THE MECHANISM OF OXIDATIVE INHIBITION OF MYOSIN

by

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The action of hydrogen peroxide on myosin has been studied in some detail by BAILEY AND PERRY[1], who found that the oxidative removal of the SH-groups was accompanied by loss of both adenosinetriphosphatase (ATP'ase) activity and ability to form actomyosin. Other oxidizing agents act similarly, particularly iodosobenzoate and iodine[1]. In isolated muscle preparations hydrogen peroxide was found by BACQ[2,3] to produce a Lundsgaard contracture, an effect characteristic of the sulphydryl reagents ("substances thioloprives"). HOBBIGER[4] has found that this action of hydrogen peroxide is reversible, like the inactivation by peroxide of myosin ATP'ase (ZIFF[5], MEHL[6]).

GOFFART[7] has recently shown that adrenochrome can cause a similar type of contracture to that produced by H_2O_2. In the present paper we report the inhibitory action of adrenochrome on the ATP'ase activity of myosin. Inactivation of this enzyme, whether by adrenochrome or by peroxide, is shown to occur via trace-metals contained in the myosin preparations.

A preliminary account of this work was presented to the British Physiological Society (DICKENS AND GLOCK[8]).

METHODS

Myosin

Rat skeletal muscle was dissected, disintegrated in 5 volumes of 0.5 *M* KCl in a blendor (M.S.E. Atomix), centrifuged and the dissolved myosin was purified by repeated (3 or 4 times) precipitation from glass-distilled water and re-solution in 0.5 *M* KCl according to BAILEY[9] but with the use of dilute NaOH to maintain the p_H at 6.8 (BAILEY[10]). This preparation from rat muscle proved considerably more stable than previously described specimens of rabbit muscle myosin. In particular, its activity remained almost unchanged on incubation at 37° even for several hours. It was stored in the refrigerator as a solution in 0.5 *M* KCl (dry wt. of myosin 5 mg/ml) without change of activity for about a month. It must not be frozen or it becomes denatured with loss of activity.

Estimation of enzymic activity of myosin

The adenosinetriphosphatase (ATP'ase) activity of the samples was determined at 37° by incubating the myosin (0.5–1.0 mg dry wt. myosin) in 0.7 ml veronal-KCl solution p_H 7.4, usually for 30 min, with 0.1 ml 0.1 *M* $CaCl_2$, 0.2 ml *M*/31 sodium adenosinetriphosphate (= 0.2 mg 7 min P) and veronal-KCl solution to a total volume of 2.0 ml. The reaction was stopped by addition of 3 ml 8% trichloroacetic acid solution, and inorganic phosphate determined (FISKE AND SUBBAROW[11]) on 3 ml of the centrifuged solution made to a final volume of 20 ml.

The veronal-KCl buffer was prepared by diluting MICHAELIS veronal-acetate buffer of the required p_H with an equal volume of *M* KCl. Most experiments were done at p_H 7.4 throughout, but in some earlier determinations the estimation was made at p_H 8.9 as has been the practice of several other workers. After we found no advantage in this, apart from the higher activity at the

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alkaline reaction, it was discontinued. The Q_p of our two preparations at pH 7.4 was Prep. I, 500, Prep. II, 1100, (cf. ENGELHARDT[12]).

Exposure of myosin to oxidants and other reagents

When the effect of a reagent on ATP'ase activity was determined, this was done by pre-incubation for 30 min at 37° of the myosin with the reagent in a total volume of 0.7 ml. After this period, the ATP'ase activity was determined as above. When reactivation by cysteine was attempted this was added in a final concentration of 0.01 *M* and the mixture incubated for a further 15 min at 37°. The control sample of enzyme was in all cases given similar treatment.

Glass-distilled water and precautions to avoid metallic contaminants were employed throughout these experiments. All reagents were neutralized to the pH of the experiment before addition.

Sodium adenosinetriphosphate was prepared from the barium salt (Boots' Pure Drug Co.) by passing the acidified solution through a column of Amberlite IR 100 as described by POLIS AND MEYERHOF[13], followed by neutralization of the eluate with NaOH. The solution (*M*/31) was stored at —10°.

