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Characterization of sarcoplasmic reticulum in skinned muscle cultures

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The plasma membranes of chick or rat skeletal muscles, grown in cell culture, were made permeable with saponin in a solution lacking calcium. The cells were then supplied with a medium resembling the cytosol and the ATP-dependent Ca^{2+} sequestration was performed. Based on the low concentration of free Ca^{2+} in the medium (below 5 μM), the presence of mitochondrial inhibitors and the effect of drugs that interfere with sarcoplasmic reticulum (SR) function, we assume that the measured Ca^{2+} accumulation expresses SR function on the saponin-treated myotubes. The development of the SR in muscle cultures is augmented as myogenesis proceeds and depends on its occurrence. Whereas creatine kinase activity is elevated immediately following cell fusion, there is a delay of at least 1 day between myoblast fusion and the increase in Ca^{2+} accumulation in the SR. Thyroxine or triiodothyronine caused an inhibition of Ca^{2+} accumulation in rat or chick muscle cultures. This inhibition could explain some of the muscle abnormalities caused by excess of thyroid hormones. A comparison was made between a white-type (fast) and heterogeneous muscle, differentiated in cell culture. There was no significant difference in SR function, indicating the important role of innervation in specifying the properties of muscle fiber types.

Introduction

The ATP-dependent uptake of Ca^{2+} by the SR is generally regarded as being the regulator of the intracellular calcium concentration, which in turn controls the contraction/relaxation cycle of the muscle [1,2]. Most data concerning SR function have been obtained from experiments performed on SR vesicles isolated from muscle homogenates. The process of isolation, however, may introduce alteration of membranes and intracellular couplings. Moreover, the SR fraction may be contaminated by membranes from other organelles, such as mitochondria, sarcolemma and T-system, which also contain Ca^{2+}-ATPase activity and therefore cause interference with the specific activity of the SR [3].

Another method proposed for studying the SR function is the 'skinning' of muscle fibers either chemically or mechanically. This method is based on the reduction or elimination of the sarcolemma, thereby allowing the access of soluble constituents to the myofilament space. This skinning procedure enables performance of functional measurements of SR in single fibers under controlled conditions, while keeping the cellular organelles intact [4]. However, the types of experiment that can be performed utilizing this method and the quantitation of these experiments is limited.

In the present study, we introduce a method for chemical skinning of muscle cultures based on the combination of Ca^{2+} elimination and the detergent saponin, which has been previously shown to potentiate the permeability of sarcolemma to large protein molecules while preserving SR function [5,6]. One of the prominent advantages of the implementation of this alternative method on muscle cultures is the ability to quantify Ca^{2+} transport through the SR, precluding the necessity to isolate large amounts of purified vesicles. Using this method, we further characterize SR function in skinned muscle developed in cell culture.

Materials and Methods

Cell cultures

Embryonic rat skeletal muscles were grown in cell culture essentially as in Ref. 7. Chick myogenic cells were

Abbreviations: SR, sarcoplasmic reticulum; T_4, thyroxine; T_3, triiodothyronine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid;

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prepared by mechanical disruption as in Ref. 8. All muscle cultures were grown in 32 mm dishes precoated with gelatin-collagen.

Measurements of Ca^{2+} uptake into SR. The growth medium of muscle cultures was replaced by 1 ml of skinning medium (140 mM KCl/20 mM Hepes/10 mM EGTA/50 μg/ml saponin/5 mM NaN_3/5 mM dipotassium oxalate/1 μM Ruthenium red (pH 7.4)) at room temperature. The cultures were then washed twice with uptake medium (140 mM KCl/20 mM Hepes/0.5 mM EGTA/0.5 mM $CaCl_2$/5 mM NaN_3/5 mM dipotassium oxalate/5 mM $MgCl_2$/1 μM Ruthenium red (pH 7.0). The uptake of Ca^{2+} was initiated by adding, at 37°C, 1 ml of radioactive uptake medium (^{45}Ca 0.2 μCi/ml) in the presence or absence of 5 mM ATP. Following incubation for 4 min, the uptake medium was removed and cultures were rinsed with fresh ice-cold uptake medium (five times). Cells were then lysed by 0.3 ml of 1% Triton X-100 and collected in counting vials containing 3 ml of scintillation fluid (Hydrofluor, National Diagnostics). Radioactivity was determined in a Kontron scintillation counter (about 85% counting efficiency).

