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MODIFICATION OF SKELETAL MUSCLE SARCOTUBULAR Ca^{2+}-STIMULATED ADENOSINE TRIPHOSPHATASE ACTIVITY BY VARIOUS AGENTS*

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Abstract—Ca^{2+}-stimulated ATPase was isolated from the sarcotubular fraction of rabbit skeletal muscle, and the effects of various interventions on its activity were studied in the presence of different concentrations of calcium. Decreasing the pH of the incubation medium from 7.5 to 6.0 or the concentration of K^+ from 100 to 40 mM decreased the enzyme activity. Different anions (I^-, SCN^-, Br^- and NO_3^-) and cations (Hg^{2+}, La^{3+}, Ni^{2+}, Sr^{2+}, Mn^{2+} and Co^{2+}) were found to inhibit Ca^{2+}-stimulated ATPase activity. Ca^{2+} antagonists (verapamil and D600) and β-adrenergic blocking agents (propranolol and practolol) also had inhibitory effects whereas cyclic AMP–protein kinase augmented the enzyme stimulation by calcium. These results implicate sarcotubular Ca^{2+}–ATPase as a possible site of action of various agents affecting the skeletal muscle function.

The sarcotubular system, due to its ability to sequester calcium, is considered to lower the cytoplasmic concentration of free calcium and thus cause relaxation of the skeletal muscle [1]. The accumulation of calcium by the sarcotubular system occurs due to an active process and involves a membrane-bound Ca^{2+}-stimulated, Mg^{2+}-dependent ATPase enzyme [2, 3]. Recently, the Ca^{2+}-stimulated ATPase has been isolated in a relatively pure form from the sarcoplasmic reticulum [4, 5]. Although some of the characteristics of this purified enzyme have been studied, little is known concerning the actions of different agents, which are known to influence muscle function and sarcotubular calcium transport, on this preparation. It was therefore the object of this study to investigate the effects of different cations, anions and pharmacological agents on the sarcotubular Ca^{2+}-stimulated ATPase enzyme activity.

MATERIALS AND METHODS

Ca^{2+}-stimulated ATPase was isolated from rabbit (New Zealand White, weighing 1.5 to 2 kg) hind leg skeletal muscle by the method of MacLennan [5], which involves deoxycholate and salt fractionation of sarcoplasmic reticulum. This preparation showed characteristics similar to those reported by MacLennan [5] with respect to pH optimum, sensitivity to low and high concentrations of calcium, and the concentrations of Mg^{2+} and ATP for optimal activity. Furthermore, gel electrophoresis of this preparation according to the method described elsewhere [6] indicated only one protein component similar to that reported by MacLennan [4, 5].

ATP-hydrolyzing ability of this purified preparation was assayed in incubation medium containing 50 mM Tris–HCl, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA [ethyleneglycol-bis-(β-aminoethylether) tetraacetic acid], enzyme (10–15 μg), and different concentrations of $CaCl_2$. It should be noted here that the assay conditions employed here are for the optimal enzyme activity and are similar to those used by MacLennan [5]. Ca^{2+}-stimulated ATPase refers to the extra amount of ATP hydrolyzed due to calcium and was calculated by subtracting ATP hydrolysis in the presence of 1 mM EGTA from that in the presence of calcium. Any changes in the composition of this medium are indicated in the text. The reaction was started by the addition of ATP (10 mM), and after 4 min of incubation at 37° the reaction was terminated by the addition of cold trichloroacetic acid. Phosphate released into the supernatant after centrifugation was determined by the method of Taussky and Shorr [7]. Interference by certain agents of the determination of phosphate was removed by treatment with activated charcoal [8]. The protein concentration was determined by the method of Lowry *et al.* [9]. All the experiments were done in duplicate on at least four different preparations.

