BIOCHEMICAL AND HISTOCHEMICAL EVIDENCE FOR STIMULATION OF MYOSIN ATPase ACTIVITY IN THYROTOXIC RABBIT HEART

Eugene MORKIN, Surath K. BANERJEE and Lawrence Z. STERN
Department of Internal Medicine, Department of Pharmacology and Department of Neurology, University of Arizona Health Sciences Center, Tucson, Arizona 85724, USA

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1. Introduction

Thyroid hormone may regulate the contractile behavior of the heart by influencing the ATPase activity of myosin. Beginning with Barany [1], it has been appreciated that myosin ATPase activity is directly related to the intrinsic speed of muscle contraction. Thyrotoxicosis induced by thyroid hormone administration has been found to increase the speed of contraction of heart muscle [2–4] and to elevate the Ca^{2+}–ATPase activity of cardiac myosin [5–8]. However, it is uncertain whether this increase in enzymatic activity accounts for the increased speed of contraction, since in living muscle Mg–ATP is hydrolyzed by myosin under the stimulating influence of actin. Attempts to measure the Mg–ATP activity of myofibrils or actomyosin from thyrotoxic animals have yielded conflicting results [3,6], possibly because of the variability in purity and activity encountered when these materials are prepared from heart muscle.

In the present study, we have compared the Mg^{2+}–ATPase activity of purified myosin from normal and thyrotoxic hearts in the presence and absence of actin. To determine whether these measurements reflect the activity of the enzyme in situ, we also have examined the myofibrillar ATPase activity in hearts by histochemical staining. Sections of skeletal muscles were taken for comparison since skeletal myosin ATPase activity is unaffected by thyroid hormone administration [7].

Address correspondence to: Eugene Morkin, M. D., Department of Internal Medicine/Section of Cardiology, University of Arizona Health Sciences Center, Tucson, Arizona 85724, USA

2. Methods

2.1. *Animal experiments*

Thyrotoxicosis was induced in three groups of six young New Zealand white rabbits weighing about 1.0–1.2 kg by injecting L-thyroxine (200 μg/kg) daily for 14 days. Animals were weighed daily and the dose of thyroxine reduced to one-half, or omitted, if the body weight fell to less than 80% of the initial value. Uninjected rabbits from the same litters were used for control experiments.

2.2. *Contractile proteins*

Myosin was isolated from hearts by the usual procedure employed in this laboratory [8]. Actin was extracted from an acetone powder prepared from the back and leg muscles of euthyroid rabbits and prepared by gel filtration according to the method of Rees and Young [9]. The methods used for assay of myosin are described in table 1.

2.3. *Histochemical methods*

Histochemical studies were performed on portions of left and right ventricular papillary muscles from the hearts of thyrotoxic and euthyroid animals. Also, portions of the extensor digitorum longus (EDL) and soleus muscles from each animal were examined by histological techniques. Specimens were quenched in isopentane cooled with liquid nitrogen and cross-sectioned at 10 μm thickness in a refrigerated microtome at –20°C. Sections were stained by the modified Gomori trichrome method [10] and the nicotinamide adenine dinucleotide tetrazolium reductase reaction [11]. The myofibrillar ATPase reaction was carried

out, at pH 9.4, on frozen sections of papillary and skeletal muscles as described by Padykula and Herman [12]. Prior to staining, sections of papillary muscles were preincubated, at pH 9.4, for 12 min at 37°C. Histochemical differentiation of skeletal muscle fibers was carried out on the basis of changes in the ATPase reaction after preincubation at either pH 9.4 or pH 4.6 [13].

3. Results and discussion

Table 1 shows the steady state rate of Mg^{2+}- and actin-activated ATPase activities of myosin from normal and thyrotoxic hearts used in this study. The Mg^{2+}–ATPase activity of cardiac myosin from thyrotoxic animals was about 300% greater than normal. This intrinsic ATPase activity was subtracted from measurements of actin-stimulated ATPase activity. Using an actin to myosin ratio similar to that found in the myofilament (4 mol actin to 1 mol myosin) the rate of actin-stimulated Mg–ATP hydrolysis by myosin from thyrotoxic hearts was about 300% greater than normal.

