGTA, 10 mM imidazole-propionate, pH 7.0), rinsed for 20–30 s in solution W (solution R without EGTA) and then challenged with the agonists. [Mg^{2+}] (either 0.09 or 2.6 mM) and [Ca^{2+}] (0.25 μM) were determined using association constants given by Orentlicher *et al.* (1977). It is likely that the addition of GTP$_\gamma$S, even at high concentrations, does not affect significantly [Mg^{2+}] since the Kd for this metal is similar to that of ATP. Furthermore, increasing concentrations of ATP, up to 5 mM, did not elicit force development. All experiments were carried out at room temperature and at an ionic strength of 0.2 M.

Isolation of sarcoplasmic reticulum fractions

Sarcoplasmic reticulum fractions were isolated from rabbit fast-twitch skeletal muscle of the hind limbs of New Zealand White male rabbits. Sarcoplasmic reticulum was fractionated by density gradient centrifugation into longitudinal tubules and terminal cisternae, as previously described (Zorzato *et al.*, 1985). After isolation, purified sarcoplasmic reticulum fractions were resuspended in 0.3 M sucrose, 5 mM imidazole, pH 7.4, and stored at −70°C until used.

Ca^{2+} uptake and release measurements

Ca^{2+} accumulation in the absence of precipitating anions is termed Ca^{2+} uptake. Ca^{2+} uptake and Ca^{2+} release were continuously monitored with a Ca^{2+}-sensitive electrode (W.Moller, Glasblaserei, Zurich, Switzerland). The assay was carried out at room temperature in a medium containing 20 mM histidine, pH 7.2, 0.1 M KCl, 5 mM $MgSO_4$, 2 mM ATP, 5 mM disodium phosphocreatine and 80 μg of creatine phosphokinase, final volume 4 ml. The response was calibrated by sequential additions of 10–50 nmol of $CaCl_2$, then, when [Ca^{2+}] was 10 μM, 200–400 μg of sarcoplasmic reticulum protein was added. GTP$_\gamma$S was added before sarcoplasmic reticulum fractions in order to determine its effect on Ca^{2+} uptake, or at peak Ca^{2+} accumulation in order to measure the effect on Ca^{2+} release.

Ca^{2+} loading

Ca^{2+} accumulation in the presence of precipitating anions, i.e. phosphate, is termed Ca^{2+} loading and was continuously monitored by following differential absorbance change of the Ca^{2+} indicator antipyrylazo III (Zorzato *et al.*, 1985) at 710–790 nm in a Perkin Elmer 356 spectrophotometer. The assay was carried out at room temperature (22–24°C) in a medium containing, in a final volume of 1 ml, 92 mM K-phosphate, pH 7.0, 1 mM $MgSO_4$, 1 mM Na_2ATP, 200 μM antipyrylazo III and 50 μM $CaCl_2$. The reaction was started by adding 30 μg of sarcoplasmic reticulum protein in the presence and absence of GTP$_\gamma$S.

Ca^{2+}-dependent ATPase

Ca^{2+}-dependent ATPase activity was measured as phosphate production as described earlier (Mitchell *et al.*, 1983) with slight modifications (Zorzato *et al.*, 1985). Briefly, sarcoplasmic reticulum fractions (10 μg of protein) were incubated in a medium containing, in a final volume of 0.5 ml, 100 mM KCl, 5 mM $MgSO_4$, 0.2 mM Tris-EGTA, 0.2 mM $CaCl_2$, 20 mM histidine, pH 7.2, in the absence and presence of GTP$_\gamma$S. After 5 min of incubation at 25°C, the reaction was started by adding 2 mM Tris-ATP and stopped at 60 s by adding 0.5 ml of 20% trichloroacetic acid, 12% ascorbic acid, 1% ammonium molybdate. Phosphate production was determined using a modification of the method of Ottolenghi (1975). Rates were determined by least squares fit.

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