Calcium Transport by Skeletal Muscle Sarcoplasmic Reticulum in the Hypothyroid Rat

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ABSTRACT The rate of calcium transport by isolated sarcoplasmic reticulum from rat skeletal muscle increases markedly during the first 4 wk of life and thereafter remains relatively constant. When animals are made hypothyroid during the first 3 wk of life, there is a marked inhibition of the increase in calcium transport by the sarcoplasmic reticulum. Production of hypothyroidism after 4 wk of age, at which time the calcium transport by sarcoplasmic reticulum has reached maximum levels, results in a depression in the rate of calcium transport. There is no clear alteration in ATPase activity of the sarcoplasmic reticulum to account for the low calcium transport in hypothyroidism. It is proposed that the decrease in calcium transport by sarcoplasmic reticulum may account for observed alterations in the intrinsic contractile properties of muscle in the hypothyroid animal.

INTRODUCTION

That there is a prolongation of contraction and relaxation of skeletal muscle in hypothyroidism has been a well known clinical finding for many years. Studies of neural conduction time of the deep tendon reflex in man support the concept that this prolongation is due to factors intrinsic to the muscle rather than those neural in origin (1). More recent studies in the cat papillary muscle (2) and in the rat skeletal muscle (3) have shown that the state of thyroid function influences intrinsic contractile properties of muscle.

The sarcoplasmic reticulum appears to play an important role in excitation-contraction coupling and may regulate both speeds of contraction and relaxation of muscle and the intensity of the active state by its ability to transport calcium in the vicinity of the myofibrillar proteins (4). Fragments of sarcoplasmic reticulum have been utilized to study calcium transport in vitro (5–7) and are thought to reflect properties of the sarcoplasmic reticulum in vivo.

Since an alteration in the activity of sarcoplasmic reticulum in transporting calcium would be at least one possible explanation for an alteration in the intrinsic contractile properties of muscle in the hypothyroid state, the present studies were undertaken to determine the influence of thyroid deficiency on calcium accumulation by isolated sarcoplasmic reticulum. These studies demonstrate a depression in calcium transport by muscle sarcoplasmic reticulum in the hypothyroid animal.

METHODS

Animals. Animals were obtained from the Charles River Laboratories at two different stages of development. For studies on newborn animals, 1 wk pregnant mothers were maintained on either Purina chow (controls) or ground Purina chow containing 0.03 g/100 mg of propylthiouracil (PTU) fed ad lib. through pregnancy and the nursing period. The only PTU that the offspring obtained postpartum was through the mother's milk. The offspring were sacrificed at 21 days of age. For studies at a later period of development, animals were obtained at a body weight of approximately 80–90 g (5½ wk of age) and were maintained on Purina chow ad lib. Some of the animals were maintained on the chow (controls), and the rest were started on 0.03 g/100 mg of PTU in ground chow when they reached a body weight of approximately 100 g (6 wk of age) and were maintained on this diet until the time of sacrifice (after 4 or 6 wk of treatment with PTU). Normal animals

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used for studies of the sarcoplasmic reticulum during the first 4 wk of life (Figs. 1 and 5) were all obtained on the same day at various ages from the Charles River Laboratories. All animals used were males, except for those studied on the day of birth when no attempt was made to separate sexes.