In some experiments, the enzyme preparation was treated with protein kinase–cyclic AMP according to the method described elsewhere [10]. The enzyme preparation (about 1 mg/ml) was incubated with and without protein kinase (about 1 mg/ml) or 1 μM cyclic AMP or both in a medium containing 5 mM $MgCl_2$, 5 mM ATP, 125 mM KCl and 40 mM histidine buffer, pH 6.8, at 25° for 10 min. The mixture was centrifuged at 100,000 *g* for 20 min, the pellet gently washed and suspended in 50 mM KCl and 20 mM Tris–HCl, pH 6.8. The activities of these treated preparations were then determined by using the above mentioned conditions for the Ca^{2+}-stimulated ATPase assay. It should be mentioned that the skeletal

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muscle protein kinase was purchased from Sigma Chemicals, St. Louis, Mo, dialyzed against 5 mM histidine-HCl buffer (pH 6.8) and stored in small aliquots at −20° before using within 10 days.

RESULTS

In view of dramatic changes in the calcium-transporting activity of the fragments of skeletal muscle sarcotubular system due to H^+, K^+ and Na^+ [11, 12], the effects of these monovalent cations on the sarcotubular Ca^{2+}-stimulated ATPase in the presence of different concentrations of calcium were studied. The results shown in Fig. 1 indicate that increasing the pH of the incubation medium from 6.0 to 7.5 increased the Ca^{2+}-stimulated ATPase activity. Likewise, increasing the concentration of K^+ from 40 to 100 mM increased the enzyme activity. However, in the presence of 100 mM K^+, addition of 40–100 mM Na^+ in the incubation medium had no effect (data not shown).

Different monovalent anions and divalent cations are known to influence the mechanical activity of skeletal muscle [13]. In this study we have observed that substituting Cl^- with anions such as I^-, SCN^-, Br^- and NO_3^- inhibited the Ca^{2+}-stimulated ATPase activity (Fig. 2). Furthermore, divalent cations such as Ni^{2+}, Sr^{2+}, Mn^{2+} and Co^{2+} in concentrations higher than 10^{-4} M were found to inhibit Ca^{2+}-stimulated ATPase (Fig. 3). La^{3+}, which is known to displace Ca^{2+} from its binding sites [14], and Hg^{2+}, a heavy metal, inhibited Ca^{2+}-stimulated ATPase in concentrations lower than 10^{-4} M (Fig. 3).

The effects of different agents, which have been shown to modify calcium movements at the sarcolemmal, mitochondrial and sarcoplasmic reticular membranes, on the Ca^{2+}-stimulated ATPase activity, were studied. Neither ruthenium red nor ouabain, which inhibit mitochondrial Ca^{2+} movements [15] and sarcolemmal Na^+-K^+ ATPase [16], respectively, had any effect on the enzyme activity (Table 1). On the other hand, well-known Ca^{2+} antagonists such as verapamil and D600 [17] and β-adrenergic blocking agents such as propranolol and practolol inhibited the Ca^{2+}-stimulated ATPase (Table 1). The inhibitory effect of propranolol and practolol was not relieved by the addition of epinephrine.

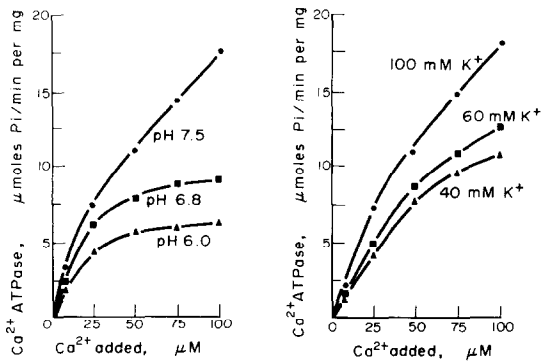

Fig. 1. Effects of pH and different concentrations of K^+ on sarcotubular Ca^{2+}-stimulated ATPase in incubation medium containing different concentrations of calcium.