Adrenochrome was prepared by the method of MACCARTHY[14].

L-*Adrenaline and* DL-*noradrenaline* were used throughout.

RESULTS

Inactivation of myosin by hydrogen peroxide and reactivation by cysteine

Fig. 1 shows the rate of inactivation of the ATP'ase activity of rat muscle myosin (Prep. I) by various concentrations of H_2O_2. With this myosin sample 50% inactivation required about $3 \cdot 10^{-3}$ *M* H_2O_2 acting for 30 min. Another sample (Prep. II) made in the same way was much more sensitive (50% inactivation by $8 \cdot 10^{-4}$ *M* H_2O_2 in 30 min).

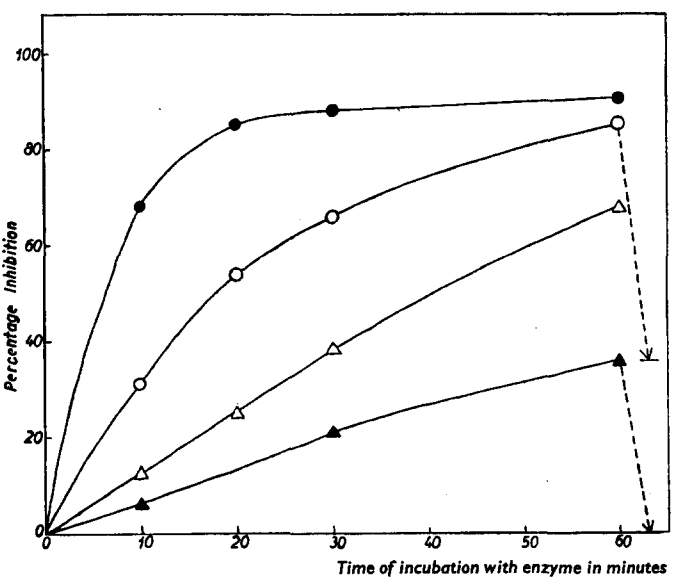

Fig. 1. Inhibition of myosin (adenosinetriphosphatase) activity by hydrogen peroxide. Myosin preparation I. Incubation with the following concentrations of H_2O_2 at 37°, pH 7.4: ▲ = 10^{-3} *M*; △ = $2 \cdot 10^{-3}$ *M*, o = $5 \cdot 10^{-3}$ *M*, ● = 10^{-2} *M*. Reactivation by cysteine is shown by the broken lines, the arrows showing the activity level after 15 min incubation with 10^{-2} *M* cysteine

Fig. 1 also shows (dotted lines) the degree of reactivation on incubation with cysteine after exposure to 10^{-3} and $5 \cdot 10^{-3}$ *M* H_2O_2: in the former case the inhibition (36%) was completely reversed by cysteine, but where the inhibition was greater (86% with $5 \cdot 10^{-3}$ *M* H_2O_2) only partial reactivation occurred.

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In other experiments excess H_2O_2 was removed by catalase before adding cysteine with similar results to those above. Addition of cysteine to the enzyme which had not been inactivated with peroxide did not increase its activity but in fact caused some decrease.

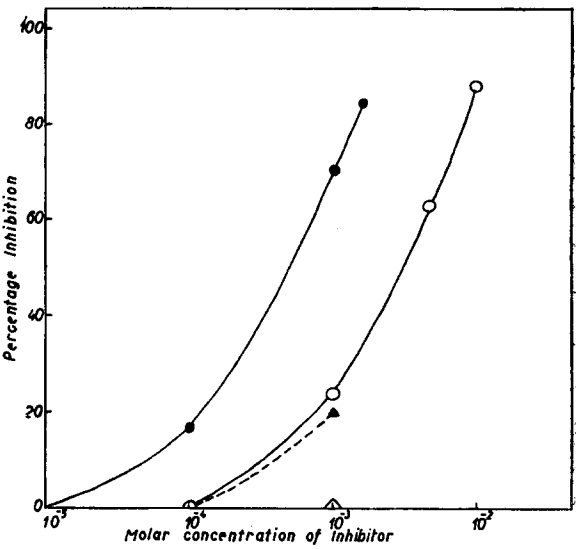

Fig. 2. Inhibition of adenosinetriphosphatase by a. adrenochrome; b. H_2O_2. Myosin preparation I. Activity measured after incubation for 30 min at 37° with the concentration of inhibitor shown on the abscissae. Inhibitor: ● = adrenochrome, o = H_2O_2, ▲ = adrenaline, △ = noradrenaline