Materials. 6-propyl-2-thiouracil (PTU) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio. Sodium triiodo-L-thyronine was from Glaxo Laboratories, Ltd., Greenford, England. Adenosine-5′-triphosphate (ATP), L-α-lecithin (soy bean), DL-α-lecithin, and lysolecithin were purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation of fragmented sarcoplasmic reticulum. The total muscle from both hind limbs was used from animals up to 4 wk of age; for the older ages, the total muscle from a single hind limb was used. In a few experiments, individual muscles were used. After decapitation of the animals, muscles were collected on ice. All subsequent procedures were carried out at 0–4°C. The muscles were homogenized in 5 volumes of 0.1 M KCl and 0.01 M Tris at pH 7.4 in a Virtis "45" homogenizer at half maximal speed for 30 sec for animals of ages up to 4 wk. For older animals, a 2 min homogenization period was used to obtain a well-dispersed homogenate. The homogenate was centrifuged at 800 *g* for 15 min. The supernatant was removed and centrifuged at 8000 *g* for 30 min. The supernatant from the second centrifugation was then passed through four layers of cheesecloth and centrifuged at 25,000 *g* for 1 hr in a Spinco Model L Ultracentrifuge. The material obtained in the 25,000 *g* pellet, which will be referred to as fragmented sarcoplasmic reticulum (FSR), was resuspended in 2½ volumes of homogenizing medium and used on the same day for measurement of calcium uptake and ATPase activity. Protein concentration was measured by the Folin method (8).

Measurement of calcium uptake. Calcium uptake by FSR was measured at 23–24°C in a medium containing 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM ATP, and 0.1 mM $^{45}CaCl_2$. There was virtually no calcium accumulation in the absence of ATP and $MgCl_2$, and uptake was depressed when oxalate was omitted. The usual volume of the medium was 2.2 ml and contained 0.05–0.1 mg of FSR protein. All reactions were started with ATP and were terminated by passing the total incubation medium through a Millipore filter (type HA, with 0.45 μ average pore diameter) (7). The remaining radioactivity in the particle-free filtrate was counted in 5% naphthalene, 0.7% 2,5-diphenyloxazole (PPO), and 0.005% 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in dioxane in a Packard Tri-Carb liquid scintillation counter. The calcium accumulated by the FSR was calculated from the difference between initial and final counts in the medium. Usually a few incubation periods were obtained for each preparation, and the rate of uptake was determined as the average uptake per minute during the first 3 min (a relatively linear part of the uptake activity).

ATPase measurements. ATPase activity was measured under the same conditions and in medium of the same composition as that used for calcium uptake. ATPase activity was also measured in a medium containing all the above ingredients except that $CaCl_2$ was omitted. In this case, 0.1 mM EGTA was added to chelate any trace amounts of calcium present. The difference in ATPase activity with and without calcium in the medium will be at times referred to as "calcium-stimulated" ATPase. Inorganic phosphorus (P_i) released during hydrolysis of ATP was determined by the Fiske-Subba-Row method (9). Values recorded in the text were obtained during an initial linear part of the ATPase activity.

RESULTS

Changes in calcium uptake by FSR during muscle development

Fig. 1 demonstrates that the rate of calcium accumulation by FSR was very low at birth (0.005 μmoles of Ca per mg of protein per min) and increased markedly during the first 4 wk of life to 0.6 μmoles of Ca per mg of protein per min. After this time, the rate of calcium uptake showed little change during at least the first 12 wk of life. The anterior tibial and extensor digitorum longus muscles showed essentially the same general pattern of increase in calcium uptake as that for whole leg muscle during the first 4 wk of life; and although calcium uptake for FSR from the isolated muscles tended to be a little higher than for FSR from total leg muscle, the difference in calcium uptake between the isolated muscle and total leg muscle was not great. This finding probably reflects the predominance of "white" muscle in the lower extremity.

Effect of PTU on animal body weight and serum protein-bound iodine (PBI)

As seen in Fig. 2, animals started on PTU during fetal life grew more slowly than controls. When animals were started on PTU at 6 wk of age (around 100 g of body weight), their growth rate began to fall off after a few days of PTU and was considerably lower than controls after 2 wk of treatment. The clear development of hypothyroidism after 4 wk of treatment was attested to by impairment in growth, a mean PBI of 1.1 ± 0.01 μg/100 ml for PTU-treated animals (six animals) as compared to 3.3 ± 0.08 μg/100 ml for controls (six animals), and by the development of reddened, enlarged thyroids.