Figure 1 shows representative cross-sections of left ventricular papillary muscles from three pairs of thyrotoxic and euthyroid animals that have been stained for myofibrillar ATPase, at pH 9.4. Sections from each pair were prepared and stained simultaneously. Although the intensity of staining was somewhat variable, the sections from thyrotoxic animals (fig.1A, 1C and 1E) were consistently darker than from the euthyroid controls (fig.1B, 1D and 1F). Sections prepared from right ventricular papillary muscles of these animals showed a similar difference in staining. The increased staining in thyrotoxic hearts appeared to be entirely myofibrillar and did not involve mitochondria or other organelles. To our knowledge, this is the first histochemical demonstration of the effect of thyroid hormone on cardiac myosin.

The pH 9.4 myofibrillar ATPase reaction is widely employed for histological classification of fiber types in skeletal muscle (see [13] for review). The procedure for demonstrating this enzyme histochemically has been validated by comparing the histochemical properties of the enzyme with those of the purified actomyosin [14]. Under the conditions usually employed in the histochemical procedure (pH 9.4 and 18 mM Ca^{2+}) the reaction product is localized to the myofibril and there is essentially no staining of the mitochondria, sarcoplasmic reticulum or the intermyofibrillar constituents [15]. As a result, the conclusion has emerged that this histochemical reaction may be considered specific for myofibrillar ATPase activity.

In cardiac muscle, however, the myofibrils stain intensely by the histochemical procedure, yet the ATPase activity of purified actomyosin is quite low. A possible explanation for this discrepancy is that cardiac actomyosin ATPase activity is much more stable than actomyosin from skeletal muscle under the alkaline pH conditions used in the histochemical procedure [16]. The alkaline stability of cardiac

Table 1
Mg^{2+}- and actin-activated ATPase activities of cardiac myosin from euthyroid and thyrotoxic rabbit

Thyroid status	Mg^{2+}–ATPase (μmol P_i/mg/min)	Actin-stimulated ATPase (μmol P_i/mg/min)
Euthyroid	0.04 ± 0.01	0.06 ± 0.02
Thyrotoxic	0.12 ± 0.01	0.18 ± 0.02

Average ± SE for 3 determinations
Assays were performed at 37°C for 2 min essentially as described by Eisenberg and Moos [20]. The reaction medium contained 15 mM Tris–Cl pH 7.5, 1.5 mM $MgCl_2$ and 1 mM ATP in final vol. 4 ml. The myosin concentration was 0.25 mg/ml and the actin concentration was 0.09 mg/ml. The assays were terminated by addition of 10% trichloroacetic acid and inorganic phosphate liberated in the reaction was measured by the method of Fiske and Subbarow [21].

