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AN EVALUATION OF THE EFFECT OF PHLOROBUTYROPHENONE DERIVATIVES ON MYOSIN AND ACTOMYOSIN ADENOSINETRIPHOSPHATASES

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Abstract—The effects of desaspidin, flavaspidic acid and nor-flavaspidic acid on myosin and actomyosin ATPases and superprecipitation of actomyosin were studied.

These substances exert a biphasic effect on myosin ATPase. 8×10^{-6} M flavaspidic acid or nor-flavaspidic acid and $1{\cdot}24 \times 10^{-4}$ M desaspidin stimulate the Ca^{2+}-activated myosin ATPase by about 25 per cent. Higher concentrations are inhibitory.

Similar results were obtained with actomyosin ATPase, and superprecipitation of actomyosin was found to be inhibited by concentrations of the phlorobutyrophenone derivatives which inhibit the ATPase.

The ATPase-stimulating effect of these substances, in contrast to that of dinitrophenol, is not dependent on the presence of Ca^{2+}. About 100 per cent stimulation is obtained in media containing 0·5 M KCl and no divalent cations.

It is concluded that the effects of the phlorobutyrophenone derivatives on mitochondrial and myosin ATPases are not dependent on the same molecular configuration and that the effect on actomyosin ATPase may play a part in the toxic effect of flavaspidic acid but not in that of desaspidin.

INTRODUCTION

FLAVASPIDIC acid, an anthelmintic from fern extract, has been suggested to act as a muscle poison by inhibiting 'myosin' adenosinetriphosphatase (ATPase).[1] Like the other phlorobutyrophenone derivatives from fern extract, flavaspidic acid also uncouples electron-transport-coupled oxidative phosphorylation[2] and stimulates mitochondrial ATPase[3] and this may also play an important part in the toxic and anthelmintic effects of these substances.[3, 4] The two suggested points of action of flavaspidic acid and the well-known similarities between mitochondrial and myosin ATPases with respect to their sensitivity to enzyme modifiers such as 2,4-dinitrophenol (DNP) and —SH reagents prompted the present study.

MATERIAL AND METHODS

Solutions of ATP and the substances tested were prepared as described earlier.[2] The formulae of the substances are given in Ref. 2.

Myosin was prepared from rabbit skeletal muscle according to the method of Mommaerts.[5] After the final precipitation myosin was dissolved in 0·5 M KCl and stored at 0° for one week at most. The preparations released only equimolar amounts of inorganic phosphate (P_i) from ATP even upon prolonged incubation and were

thus devoid of myokinase activity. Kjeldahl nitrogen was determined in some preparations.

Actomyosin was prepared according to Mommaerts,[5] except that the step involving addition of ATP during the preparation was omitted. The actomyosin was dissolved in 0·5 M KCl, reprecipitated twice by dilution and finally dissolved in 0·5 M KCl. It was stored at 0° and used within 2 days.

Myosin ATPase was determined in media with a final volume of 2 ml containing 8 or 10 μmoles ATP, 100 μmoles tris buffer, pH 7·5 and $CaCl_2$ and KCl as indicated. The myosin stock solution was diluted with 0·5 M KCl to give a convenient enzymic activity and 0·2 ml of this ice-cold solution was added to each prewarmed tube to start the reaction. The temperature was 25° and the reaction time 7–10 min. The reaction was stopped with 2 ml 1 M perchloric acid and P_i determined according to the method of Ernster *et al.*[6]

Actomyosin ATPase was assayed in a medium with a final volume of 2 ml containing 20 μmoles tris buffer, pH 7·5, 100 μmoles KCl, 4 μmoles $MgCl_2$ and 8 μmoles ATP. The experimental conditions were otherwise the same as for myosin ATPase but incubation times of 3–5 min were employed.

Superprecipitation of actomyosin was determined according to the method of Blum[7] in the medium described above.