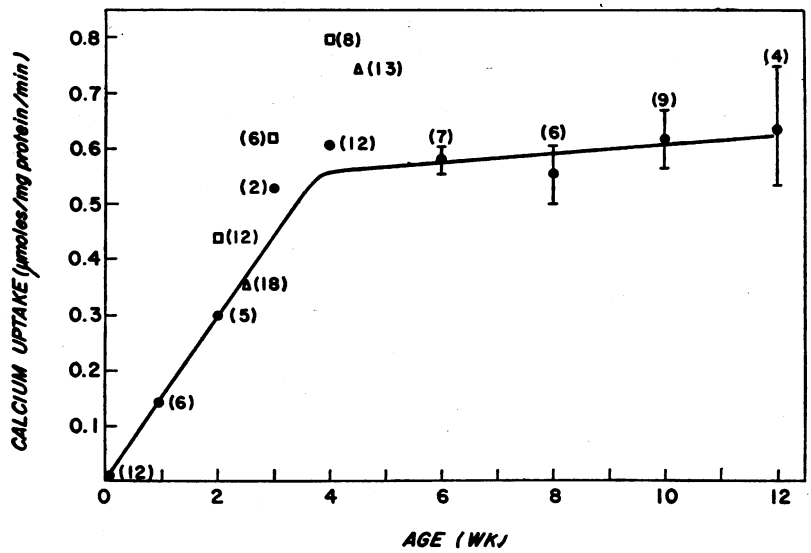

FIGURE 1 Calcium uptake by fragmented sarcoplasmic reticulum (FSR) from muscle at various animal ages. Calcium uptake was determined under the conditions noted in Methods. At times up to and including 4 wk of age, single FSR preparations from the number of animals noted in parenthesis were used. After 4 wk of age, the numbers indicated in parenthesis represent the number of preparations made, using muscle from one animal for each preparation. Symbols: ●——●, whole hind leg muscle; □, anterior tibial muscle; △, extensor digitorum longus muscle.

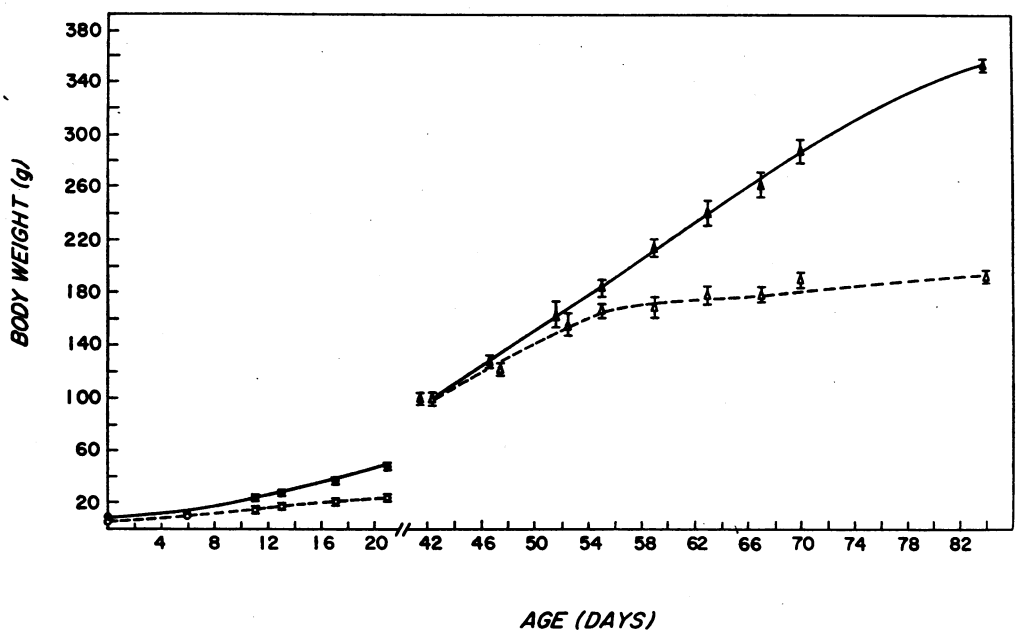

FIGURE 2 Body weights of animals when propylthiouracil (PTU) treatment was begun either (*a*) after 1 wk of fetal life or (*b*) at 6 wk of age. 10–40 animals were used for each time. Symbols: (*a*) ●——●, control animals; ○----○, treated with PTU; (*b*) ▲——▲, control animals; △----△, treated with PTU. The range for 1 SE is indicated by brackets.