Inactivation by adrenochrome

Although adrenochrome is known to inhibit tissue glycolysis *in vitro* (RANDALL[15]; MEYERHOF AND RANDALL[16]), the action of this substance on ATP'ase has not been reported. Fig. 2 shows that adrenochrome is a powerful inhibitor of myosin ATP'ase, causing 50% fall of activity in a concentration of $5 \cdot 10^{-4}$ *M*. Comparison with the curves for H_2O_2 inactivation of the same preparation of myosin, which is also included in Fig. 2, shows that adrenochrome is about 8 times as powerful an inhibitor as H_2O_2. Adrenaline in 10^{-4} *M* concentration is without any effect, but 10^{-3} *M* is slightly (20%) inhibitory. Noradrenaline even in 10^{-3} *M* concentration has no effect on the ATP'ase activity.

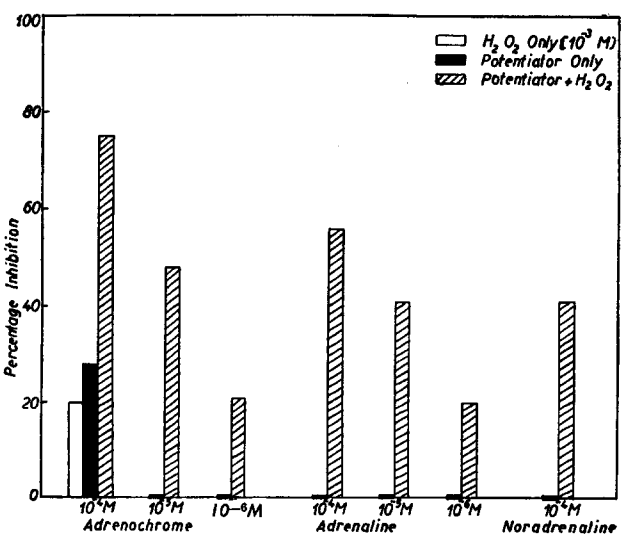

Fig. 3. Potentiation of H_2O_2 inhibition of adenosinetriphosphatase by adrenochrome, adrenaline and noradrenaline. Myosin preparation I. Concentration of H_2O_2, 10^{-3} *M*, which alone gave 20% inhibition. The molar concentrations of potentiator are shown at the foot of the columns

Potentiation of H_2O_2 inactivation of myosin by adrenaline and its derivatives

Fig. 3 shows that concentrations of adrenochrome, adrenaline and noradrenaline which are themselves too low to have any effect on the enzyme activity, powerfully potentiate the inactivation by H_2O_2 (10^{-3} *M*, 20% inhibition alone). Thus, for example, 10^{-5} *M* adrenochrome increases the inhibition to 48%, 10^{-5} *M* adrenaline to 42%, and 10^{-4} *M* noradrenaline to 43%, although none of these substances in the above concentrations had any inhibitory effect without peroxide (Fig. 3).

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Effect of metal-binding reagents on the oxidative inactivation of myosin

Hitherto, the effect of cyanide and other metal-binding agents on the oxidative inactivation of myosin has not been investigated, although cyanide is known to increase the activity, particularly of older preparations in which atmospheric oxidation of -SH groups is presumed to have occurred (BINKLEY, WARD, AND HOAGLAND[17]). Such an action could be due either to the reducing action of cyanide or to the removal of an inhibitory metal. We, therefore, studied the action not only of cyanide and azide but also of some metal-chelating reagents.

Table I shows, in the column headed 'Control', the effect of adding these reagents to myosin before estimating its ATP'ase activity in the usual way. Cyanide, 10^{-3} *M*, caused 40%, and 10^{-4} *M* 20% increase of the original activity of the enzyme (*i.e.* the activity without reagent). Insignificant alterations of activity were caused by azide (10^{-3} *M*), *aa'*-dipyridyl (10^{-3} *M*) or 8-hydroxyquinoline (saturated). Diethyl-dithiocarbamate (10^{-3} *M*) was slightly inhibitory.