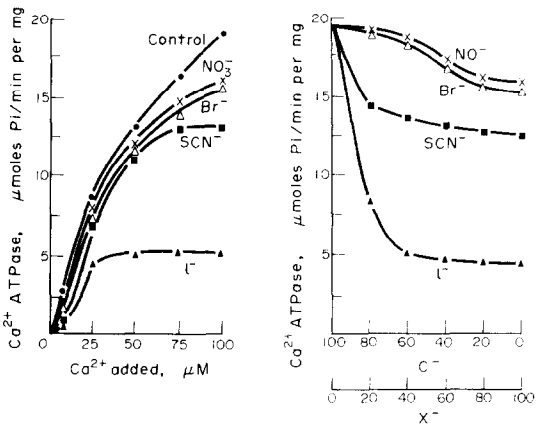

Fig. 2. Effects of substitution of Cl^- with different anions (100 mM) on sarcotubular Ca^{2+}-stimulated ATPase in incubation medium containing different concentrations of calcium (left panel) or medium containing 0.1 mM calcium but different concentrations (mM) of anions (right panel).

Since epinephrine and glucagon are known to increase the intracellular cyclic AMP concentration [18], it was of interest to study the effects of these agents as well as cyclic AMP on the Ca^{2+}-stimulated ATPase activity. The data given in Table 1 indicate that epinephrine, glucagon, cyclic AMP and dibutyryl cyclic AMP had no effect. These agents in lower or higher concentrations than those indicated in Table 1 were also ineffective.

Because the effects of cyclic AMP are thought to be mediated via the phosphorylation due to protein kinase [19], the action of cyclic AMP-dependent protein kinase was tested on the Ca^{2+}-stimulated ATPase activity. The results shown in Fig. 4 demonstrate that treatment of the enzyme preparation with protein kinase (1 μg/μg of enzyme) plus cyclic AMP (10^{-6} M) increased ATP hydrolysis at different concentrations of calcium. The stimulant effect of cyclic AMP-dependent protein kinase was greater at low concentrations of calcium in the incubation medium. It could also be seen from Fig. 4 that treatments of the enzyme preparations with either cyclic AMP or protein kinase also increased the enzyme activity

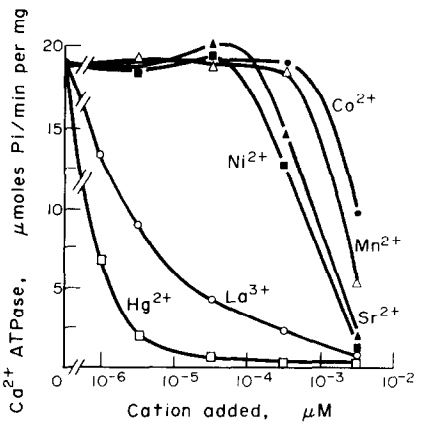

Fig. 3. Effects of different concentrations of various cations on sarcotubular Ca^{2+}-stimulated ATPase in incubation medium containing 0.1 mM calcium.

Table 1. Effect of various agents on sarcotubular Ca^{2+}-stimulated adenosine triphosphatase*

Agent	Concn (M)	Ca^{2+}-ATPase activity (μmoles P_i/min/mg protein
Control		18.5 ± 0.54
Ruthenium red	2.5×10^{-6}	19.2 ± 0.53
Ouabain	10^{-3}	18.1 ± 0.82
Verapamil	10^{-4}	13.9 ± 1.07†
D600	10^{-4}	14.1 ± 0.41†
Propranolol	10^{-4}	13.5 ± 0.81†
Practolol	10^{-4}	14.9 ± 1.01†
Epinephrine	10^{-5}	19.7 ± 0.67
Glucagon	10^{-6}	20.6 ± 0.99
Cyclic AMP	10^{-5}	19.0 ± 0.90
Dibutyryl cyclic AMP	10^{-5}	19.8 ± 0.37

* Ca^{2+}-stimulated ATPase activity was determined as described in Materials and Methods in incubation medium containing 0.1 mM calcium. Each value represents the mean ± S. E. of four experiments.