Ca uptake by FSR from hypothyroid animals

Because of changes in calcium uptake by FSR during the first 4 wk of life, the effect of hypothyroidism on calcium transport was studied at two different stages of development. In the first case, animals were made hypothyroid during the first 3 wk of life, a period during which there was a rapid increase in the ability of normal FSR to accumulate calcium (Fig. 1). In the second case, animals were not started on PTU until the 6th wk of life at which time the rate of calcium accumulation by the FSR had reached a maximum or near maximum level.

Ca accumulation by FSR of animals treated with PTU started during gestation

When animals were treated with PTU from the 1st wk of fetal life and were sacrificed at 3 wk of age, there was a marked reduction in the rate of calcium accumulation by the FSR. Fig. 3 *a* shows a typical kinetic pattern of calcium accumulation by the FSR from this group of animals at 3 wk of age. The initial rate of calcium accumulation for FSR from hypothyroid animals was 0.2 μmoles of Ca per mg of protein per min as compared to 0.62 μmoles of Ca per mg of protein per min for controls. Treatment with triiodothyronine (T3) (1 μg/animal per day) for 5 days before sacrifice allowed calcium uptake to reach a rate about halfway between that of the PTU-treated animals and controls. Treatment with the same amount of T3 for 10 days before sacrifice, however, allowed uptake to reach control levels. The reversibility by T3 of the low calcium uptake of the PTU-treated animals at 3 wk of age is demonstrated in Fig. 3 *b*. While a single injection of 50 μg/animal of T3 16 hr before sacrifice had no effect on the low calcium uptake, treatment of the hypothyroid animals for 2 wk with T3 (1 μg/animal per day) completely restored the ability of the FSR to accumulate calcium to normal levels.

Ca accumulation by FSR of animals treated with PTU started at 6 wk of age

The mean calcium accumulation by FSR of animals treated with PTU from 6 wk of age and