Preparation for scanning electron microscopy (SEM). Cells were fixed by 2.5% glutaraldehyde in phosphate buffer (pH 7.2), then washed in the same buffer and post-fixed with 2% osmium tetroxide. The third fixation was by a solution of 2% tannic acid-guanidine hydrochloride. The triple-fixed cells were dehydrated in graded alcohol solutions, thereafter the alcohol was exchanged for freon-112 by graded freon solutions. The cells were air-dried, gold-coated and examined by a JEOL 840 SEM.

Hormone treatment. Thyroxine (T_4) (Sigma, St. Louis, MO, U.S.A.) was dissolved in 0.05 M NaOH and applied to muscle cultures.

The protein content of samples was measured by the procedure of Lowry et al. [36].

Reproducibility of data

Each experiment was carried out on sister cultures in triplicate. Variations were within 10%. In some cases, variations between different batches of cultures were larger, but trends and proportions of various phenomena were maintained.

Results

In order to find the optimal conditions for the measurements of Ca^{2+} accumulation in SR, the effects of several variables were tested.

Duration of skinning procedure

7-day-old chick myotubes were exposed to 1 ml of skinning medium for various periods of time, keeping the time-course of Ca^{2+} uptake constant (4 min). The results shown in Fig. 1 indicate two main points: (1) Ca^{2+} accumulation is an active process, i.e., ATP-dependent. (2) The active Ca^{2+} accumulation is increased significantly in a time-dependent manner, compared to the passive accumulation in the absence of ATP, and it reaches saturation after about 30 min of skinning. We preferred to use 30 min skinning for most of our experiments, since we noticed that following this duration of saponin treatment, the myotubes resumed their ability to contract spontaneously when supplied with normal medium, whereas after 1 h of skinning they had lost it.

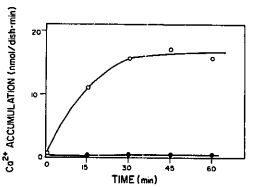

Fig. 1. Duration of skinning procedure. 7-day-old chick muscle cultures were exposed to skinning medium for the indicated time; then the accumulation of Ca^{2+} in 4 min was measured in the presence (○) or absence (●) of ATP. The values are means of triplicate determinations. The average amount of protein in a dish was 1.15 mg.

Time-course of Ca^{2+} accumulation in skinned-muscle cultures

Fig. 2 shows the time-course of Ca^{2+} accumulation at 37°C in 7-day-old chick muscle fibers following 30 min of skinning. While the passive Ca^{2+} accumulation (in the absence of ATP) remains low and constant, ATP-dependent Ca^{2+} loading increases linearly with time for at least 30 min. 4 min exposure to uptake medium is a convenient and sufficient time for the implementation of the experimental procedures. The advantage of measuring Ca^{2+} accumulation over such a

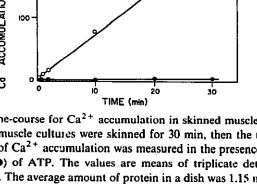

Fig. 2. Time-course for Ca^{2+} accumulation in skinned muscle. 7-day-old chick muscle cultures were skinned for 30 min, then the time-dependence of Ca^{2+} accumulation was measured in the presence (○) or absence (●) of ATP. The values are means of triplicate determinations. The average amount of protein in a dish was 1.15 mg.

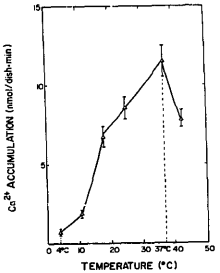

Fig. 3. Temperature-dependent Ca^{2+} accumulation in skinned muscle. 7-day-old chick muscle cultures were skinned for 30 min at room temperature. Then Ca^{2+} accumulation was measured at different temperatures. The values are means ± S.D. of triplicate determinations.

short time is that the addition of a regeneration system for production of ATP is unnecessary.