TABLE I

ACTION OF COMPOUNDS FORMING METAL COMPLEXES ON INHIBITION OF MYOSIN BY PEROXIDE AND ADRENOCHROME

Myosin preparation I

Complex-forming compound	Percentage of original activity		
	Control	+ H_2O_2 (10^{-2} *M*)	+ Adrenochrome (10^{-3} *M*)
None	100	44	17
NaCN 10^{-3} *M*	140	143	140
10^{-4} *M*	120	108	113
NaN_3 10^{-3} *M*	102	50	—
aa'-Dipyridyl 10^{-3} *M*	108	116	101
10^{-4} *M*	100	101	
8-Hydroxyquinoline	103	103	—
Diethyldithiocarbamate 10^{-3} *M*	87	30	—

These reagents were then added to the enzyme before the addition of 10^{-2} *M* hydrogen peroxide and 10^{-3} *M* adrenochrome (Table I). Cyanide in 10^{-3} *M* concentration protected the enzyme completely against the toxic effect of both these oxidants, and even 10^{-4} *M* cyanide gave virtually complete protection. On the other hand azide (10^{-3} *M*) gave no significant protection. The experiments with azide included in Table I were made at p_H 6.4, since azide is most active at this reaction as an inhibitor of metal-catalysed reactions (KEILIN[18]). Azide was also inert at p_H 7.4 in this system.

aa'-Dipyridyl (10^{-3} and 10^{-4} *M*) was as effective as cyanide in blocking the oxidative inhibition of myosin by either H_2O_2 or adrenochrome (Table I). 8-Hydroxyquinoline also protected the enzyme against H_2O_2. Both of these reagents form complexes with iron, as well as with some other metals. On the other hand, diethyldithiocarbamate which forms complexes with many metals including copper does not give any protection against H_2O_2 (Table I).

These results indicate that a metal is concerned in the inhibition of myosin ATP'ase activity caused by either peroxide or adrenochrome. Although it is not possible to state definitely which metal is responsible, the nature of the inhibitors suggests iron. Spectro-

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graphic examination of this specimen of myosin (Preparation I) was kindly carried out for us by Messrs. BIRCH AND WEBB of Hilger & Watts Ltd., who detected the presence of the following metals: K, Na, Ca, Mg, Cu and Fe. Estimation of the iron content of the myosin preparation with $\alpha\alpha'$-dipyridyl after ashing 50 mg (dry wt.) with iron-free nitric acid, showed that the Fe content was less than 0.004% or a concentration of below $7 \cdot 10^{-5}$ g atoms Fe/100 g myosin. Since the apparent cysteine content of myosin[1] (1.7 g cysteine/100 g) is about 10^{-2} g mol cysteine/100 g myosin, the ratio of atoms Fe/mol cysteine is less than 1:100.

Effect of added metals on inactivation of myosin by peroxide

Even if iron is the catalytically active metal present in myosin, it is not the most active of those which, when added in the form of inorganic salts, bring about the inactivation of myosin by peroxide. Fig. 4 shows that Cu^{++}, Zn^{++}, Co^{++} are more strongly active than iron added in the form of ferrocyanide. Ferrous sulphate behaves similarly to ferrocyanide. Mn^{++} shows only feeble activity even in $3 \cdot 10^{-4}$ *M* concentration.