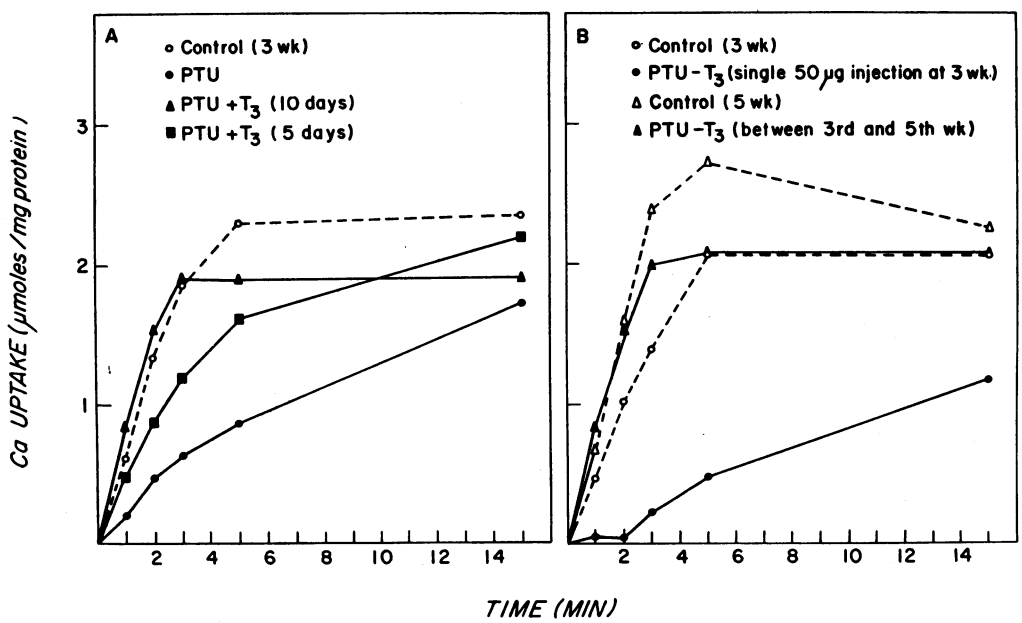

Figure 3 *a* Calcium uptake by FSR from 3-wk old animals treated with PTU from the 1st wk of fetal life. Calcium uptake was determined as noted in Methods. Triiodothyronine (T3) (1 μg/animal per day) was given for either 5 or 10 days before sacrifice at 3 wk of age. *b*) Reversibility with T3 of depression of calcium uptake in the hypothyroid animal. Calcium uptake was determined as noted in Methods. The single injection with T3 was given 16 hr before sacrifice at 3 wk of age. The T3 given between the 3rd and 5th wk of life was in doses of 2 μg/animal per day, and animals were sacrificed at 5 wk of age.

sacrificed at 10 wk of age was 0.42 ± 0.03 μmoles/mg of protein per min as compared to 0.62 ± 0.05 μmoles/mg of protein per min for controls (Fig. 4). A few preparations were made to determine if calcium uptake became any lower after a longer duration of treatment. After 6 wk of PTU, the calcium uptake by FSR was no lower than at 4 wk (0.45 ± 0.04 μmoles of Ca per mg of protein per min as compared to controls of 0.64 ± 0.13 μmoles of Ca per mg of protein per min for four preparations each). These changes are less marked than those obtained when animals were treated during the earlier period of development.

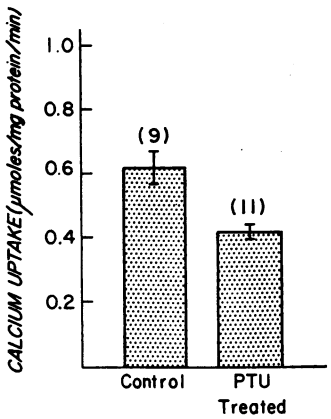

FIGURE 4 Calcium uptake by FSR from animals begun on PTU at 6 wk of age and sacrificed at 10 wk of age. Animals were treated with PTU, and calcium uptake was determined as noted in Methods. Values represent mean calcium uptake ± 1 SE of the mean with the number of preparations indicated in parenthesis. $P = 0.002$ for significance of the difference of the means.

ATPase activity of FSR

Since calcium accumulation by FSR is an energy-dependent process and appears to be linked to ATPase activity of the FSR (6), it seemed profitable to study the ATPase activity, as well as calcium uptake, of FSR from developing and hypothyroid muscle. These studies were carried out concomitantly with and on the same preparations as the calcium uptake studies.

DURING MUSCLE DEVELOPMENT

ATPase activity of the FSR in the presence of calcium was low at the time of birth (0.45 μmoles of P_i per mg of protein per min) and reached a peak value of 1.7 μmoles of P_i per mg of protein per min at 2 wk of age (Fig. 5). Thereafter, there was a decrease in activity until it reached a stable level of 1.1 to 1.2 μmoles of P_i per mg of protein per min by 6 wk of age. The ATPase activity at birth and at 1 wk of age was slightly stimulated by the removal of calcium from the medium. At 2 wk of age, the activity was slightly inhibited by the removal of calcium; and by 3 wk of age, and thereafter, there was a 25–30% inhibition in ATPase activity by the removal of calcium.