RESULTS

Desaspidin (Fig. 1), flavaspidic acid and nor-flavaspidic acid (Fig. 2) exert a biphasic effect on the Ca^{2+}-activated myosin ATPase, since they cause stimulation of the enzymic activity at low concentrations ($1{\cdot}25 \times 10^{-4}$ M for desaspidin and 8×10^{-6} M for flavaspidic acid and nor-flavaspidic acid) and inhibition at higher concentrations. While this effect resembles that exerted by DNP,[8–11] the classical enzyme modifier in this field, it is interesting to note that the effects of the phlorobutyrophenone derivatives on mitochondria[2, 3] on the one hand and on myosin on the other are

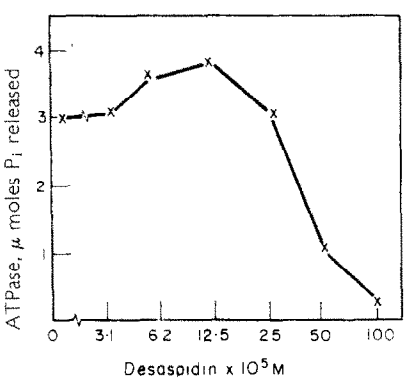

FIG. 1. The effect of desaspidin on Ca^{2+}-activated myosin ATPase.

Each sample contained 0·0824 mg myosin nitrogen, 10 μmoles ATP, 100 μmoles tris buffer, pH 7·5, 100 μmoles KCl and 20 μmoles $CaCl_2$. The final volume was 2 ml. The reaction was started by the addition of myosin in 0·2 ml ice-cold 0·5 M KCl to the prewarmed reaction mixture. The temperature was 25° and the reaction time 7 min.

The ordinate gives the ATPase activity as μmoles P_i released, the abscissa gives the concentration of desaspidin $\times 10^5$ M.

apparently not dependent on exactly the same molecular configuration. Thus desaspidin is about 25 times more effective than flavaspidic acid when tested on mitochondria,[2, 3] but at the same time about 16 times less effective when tested on myosin. Since nor-flavaspidic acid resembles flavaspidic acid in its effects, the difference in activity between desaspidin and flavaspidic acid, which are the most commonly used anthelmintics from fern extract, must be due to the presence of the methoxy group in the former compound.[2]

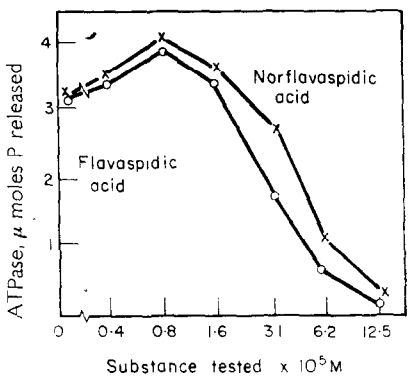

FIG. 2. The effect of flavaspidic acid and nor-flavaspidic acid on Ca^{2+}-activated myosin ATPase. Medium and experimental conditions as in Fig. 1.

In the same way as with DNP,[10, 11] the stimulating effect of desaspidin on myosin ATPase is temperature-dependent and no stimulation is obtained at 0° (Fig. 3).

The maximal stimulation obtained at 25° with the phlorobutyrophenone derivatives tested is only about 25 per cent of the basal activity, however, which is considerably less than the about 80 per cent stimulation obtained with DNP (Fig. 5). The effects

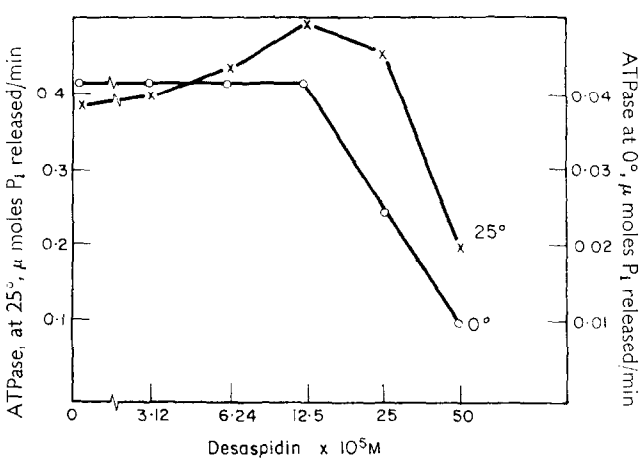

FIG. 3. The effect of desaspidin on Ca^{2+}-activated myosin ATPase at 0° and 25°.

The medium was as in Fig. 1. Of two parallel samples one was allowed to react for 8 min at 25° and the other for 95 min at 0°.