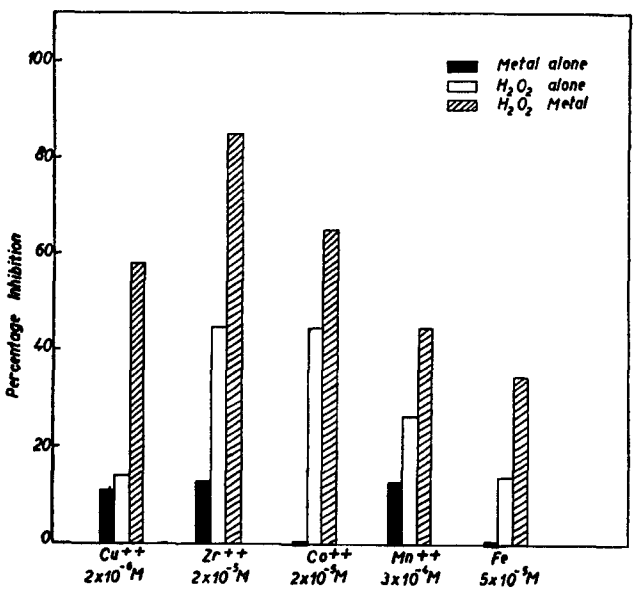

Fig. 4. Effect of metal ions on the inhibition of adenosinetriphosphatase by H_2O_2. Myosin preparation II. Concentrations of H_2O_2 for the various experiments: with Cu, $3 \cdot 10^{-4}$; Zn, $5 \cdot 10^{-4}$, Co, $5 \cdot 10^{-4}$; Mn, $4 \cdot 10^{-4}$, Ferrocyanide, $3 \cdot 10^{-4}$ *M* H_2O_2

The striking increases in the action of peroxide shown in the presence of as little as $2 \cdot 10^{-6}$ *M* Cu and $2 \cdot 10^{-5}$ *M* Zn are evidence of the catalytic actions of these two metals, in view of the fact that the concentration of myosin –SH groups in the digest was approximately 10^{-4} *M*.

To investigate the possibility that haematin compounds might be more active than the above inorganic iron compounds, haematin, haemoglobin, methaemoglobin, cytochrome c and catalase were added to myosin before treatment with H_2O_2. In no instance was there any increased inactivation.

DISCUSSION

The present work has shown that trace metals are involved in the oxidative inactivation of myosin by H_2O_2. Thus this inactivation is prevented by low concentrations of cyanide, $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline, all of which form complexes with heavy metals, and is potentiated by the addition of various metal salts, particularly by salts of Cu and Zn but also by Co, Fe and Ni.

In contrast to cyanide, azide does not protect myosin from peroxide inactivation even at p_H 6.4, at which p_H azide is known to combine with respiratory metallo-

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enzymes[18]. Haematin derivatives, however, have generally a much higher affinity for cyanide than for azide[19].

It is of interest that myosin is inactivated by adrenochrome and that this effect is presumably also dependent on the presence of trace-metals, since it is blocked by both cyanide and $\alpha\alpha'$-dipyridyl. This suggests that some of the pharmacological effects of adrenochrome[20] may also depend on metal catalysed systems. The same might also apply to the inhibitory action of adrenochrome on other SH enzymes[15,16].

Although adrenaline and noradrenaline in low concentrations (10^{-5} *M*) do not directly affect myosin ATP'ase activity they potentiate markedly its inactivation by H_2O_2. This opens up the interesting speculation that these hormones might be concerned in the physiological control of the oxidative state of myosin *in vivo*, by acting as hydrogen carriers between the cytochrome system and myosin. Adrenaline is, indeed, known to be oxidized to adrenochrome by the cytochrome system[21,22] and its autoxidation is catalysed by trace metals[20] and by cytochrome c and methaemoglobin, which are considered to react peroxidatively with the H_2O_2 formed during autoxidation[21,23].

Adrenochrome can also act as a hydrogen carrier in the malic and lactic dehydrogenase systems[21] and in the oxidation of ascorbic acid[23].

However, no proof yet exists that muscular activity involves a physiological alteration in the oxidative state of myosin, an important matter requiring much further investigation.

ACKNOWLEDGEMENT

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SUMMARY

1. Rat myosin preparations were stable at 37° in the absence of substrate, unlike similar preparations made from rabbit muscle by other workers.

2. The ATP'ase activity of such preparations can be inhibited at p_H 7.4 by H_2O_2 and restored by incubation with cysteine.

3. Adrenochrome is about 8 times as powerful an inactivator of myosin as H_2O_2.

4. Adrenaline, adrenochrome and noradrenaline in low concentrations potentiate the inactivation of myosin by H_2O_2.

5. Cyanide (10^{-4} *M*) protects the enzyme against inactivation by H_2O_2, whereas azide (10^{-3} *M*) has no such effect.