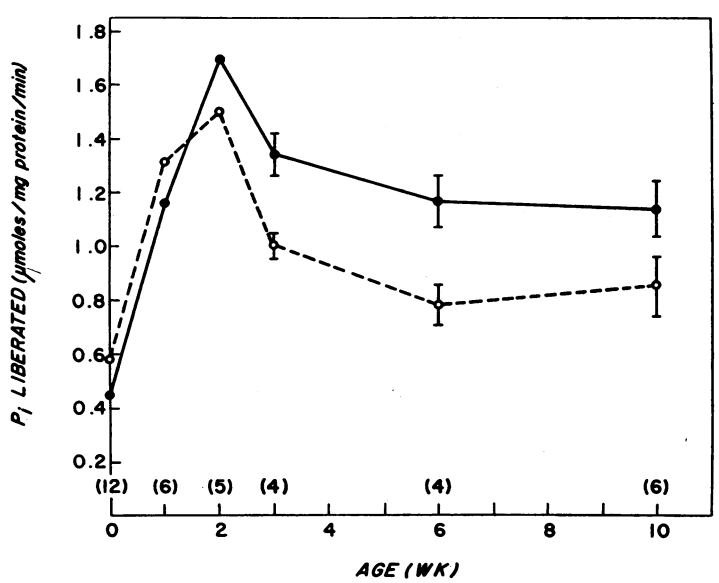

FIGURE 5 ATPase activity of FSR at various animal ages. For animals up to and including 2 wk of age, the values represent the results of a single determination on the number of animals indicated in parenthesis. After 2 wk of age, the values represent mean ± 1 SE for the number of preparations noted in parenthesis. Symbols: ●——●, ATPase activity in the presence of 0.1 mM $CaCl_2$; ○----○, ATPase activity in the absence of Ca. P_i, inorganic phosphorus.

TABLE I

Effect of PTU on ATPase Activity of FSR

Treatment started	Ca uptake	ATPase (1) 0.1 mM Ca^{++}	ATPase (2) 0 Ca^{++}	ATPase "Ca-stimulated" [(1)−(2)]	Percent of inhibition with removal of Ca^{++}	Ratio of Ca uptake: "Ca-stimulated" ATPase
	μmoles/mg per min			*μmoles/mg per min*		
Group 1: prenatal						
Control	0.67	1.1	0.7	0.4	36	1.68
PTU	0.23	1.43	1.2	0.23	16	1.0
Group 2: age 6 wk						
Control	0.62 ± 0.05*	1.14 ± 0.1	0.87 ± 0.12	0.19 ± 0.03	19 ± 2.8	2.96 ± 0.2
PTU	0.42 ± 0.03*	1.07 ± 0.1	0.80 ± 0.07	0.27 ± 0.045	25 ± 2.5	1.59 ± 0.2

PTU, propylthiouracil; ATPase, adenosine triphosphatase; FSR, fragmented sarcoplasmic reticulum.
Calcium uptake and ATPase activities were measured as noted in Methods. Animals in group 1 were sacrificed at 3 wk of age. Animals in group 2 were sacrificed at 10 wk of age. The results in group 1 were obtained from preparations in which three control and six PTU-treated animals were used. For group 2, means were determined from six control and eight PTU-treated preparations. ± 1 SE of the mean is recorded.
* P value for significance of difference of means = 0.002.

IN HYPOTHYROID ANIMALS

ATPase activity of FSR from animals treated during the newborn period and sacrificed at 21 days of age was higher both in the presence and absence of calcium (Table I) than in controls. The per cent of inhibition of ATPase activity by removal of calcium from the medium was lower for treated animals than for controls. Hence, the pattern of ATPase activity of the FSR from animals treated with PTU was similar to that of FSR from animals about 1 wk younger in development (Fig. 5).

ATPase activity of FSR from animals started on PTU treatment at the older age (6 wk), on the other hand, was similar to that of control animals both in the presence and absence of calcium. Treatment of both the newborn and older animals resulted in lowering of the calcium uptake: "calcium-stiumulated" ATPase ratio (Table I).