Effect of temperature on Ca^{2+} accumulation in myotubes

Fig. 3 shows the dependence of Ca^{2+} accumulation in skinned muscle fibers on temperature. At 37 °C, ATP increases Ca^{2+} accumulation in the myotubes about 6-fold in comparison to that at 10 °C. At 4 °C, hardly any accumulation of Ca^{2+} is observed.

Morphology of skinned muscle

Examination of chick myotubes that were exposed to skinning medium and then processed for scanning electron microscopy reveals that although there is an increase in longitudinal folding of the sarcolemma, its structure remains undisrupted. The fibroblasts present in the culture seem to be unaffected (Fig. 4), like those of control myotubes (without skinning).

Specificity of Ca^{2+} accumulation

Two major intracellular ATP-dependent Ca^{2+}-accumulating activities were identifiable within muscle cells. These activities were defined as mitochondrial and nonmitochondrial Ca^{2+} uptake. The nonmitochondrial component was estimated by measuring the residual activity of Ca^{2+} accumulation in the presence of mitochondrial inhibitors. We have used, in our system, a combination of 5 mM sodium azide and 1 μM Ruthenium red [9] in the uptake medium to inhibit Ca^{2+} uptake by the mitochondria. The accumulation of Ca^{2+} in the nonmitochondrial component accounts for about 60–70% of the total Ca^{2+} uptake in 7-day-old chick muscle cultures (Table I). This proportion is dependent on the culture age. As the assay conditions were not optimal for mitochondrial Ca^{2+} accumulation, the contribution of these organelles may be somewhat underestimated.

The nonmitochondrial ATP-dependent accumulation of Ca^{2+} within the myotubes could be reversed by addition of the Ca^{2+} ionophore, A23187, 3 μM of A23187 completely released the Ca^{2+} accumulated by the myotubes (Table II). On the other hand, the Ca^{2+} antagonist, D600 (50 μM), had almost no effect on Ca^{2+} accumulation (Table II).

A significant functional distinction between the sarcolemmal Ca^{2+} pump and the ATP-dependent intracellular Ca^{2+} pump is the divergence in their sensitivity towards th : vanadate anion [10,11]. The sarcolemma is very sensitive to vanadate ($IC_{50} = 0.5$ μM). Therefore, we analyzed our permeabilized myotubes for the nonmitochondrial Ca^{2+} uptake activity in the presence of 2–200 μM Na_3VO_4. It was found that these perforated myotubes accumulated Ca^{2+} at the same rate as the control, in spite of 2 μM vanadate, indicating that ATP-dependent Ca^{2+} accumulation by the sarcolemma or pinocytotic vesicles was negligible (Fig. 5).

To further characterize Ca^{2+} accumulation, we used caffeine, which is known to induce Ca^{2+} release from the SR [12,19]. This agent reduced Ca^{2+} accumulation in the skinned myotubes in a concentration-dependent manner (Fig. 6). At a concentration of 2 mM, Ca^{2+} accumulation was about 50% of control in chick culture, whereas in rat cultures, 10 mM of caffeine reached similar values (Fig. 6). Quercetin, an ATPase inhibitor that inhibits Ca^{2+} uptake by SR [13], also showed a dose-dependent inhibition effect on Ca^{2+} accumulation (Table II).

Since it has been shown that calmodulin plays a functional role in stimulating Ca^{2+} accumulation in isolated SR [14,15], we have measured the influence of anticalmodulin drugs in our system. Fig. 7 shows the effect of trifluoperazine and 48/80 on Ca^{2+} accumulation in saponin-perforated myotubes. 15 and 7 μg/ml of trifluoperazine and 48/80, respectively, inhibited about 50% of Ca^{2+} accumulation.