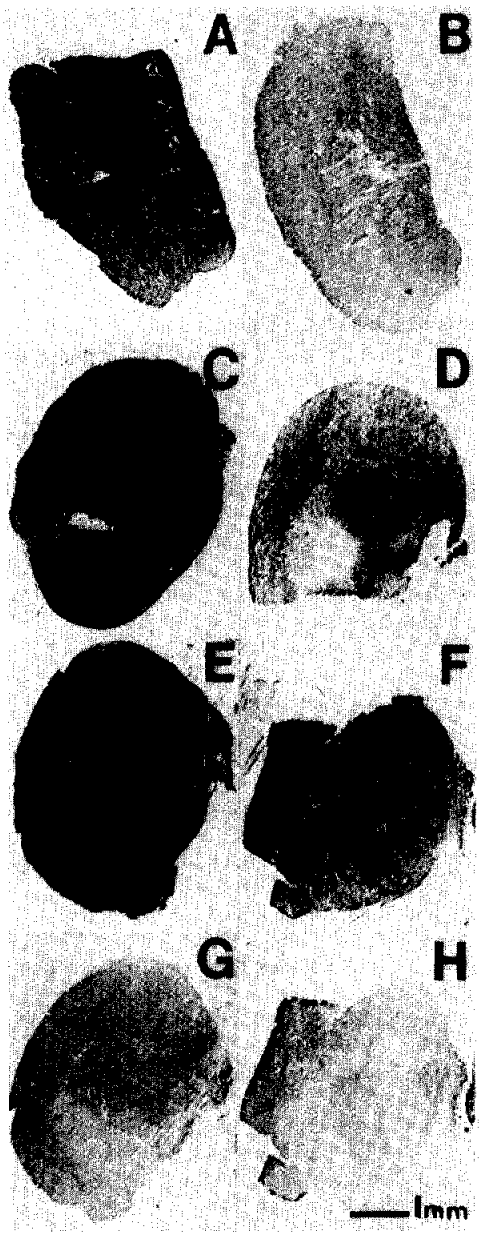

Fig.1. Transverse sections of papillary muscles: alkaline preincubated pH 9.4 ATPase reaction. (A, C, E) Papillary muscles from three thyrotoxic rabbits showing increased staining intensity. (B, D, F) Papillary muscles from three euthyroid rabbits. Each section of thyrotoxic tissue was processed simultaneously with the section of euthyroid tissue shown on the right. (G) Section of thyrotoxic papillary muscle incubated for ATPase reaction in the presence of 0.003 M NEM. (H) Section of euthyroid papillary muscle incubated for ATPase reaction in the presence of 3 mM NEM. The staining intensity of thyrotoxic and euthyroid tissue was greatly diminished by addition of NEM to the incubation medium. The black bar on fig.1H = 1 mm.

actomyosin is related to some protective effect of actin on this form of myosin, as dissociation of cardiac actomyosin results in increased alkali lability.

Additional control experiments were performed to establish that the intensity of the ATPase staining in thyrotoxic tissue was, in fact, myofibrillar and not mitochondrial in origin. First, we examined the effect of addition of 3 mM *N*-ethylmaleimide (NEM), a sulfhydryl blocking reagent, to the incubation medium. When thiol reagents are added prior to histochemical staining they depress the pH 9.4 myofibrillar ATPase reactivity, but produce an increase in the reactivity of mitochondrial ATPases [15]. In the present experiments, NEM appeared to markedly depress the ATPase reaction in papillary muscles from both normal and thyrotoxic animals (fig.1G and 1H), which would be consistent with a myofibrillar origin for this activity.

Secondly, we evaluated the possibility that myofibrillar ATPase staining might result, in part, from the contractile proteins binding inorganic orthophosphate released by mitochondrial ATPases [17]. In this experiment, 0.02 M to 5 mM K_2HPO_4 was substituted for ATP in the incubation medium. The reaction was then continued with the steps usually used for staining the reaction product after the ATPase procedure. Microscopic examination of these sections revealed only slight staining at the highest phosphate concentrations employed (0.02 M), which was about five times greater than the concentration of ATP used in the ATPase reaction. Even after incubation in this high concentration of K_2HPO_4 the sections were too faintly stained to photograph satisfactorily.

Rabbits treated with thyroxine lost about 10% of their initial body weight and there was some gross evidence of skeletal muscle atrophy. However, the microscopic and histochemical changes in these muscles were minimal. They consisted only of mild atrophy of both Type I and Type II fibers in some, but not all, of the EDL muscles and in none of the soleus muscles. Using the histochemical techniques described above, no other abnormalities could be

identified. Specifically, we did not observe differences in intensity of the pH 9.4 myofibrillar ATPase stain in euthyroid and thyrotoxic muscle fibers. Moreover, we did not see any of the architectural or other changes associated with neuromuscular diseases, such as, fiber type grouping or predominance, small angular fibers, inflammatory infiltrates or increased number of fibers with internal nuclei. Thus the morphological evidence of myopathy in these rabbits was less striking than has been reported in rats given thyroxine, in which a more marked and specific Type I fiber atrophy was described [18]. Possibly, this is related to variations between species in responsiveness to thyroid hormone or to differences in the dosage of thyroxine administered.

The effect of thyrotoxicosis on mitochondrial ATPase and myosin ATPase activities in skeletal muscle provide an interesting contrast with the effects on these activities in heart. In skeletal muscle, mitochondrial respiratory activity is increased in response to thyroid hormone administration [19], but myosin ATPase activity is unaffected [7]. In heart muscle, both mitochondrial ATPase and myosin ATPase activies are increased. Thus the fact that the 9.4 myofibrillar ATPase reaction was unchanged in thyrotoxic skeletal muscle can be taken as further evidence that the increased staining in thyrotoxic heart was myofibrillar in origin.

The present results indicate that thyroxine treatment results in increased actin-stimulated cardiac myosin ATPase activity. Moreover, this increased activity can be demonstrated histochemically using the pH 9.4 myofibrillar ATPase reaction. These findings strongly suggest that changes in enzymatic properties of myosin are responsible for the increased speed of contraction observed in the hearts of thyrotoxic animals.

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