DISCUSSION

Physiologic studies of both skeletal and cardiac muscle have demonstrated that alterations in the intrinsic contractile properties of muscle are associated with changes in the state of thyroid activity (2, 3). In the case of skeletal muscle, it has been demonstrated that depression of thyroid function results in decreased maximum tension, decreased rate of tension development, prolongation of the active state, and delayed relaxation in the isometrically contracting muscle (3). Similar findings without data on rates of relaxation were reported for cardiac muscle (2). Studies for specific factors to account for the physiologic alterations included measurements of norepinephrine and nucleotide levels in heart muscle; there was no relationship between these and the alterations in contractile properties.

The present studies demonstrate lower calcium-transporting activity of sarcoplasmic reticulum from skeletal muscle of hypothyroid animals and suggest this as a possible mechanism for the observed alterations in contraction. The low calcium transport by FSR in the PTU-treated newborn animals may be the result of a block in maturation of the FSR, since calcium transport increases markedly in normal animals during the first 4 wk of life. However, by 4 wk of age, the rate of calcium uptake by FSR has reached a maximum or near maximum level, and depression of calcium uptake by the FSR in animals treated from the 6th wk of life shows that hypothyroidism does not affect only the maturation process.

Since calcium uptake appears to be linked to ATPase activity (6), it may be expected that the low calcium uptake by FSR in hypothyroid animals would be accompanied by depressed ATPase activity. However, ATPase activity of the PTU-

treated newborn animals, sacrificed at 3 wk of age, actually increased above controls both in the presence and absence of calcium. Also, "calcium-stimulated" ATPase activity in the PTU-treated animals was decreased to a lesser extent than the calcium uptake. In the case of the animals started on treatment at 6 and sacrificed at 10 wk of age, there was no significant difference from controls in ATPase activity either in the presence or absence of calcium. "Calcium-activated" ATPase, in this group as opposed to the newborn animals, was slightly stimulated in the hypothyroid animals. Therefore, there is no obvious relationship between alterations in calcium transport and ATPase activity of FSR in hypothyroid animals. Similarly, there is no clear correlation of these activities in normal FSR during the first 4 wk of development (Figs. 1 and 5). The difficulty in making this correlation could result from a heterogeneity in ATPase activity of the subcellular fraction studied. Separation of the soluble enzyme by methods such as those used by Martonosi, Donley, and Halpin (10, 11) may be helpful in this clarification. It is of interest that there is a consistent depression in the calcium uptake: "calcium-stimulated" ATPase ratio in the case of FSR from animals treated with PTU, and this finding may suggest a decrease in the "efficiency" of the sarcoplasmic reticulum of hypothyroid animals to transport calcium.

Thyroid hormone has been shown to play an important role in both protein and phospholipid synthesis (12–15). It is possible, therefore, that thyroid affects calcium transport of FSR by altering the structure of the sarcoplasmic reticulum. The necessity to administer T3 for several days to prevent or reverse the calcium-transporting defect in hypothyroid animals would be consistent with such an alteration. Martonosi has shown that phospholipids are critical to calcium transport by FSR and has been able to restore calcium transport in phospholipase-treated FSR by the addition of phospholipids in vitro (10, 16). In the present study, calcium transport was not restored to control levels by the addition of lecithin or lysolecithin to FSR of hypothyroid animals.

The finding of decreased calcium uptake by FSR in the hypothyroid animal provides another case in which the rate of calcium transport by the isolated SR may be associated with speeds of contraction and relaxation of intact muscle. There is a slower rate of calcium transport by isolated SR from "red" muscle than by SR from "white" muscle which has faster speeds of contraction and relaxation (17). Similarly, there is an increase in rate of calcium transport during muscle maturation (18), and a close temporal correlation between the increase in rate of calcium transport by the isolated SR in this study and the increase in speeds of contraction and relaxation of rat skeletal muscle during the first 3–4 wk of development as reported by Close (19). The specific alteration(s) in the sarcoplasmic reticulum for all of these situations requires further evaluation.

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