The left-hand ordinate gives the ATPase activity at 25°, the right-hand ordinate gives the activity at 0°.

of the phlorobutyrophenone derivatives and DNP also differed with respect to their dependence on ionic conditions. Thus high concentrations of Ca^{2+} potentiated the inhibitory effect of desaspidin but not that of DNP (Figs. 4 and 5). It is also noted from these figures that the slight basal enzymic activity measured at low ionic strength in the absence of divalent cations is also somewhat stimulated by desaspidin, while

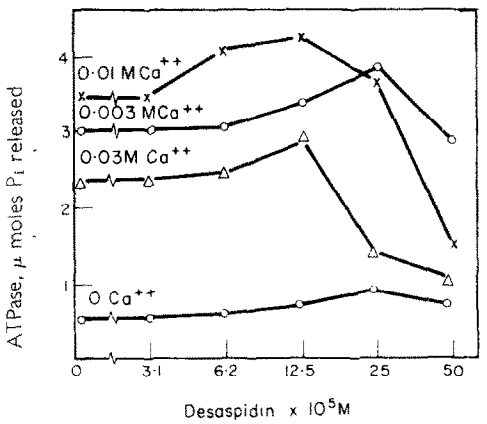

FIG. 4. The effect of desaspidin on myosin ATPase at different Ca^{2+} concentrations. Each sample contained 10 μmoles ATP, 100 μmoles tris buffer, pH 7·5, 100 μmoles KCl and either 0, 6, 20 or 60 μmoles $CaCl_2$. The final volume was 2 ml. Experimental conditions as in Fig. 1.

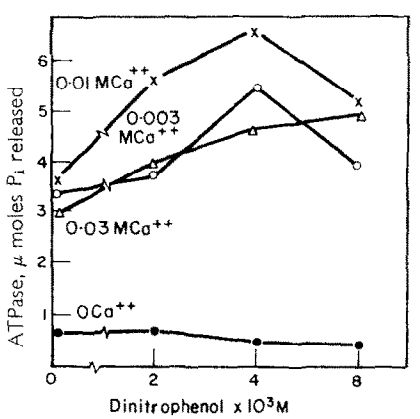

FIG. 5. The effect of dinitrophenol on myosin ATPase at different Ca^{2+} concentrations. Medium and experimental conditions as in Fig. 4.

the solely inhibitory effect of DNP under such conditions reported by others[8] was confirmed in the present study.

The stimulating effect of desaspidin on myosin ATPase was considerably greater, and somewhat smaller concentrations were needed when the effect of the substance was tested at high ionic strength (0·5 M KCl) in the absence of divalent cations. About 100 per cent stimulation was achieved (Fig. 6). In the presence of 10^{-3} M EDTA, which strongly stimulates myosin ATPase under these conditions,[12] desaspidin causes

a minimal further stimulation which rapidly turns into inhibition with higher concentrations of desaspidin (Fig. 6). The solely inhibitory effect of DNP in the absence of divalent cations is seen from Table 1.

Variation of the myosin content of the samples by a factor of 4 did not alter the effective concentrations of the phlorobutyrophenone derivatives and there was thus no such titration effect as was observed with mitochondria.[3]

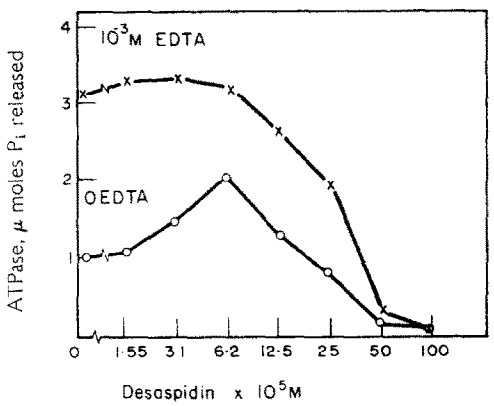

FIG. 6. The effect of desaspidin on myosin ATPase at high ionic strength with and without EDTA
Each sample contained 10 μmoles ATP, 100 μmoles tris buffer, pH 7·5, 1000 μmoles KCl, 0 or 2 μmoles EDTA. The final volume was 2 ml. Experimental conditions as in Fig. 1 except that the reaction time was 4·5 min.