TABLE I

Effect of antimitochondrial agents on Ca^{2+} accumulation

Ca^{2+} accumulation was determ ed in 7-day-old chick muscle cultures, in the presence or absence (control) of 5 mM NaN_3 and 1 μM Ruthenium red (RR) in the uptake medium. The results are means of triplicate determinations ± S.D. The average amount of protein in a dish was 0.78 mg.

Treatment	Ca^{2+} accumulation (nmol/dish per min)		
	with ATP	without ATP	net Ca^{2+} uptake
Control	11.4 ± 0.35	1.35 ± 0.06	10.05
NaN_3 + RR	8.5 ± 0.03	0.83 ± 0.06	6.67

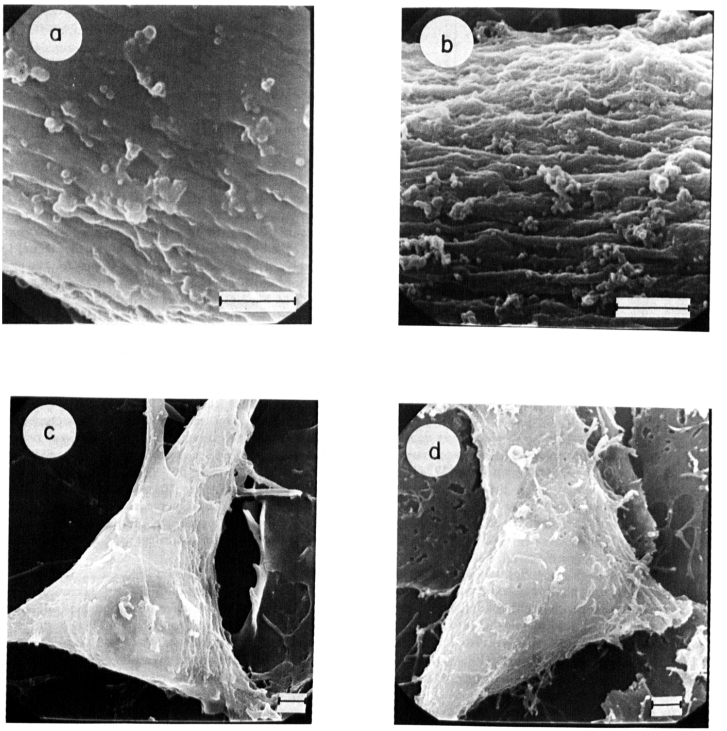

Fig. 4. Scanning electron micrographs of 7-day-old chick muscle cultures (bar = 1 μm in all cases). (a) Control muscle × 18000. (b) Skinned muscle × 18000. (c) Control fibroblast × 6600. (d) Skinned fibroblast × 6600.

Since muscle cultures consist of mixed population of cells (myoblasts, fibroblasts, myotubes), the experiments described above do not define the type of cells in which Ca^{2+} accumulation took place. To determine which types of cell were affected, fibroblast-like cells derived from several subcultivations of primary muscle cultures were treated with saponin and analyzed for Ca^{2+} accumulation. These fibroblasts exhibited less than 10% of the total Ca^{2+} uptake in control 7-day-old muscle cultures (Table II). These results suggest that Ca^{2+} accumulation occurs predominantly in the developed myotubes in cultures.

Because of the analogy between nonmitochondrial ATP-dependent Ca^{2+} accumulation into saponin-treated myotubes and the SR function with regard to the influences of various agents, we assume that we are actu-

TABLE II

Effect of various agents on Ca^{2+} accumulation

Ca^{2+} accumulation was determined in 5-day-old chick muscle cultures in the presence of the above agents. These agents were applied during the skinning and uptake procedures. The results are means of triplicate determinations ± S.D. The amounts of total cell proteins in the control and fibroblast were 0.83 and 0.52 mg/dish, respectively.