† Significant differences from control activity at $P < 0.05$.

slightly; the reasons for such increases are not clear at present because these effects were not apparent in untreated preparations. In another set of experiments, the enzyme preparations were preincubated with cyclic AMP (10^{-7} to 10^{-5} M), protein kinase (100–500 μg/mg of enzyme) and cyclic AMP plus protein kinase for 2 min before starting the reaction. A marked (30–70 per cent) increase in the Ca^{2+}-stimulated ATPase activity was seen when protein kinase and cyclic AMP were added in the incubation medium whereas neither protein kinase nor cyclic AMP, when added alone, had any significant effect on the enzyme activity. The concentrations of protein kinase and cyclic AMP for the optimal effect were 200 μg/mg of enzyme and 10^{-6} M respectively.

DISCUSSION

Sarcotubular Ca^{2+}-stimulated ATPase has now been established to participate in calcium transport and thereby influence muscle performance [1–3]. For example, augmentation of Ca^{2+}-stimulated ATPase activity can be conceived to be associated with faster muscle relaxation, whereas its inhibition will make it more difficult for the muscle to relax. It was interesting to observe that the enzyme activity at different concentrations of calcium was elevated by increasing the concentration of K^+ from 40 to 100 mM. The significance of this finding is not clear at present since the magnitude of changes in the cytoplasmic concentration of K^+ during muscle contraction and relaxation under normal conditions is relatively small. However, changes in pH during muscle contraction and relaxation [20] may be important in determining the activation state of this enzyme because its activity was found to be highly sensitive to variations in the pH of the incubation medium. Since marked alterations in dystrophic muscle metabolism and electrolyte composition have been seen [21, 22], it is likely that both pH and K^+ may play a crucial role in regulating the activity of Ca^{2+}-stimulated ATPase under pathological conditions.

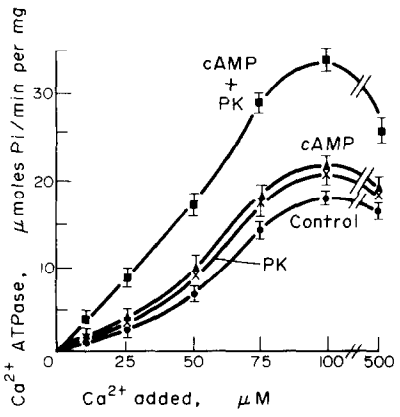

Fig. 4. Effects of cyclic AMP, protein kinase and cyclic AMP plus protein kinase treatments on sarcotubular Ca^{2+} stimulated ATPase in incubation medium containing different concentrations of calcium. The concentrations of cyclic AMP and protein kinase were 10^{-6} M and 1 μg/μg of enzyme respectively. The preparations were used immediately after treatments as described in Materials and Methods. Each value is a mean ± S. E. of four experiments.

Various cations such as Ni^{2+}, Co^{2+}, Mn^{2+} and Sr^{2+} as well as anions such as I^-, SCN^-, Br^- and NO_3^- were found to inhibit Ca^{2+}-ATPase activity. This effect may be explained on the basis of a complex formation of these cations with ATP or the enzyme preparation *per se*. Since these agents have been shown to potentiate muscle contraction and prolong the contractile state [13], it is plausible that their effect on sarcotubular Ca^{2+}-ATPase may contribute to their contraction-potentiating action through inhibition of calcium transport [23–26]. Furthermore, Hg^{2+}, which has been shown to produce irreversible inhibition of muscle contraction [27], was found to exert a marked depressant effect upon the Ca^{2+}-ATPase. The mechanism of inhibition of these divalent cations and monovalent anions, however, remains to be elucidated.

Different calcium antagonists such as verapamil, D600 and Lanthanum [14, 17], which have been shown to act at the plasma membrane, were found to inhibit sarcotubular Ca^{2+}-ATPase activity. Although these agents can be conceived to compete for the Ca^{2+} sites on the enzyme preparation, extensive work is necessary to establish this point. At any rate, other membrane inhibitors such as ruthenium red and ouabain, which influence mitochondrial calcium transport [15] and sarcolemmal Na^+-K^+ ATPase activity [16], respectively, were observed to have no effect on sarcotubular Ca^{2+}-stimulated ATPase. We have demonstrated that β-adrenergic blocking agents such as propranolol and practolol inhibited the sarcotubular Ca^{2+}-ATPase, but their action appears to be of a nonspecific nature since a β-receptor agonist, epinephrine, failed to relieve this inhibition.