6. Heavy metal reagents ($\alpha\alpha'$-dipyridyl, 8-hydroxyquinoline) protect the enzyme against inactivation by H_2O_2.

7. $\alpha\alpha'$-dipyridyl also prevents inactivation by adrenochrome.

8. Added metallic ions catalyse the inactivation of myosin by H_2O_2, especially active metals being Cu ($2 \cdot 10^{-6}$ *M*) and Zn ($2 \cdot 10^{-5}$ *M*).

9. It is considered that a metallic catalyst present in myosin preparations is required for their oxidative inactivation, and it is suggested that such a reversible inactivation of myosin may play some part in the physiological control of muscular activity.

RÉSUMÉ

1. Des préparations de myosine de Rat étaient stables en l'absence de substrat, contrairement à ce qui a été trouvé par d'autres chercheurs pour des préparations semblables de muscle de Lapin.

2. L'activité ATP-asique de telles préparations peut être inhibée à p_H 7.4 par H_2O_2 et rétablie par incubation avec de la cystéine.

3. L'adrénochrome est, vis-à-vis de la myosine, un inhibiteur 8 fois plus puissant que l'H_2O_2.

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4. L'adrénaline, l'adrénochrome et la noradrénaline en faibles concentrations potentialisent l'inactivation de la myosine par l'H_2O_2.

5. Le cyanure (10^{-4} *M*) protège l'enzyme contre l'inactivation par l'H_2O_2, tandis que l'azoture (10^{-3} *M*) est sans action.

6. Les réactifs des métaux lourds (*α.α'*-dipyridyl, 8-hydroxyquinoléine) protègent l'enzyme contre l'inactivation par l'H_2O_2.

7. L'*α.α'*-dipyridyl empêche aussi l'inactivation par l'adrénochrome.

8. Des ions métalliques ajoutés au mélange catalysent l'inactivation de la myosine par l'H_2O_2, les métaux particulièrement actifs étant le Cu ($2 \cdot 10^{-6}$ *M*) et le Zn ($2 \cdot 10^{-5}$ *M*).

9. Nous considérons qu'un catalyseur métallique présent dans les préparations de myosine soit nécessaire à l'inactivation oxydative de ces préparations et nous suggérons l'idée qu'une telle inactivation réversible de la myosine pourrait jouer quelque rôle dans la régularisation physiologique de l'activité musculaire.

ZUSAMMENFASSUNG

1. Rattenmyosin-Präparate waren bei 37° in Abwesenheit von Substrat beständig, zum Unterschied von ähnlichen Präparaten welche von anderen Forschern aus Kaninchenmuskel hergestellt worden waren.

2. Die ATP-ase Aktivität solcher Präparate kann bei p_H 7.4 durch H_2O_2 gehemmt und durch Inkubation mit Cystein wiederhergestellt werden.

3. Adrenochrom ist in Bezug auf Myosin ein 8-mal so starker Hemmstoff wie H_2O_2.

4. Adrenalin, Adrenochrom und Noradrenalin in niedrigen Konzentrationen potentialisieren die Inaktivierung von Myosin durch H_2O_2.

5. Cyanid (10^{-4} *M*) schützt das Enzym gegen Inaktivierung durch H_2O_2, während Azid (10^{-3} *M*) keine solche Wirkung hat.

6. Schwermetallreagentien (*α.α'*-Dipyridyl, 8-Hydroxychinolin) schützen das Enzym gegen Inaktivierung durch H_2O_2.

7. *α.α'*-Dipyridyl verhindert auch die Inaktivierung durch Adrenochrom.

8. Zugefügte Metallionen katalysieren die Inaktivierung von Myosin durch H_2O_2; Cu ($2 \cdot 10^{-6}$ *M*) und Zn ($2 \cdot 10^{-5}$ *M*) sind besonders aktive Metalle.

9. Es wird erwogen, dass ein metallischer Katalysator, welcher sich in Myosinpräparaten vorfindet, für die oxydative Inaktivierung dieser Präparate notwendig sein könnte, und die Vermutung wird geäussert, dass so eine reversible Inaktivierung des Myosins bei der physiologischen Regulierung der Muskelaktivität eine Rolle spielen könnte.

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