Treatment		Ca^{2+} accumulation (nmol/dish per min)		
		with ATP (nmol/dish per min)	without ATP (nmol/dish per min)	net uptake (nmol/dish per min)
Control		8.815 ± 1.36	0.44 ± 0.02	8.375
A23187	(1 μM)	0.77 ± 0.004	0.16 ± 0.016	0.61
	(3 μM)	0.20	0.14 ± 0.016	0.06
D600	(50 μM)	7.96 ± 0.68	0.19 ± 0.02	7.77
	(120 μM)	5.15 ± 0.42	0.19 ± 0.03	4.96
Quercetin	(100 μM)	1.77 ± 0.14	0.18 ± 0.02	1.59
	(250 μM)	1.35 ± 0.13	0.18 ± 0.02	1.17
Fibroblasts		0.26 ± 0.01	0.01	0.25

ally measuring Ca^{2+} accumulation in the SR. Therefore, we may refer to Ca^{2+} accumulations as a Ca^{2+} uptake by SR in the muscle cultures.

It was shown that chronic administration of excess of T_4 or T_3 to animals gave rise to muscle abnormalities.

TABLE III

Ca^{2+} accumulation in different types of rudiment muscle developed in culture

Ca^{2+} accumulation of skinned muscle fibers differentiated in vitro from myogenic cells of pectoralis or thigh muscles. Results are means of triplicate determinations ± S.D.

Muscle type	Ca^{2+} accumulation (nmol/mg per dish)	
Culture age (days):	3	4
Pectoralis cultures	3.5 ± 0.28	6.63 ± 0.54
Thigh cultures	3.73 ± 0.30	6.35 ± 0.48

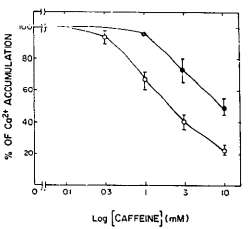

Fig. 6. Effect of caffeine on Ca^{2+} accumulation. 5-day-old chick (○) and 7-day-old rat (●) muscle cultures were exposed to different concentrations of caffeine in the skinning and the uptake medium. The results of net Ca^{2+} accumulation are means of triplicate determinations expressed as percentages of control (without caffeine).

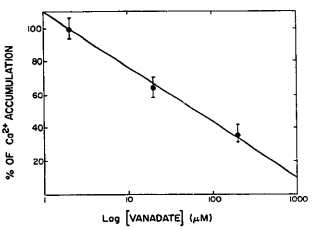

Fig. 5. Effect of vanadate on Ca^{2+} accumulation. 5-day-old chick muscle cultures were exposed to different concentrations of Na_3VO_4, in the uptake medium. The results of net Ca^{2+} accumulation are means of triplicate determinations expressed as percentages of control (without vanadate).

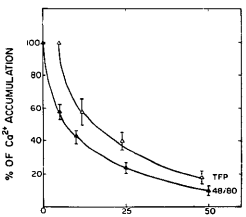

Fig. 7. Effect of anticalmodulin drugs on Ca^{2+} accumulation. 5-day-old chick muscle cultures were exposed to TFP (△) or 48/80 (▲) at different concentrations in the skinning and the uptake medium. The results of net Ca^{2+} accumulation are means of triplicate determinations expressed as percentages of control (without any of the drugs).

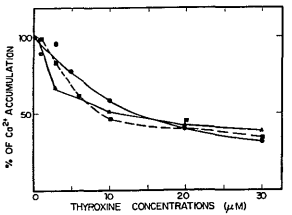

Fig. 8. Effect of T_4 on Ca^{2+} accumulation. 9-day-old chick (●) 16-day-old chick (■) or 9-day-old rat (▲) muscle cultures were exposed to T_4 at different concentrations in the uptake medium. The results of net Ca^{2+} accumulation are means of triplicate determinations expressed as percentages of control (without T_4).

which were expressed in reduced Ca^{2+} uptake by isolated SR [16]. In contrast, Kim et al. [17] and Simonides and Van Hardveld [18] have reported that, in thyrotoxic animals, Ca^{2+} uptake by the SR of slow muscle is increased, while the fast muscle is not altered. Therefore, the effect of T_4 on the SR function in skinned muscle cultures was analyzed. Such treatment caused an inhibition of Ca^{2+} uptake (Fig. 8), confirming our studies on isolated SR (Shainberg, A. and Shoshan-Barmatz, V., unpublished data).