The results reported in this study reveal that cyclic AMP in the presence of protein kinase markedly augments the sarcotubular Ca^{2+}-stimulated ATPase activity whereas cyclic AMP or protein kinase alone had only a slight effect. Such a dramatic augmentation may be due to phosphorylation of the skeletal muscle enzyme preparation in a manner similar to that shown to occur in heart sarcotubular membranes [19]. It was interesting to find that hormones such as epinephrine and glucagon, which increase the intracellular concentration of cyclic AMP [18], had

no direct effect on Ca^{2+}–ATPase activity. It is thus possible that various hormones regulate the activity of the skeletal muscle sarcotubular calcium pump via cyclic AMP-dependent protein kinase. This mechanism may be operating in conditions such as exercise where circulating epinephrine is elevated and the muscle is undergoing repeated contraction-relaxation cycles at a rapid rate.

REFERENCES

1. S. Ebashi and M. Endo, *Prog. Biophys. molec. Biol.* **18,** 123 (1968).
2. W. Hasselbach, *Prog. Biophys. molec. Biol.* **14,** 167 (1964).
3. G. Inesi, *A. Rev. Biophys. Bioengin.* **1,** 191 (1972).
4. D. H. MacLennan, *J. biol. Chem.* **245,** 4508 (1970).
5. D. H. MacLennan, C. C. Yip, G. H. Iles and P. Seeman, *Cold Spring Harb. Symp. Quant. Biol.* **37,** 469 (1972).
6. K. Weber and M. Osborn, *J. biol. Chem.* **244,** 4406 (1969).
7. H. H. Taussky and E. Shorr, *J. biol. Chem.* **202,** 675 (1953).
8. F. Fuchs, E. W. Gertz and F. N. Briggs, *J. gen. Physiol.* **52,** 955 (1968).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
10. M. Tada, M. A. Kirchberger, D. I. Repke and A. M. Katz, *J. biol. Chem.* **249,** 6147 (1974).
11. B. B. Rubin and A. M. Katz, *Science, N.Y.* **158,** 1189 (1967).
12. Y. Nakamaru and A. Schwartz, *J. gen. Physiol.* **59,** 22 (1972).
13. A. Sandow, *Pharmac. Rev.* **17,** 265 (1965).
14. W. G. Sanborn and G. A. Langer, *J. gen. Physiol.* **56,** 191 (1970).
15. J. H. Thakar, K. Wrogemann and M. C. Blanchaer, *Biochim. biophys. Acta* **314,** 8 (1973).
16. D. B. McNamara, P. V. Sulakhe and N. S. Dhalla, *Biochem. J.* **125,** 525 (1971).
17. A. Fleckenstein, in *Calcium and the Heart* (Eds. P. Harris and L. Opie), p. 135. Academic Press, London (1971).
18. G. A. Robison, R. W. Butcher and E. W. Sutherland, *A. Rev. Biochem.* **37,** 149 (1968).
19. M. A. Kirchberger, M. Tada and A. M. Katz, *J. biol. Chem.* **249,** 6166 (1974).
20. M. Dubuisson, *J. Physiol., Lond.* **94,** 461 (1939).
21. N. S. Dhalla, M. Fedelesova and I. Toffler, *Can. J. Biochem.* **50,** 550 (1972).
22. I. F. M. Borowski, J. A. Harrow, E. T. Pritchard and N. S. Dhalla, *Res. Commun. Chem. Path. Pharmac.* **7,** 443 (1974).
23. J. E. Chimoskey and J. Gergely, *Archs Biochem. Biophys.* **128,** 601 (1968).
24. A. P. Carvalho, *J. gen. Physiol.* **51,** 427 (1968).
25. A. P. Carvalho, *Eur. J. Biochem.* **27,** 491 (1972).
26. R. The and W. Hasselbach, *Eur. J. Biochem.* **53,** 105 (1975).
27. G. B. Frank, *J. Physiol., Lond.* **163,** 254 (1962).