There is a debate as to whether the fusion of myoblasts and the appearance of characteristic muscle proteins are causally linked [8,20,21]. To elucidate this, we have studied the development of SR function in myotubes grown in cell cultures. We compared Ca^{2+} uptake by SR in myoblasts grown in normal medium with myoblasts grown in Ca^{2+}-deficient medium, which we have previously shown to inhibit myogenesis [7,8]. We found that inhibition of cell fusion in rat muscle cultures depressed the development of SR function in a reversible manner. Upon addition of Ca^{2+} (1.8 mM) to fusion-arrested cells, fusion immediately proceeded, accompanied by increased Ca^{2+} accumulation (Fig. 9). Similar results were also seen when the activity of creatine kinase was measured. Thus, it seems that there is a linear correlation between rat myoblasts fusion and the development of SR function, confirming previous studies in chick muscle cultures [22,23]. Furthermore, a rise in the activity of creatine kinase precedes the increased activity of SR in normal medium, indicating that fusion is a prerequisite but is not sufficient for the expression of SR function (Fig. 9). In contrast, others have found that fusion of myoblasts is not essential for this phenomenon, although it is normally concomitant [23].

Two main avian muscle fiber types, 'slow' and 'fast,' can be distinguished by SR function. Fast muscle is much richer in SR. We compared the development of

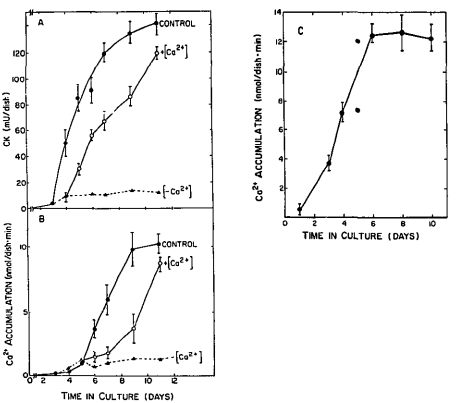

Fig. 9. Time-course of appearance of creatine kinase activity and changes in Ca^{2+} accumulation in rat muscle cultures. Each point is an average of triplicate determinations ± S.D. ●, control cultures (normal Ca); ▲, Ca^{2+}-deficient medium and ○, cells grown in Ca^{2+}-deficient medium until the 3rd day when (1.8 mM) Ca^{2+} was added. (A) Development of creatine kinase in rat muscle cultures. (B) Ca^{2+} accumulation in rat muscle cultures. (C) Development of Ca^{2+} accumulation in chick muscle cultures.

SR function in myotubes grown in cultures between those originating either from breast (fast) or thigh (mixed) muscle cultures of chick embryos. The results of these experiments can indicate whether the difference between fast and slow muscle, in SR content, is a result of innervation, or if they differ in gene expression. Our results show that there is no significant difference in Ca^{2+} accumulation between these two types of muscle, indicating that innervation exerts an important influence upon muscle-fiber differentiation, particularly with regard to characteristic properties of the fiber type.

Discussion

The results described above have identified and characterized an intracellular Ca^{2+} pumping mechanism that functions within the myotubes. This characterization of the high-affinity ATP-dependent Ca^{2+} pump is based on skinning of the muscle grown in vitro with plant glycoside, saponin, in a solution lacking Ca^{2+}. Although the exact mechanism of saponin action is unclear, it is believed to permeabilize the plasma membrane by complexing with cholesterol to form pores [24]. Thus, SR and mitochondria, which contain very little cholesterol compared to the plasma membrane, are probably not damaged by saponin [6]. Morphological and biochemical evidence in muscle and other tissues, support the assumptions that the intracellular organelles of saponin-treated cells are present and intact. Furthermore, the technique of differential saponin permeabilization has been extensively applied to study some properties of SR within muscle tissue [12], and to identify nonmitochondrial Ca^{2+} sequestering organelles within other cells: macrophages [27], pancreatic acini [25], neuronal cell lines [11] and smooth-muscle cell lines [29]. It is probable that the identity of Ca^{2+}-accumulating organelles within these cell types is the smooth endoplasmic reticulum, which may have a functional analogy to the SR in the muscle cells.

Some of the advantages of the saponin-treated cell preparation are the following. (1) The potential artifacts of contaminations from other organelles are minimal. (2) The process is reversible; upon replacing the saponin with normal medium, the myotubes resume their ability to contract spontaneously within 15 min. (3) It is possible to introduce into the myotubes large molecules like proteins and even viruses, to which the myotubes are generally impermeable, and then close the pores by growing the cells in normal medium. (4) The internal function of the myotubes is apparently intact, yet the environment surrounding the organelles can be precisely controlled. In this way, the addition of a metabolic inhibitor such as sodium azide and Ruthenium red, which are known to inhibit mitochondrial Ca^{2+} accumulation [9,27], does not compromise Ca^{2+} retention by SR o plasma membrane. The mitochondria are able to sequester Ca^{2+} only when the free Ca^{2+} concentration in the medium is greater than 1 μM [25,31]. According to Fabiato and Fabiato [31], our calculated free Ca^{2+} is expected to be almost 5 μM. Therefore, we had to add mitochondrial inhibitors to our uptake medium.

The nonmitochondrial ATP-dependent Ca^{2+} accumulation within the myotube provides an analogy with the Ca^{2+} pumping activity in the SR, and yet is distinct from the plasma membrane Ca^{2+} pumping activity. They can be distinguished by a number of criteria. First, caffeine, which causes a release of sequestered intracellular Ca^{2+}, also reduced the accumulation of Ca^{2+} in the permeabilized myotubes. Second, there is the similar sensitivity of both systems to anticalmodulin drugs. Finally, SR is relatively insensitive to vanadate anions, whereas the sarcolemma was shown to be very sensitive to them [10,11]. On the basis of these parameters, we classified our nonmitochondrial ATP-dependent Ca^{2+} accumulation as SR pumping system, especially since Ca^{2+} blockers produced little inhibition of Ca^{2+} uptake on the one hand and Ca^{2+} ionophore caused a release of the sequestered Ca^{2+} on the other. Nevertheless, this result does not rule out the possibility that a small fraction of the Ca^{2+} may be taken up into organelles other than SR.

The expression of SR Ca^{2+} pumping activity, like that of a number of other muscle-specific proteins, is greatly accelerated as myoblasts fuse and differentiate into myotubes. However, whereas creatine kinase activity is promptly increased upon cell fusion, there is a lag of at least 1 day until Ca^{2+} accumulation in the SR is accelerated in rat muscle cultures. The lag in expression of Ca^{2+} uptake by SR following cell fusion could be the reason for the lack of a significant difference between pectoralis and thigh chick muscle cultures. It is possible that the accumulation of Ca^{2+} in these two types of muscle required a longer duration of cultivation until a difference would be detected in aneural cultures. Thus, the suggestion we made with regard to the role of innervation determination of muscle type is not conclusive. This finding could explain the results obtained from quail embryos, showing that myoblasts from slow- and fast-muscle rudiment can express in vitro some of the characteristic features of slow and fast muscle fibers, independently of motor innervation [33].

The observation that T_4 (or T_3) decreased Ca^{2+} accumulation by the SR suggests that the hormones might increase the myoplasmic free Ca^{2+} concentration. Indeed, it has been shown that there is an increased intracellular Ca^{2+} level in skeletal muscle treated with T_4 [33,34]. This might partially explain the alteration of muscle contractions observed in thyrotoxicosis. Furthermore, it was shown that increased intracellular Ca^{2+} levels appear to favor muscle catabolism [35]. It is tempting to speculate that the increased rate of protein

degradation observed in excess of thyroid hormone is a result of the elevated intracellular Ca^{2+} concentration.

We have tried to elucidate the mechanism by which thyroid hormones bring about the inhibition of Ca^{2+} accumulation. However, this has been done on isolated SR vesicles and it justifies a separate communication (unpublished data).

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