Desaspidin and flavaspidic acid exert the same type of effect on the ATPase activity of natural actomyosin as they do on myosin ATPase. The effect of the substances on the superprecipitation of actomyosin, considered to be a kind of gel analogy to muscular contraction,[13, 14] was tested in parallel samples (Figs. 7 and 8). It is seen that superprecipitation is inhibited by those concentrations of the substances which are also inhibitory to the enzymic activity of actomyosin, while at concentrations at

TABLE 1. THE EFFECT OF DINITROPHENOL ON MYOSIN ATPASE AT HIGH IONIC STRENGTH IN THE PRESENCE AND ABSENCE OF Ca^{2+} AND EDTA

The reaction medium contained 100 μmoles tris buffer, pH 7·5, 1000 μmoles KCl, and additions as indicated in a final volume of 2 ml. The experimental conditions were as in Fig. 1.

	Additions	ATPase, μmoles P_i released
Expt. 1	none	1·60
	4×10^{-4} M DNP	1·25
	4×10^{-3} M DNP	0·89
	10^{-3} M EDTA	3·70
	10^{-3} M EDTA + 4×10^{-4} M DNP	3·55
	10^{-3} M EDTA + 4×10^{-3} M DNP	1·70
Expt. 2	none	1·36
	4×10^{-3} M DNP	1·22
	10^{-2} M $CaCl_2$	1·71
	10^{-2} M $CaCl_2$ + 4×10^{-3} M DNP	3·20

which ATPase activity is stimulated there is no interference with superprecipitation. The fact that both myosin and actomyosin ATPases are inhibited by the same concentrations as superprecipitation indicates that inhibition of superprecipitation is the result of ATPase inhibition and not of the interaction-inhibitor type described by Barany and Barany.[16]

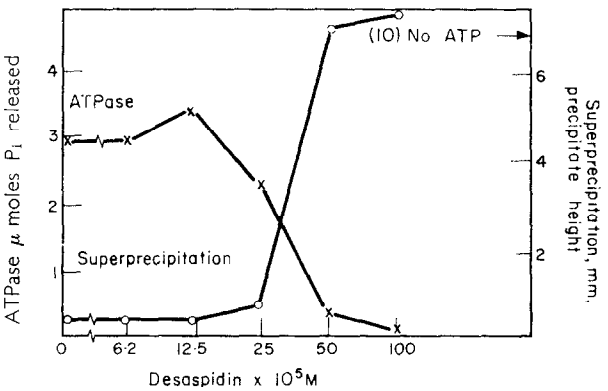

FIG. 7. The effect of desaspidin on acetomyosin ATPase and superprecipitation of actomyosin. Each sample contained 8 μmoles ATP, 20 μmoles tris buffer, pH 7·5, 100 μmoles KCl and 4 μmoles $MgCl_2$ in a final volume of 2 ml. Of the two duplicate samples one was incubated for 3·5 min at 25° for assay of ATPase activity under experimental conditions otherwise the same as in Fig. 1 for myosin ATPase. In the other sample superprecipitation was determined at room temperature.

The left-hand ordinate gives the ATPase activity as μmoles P_i released, the right-hand ordinate gives the superprecipitation as the height of the actomyosin precipitate after centrifugation. The abscissa gives the concentration of desaspidin × 10^5 M.

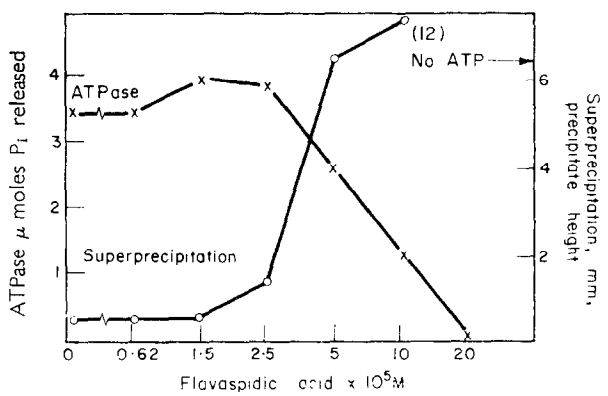

FIG. 8. The effect of flavaspidic acid on actomyosin ATPase and superprecipitation of actomyosin. Medium and experimental conditions as in Fig. 7.

DISCUSSION

The biphasic effect of phlorobutyrophenone derivatives on both mitochondrial[3] and myosin ATPases resembles that of DNP and it further emphasizes the similarities between these two ATPases with respect to their sensitivity to enzyme modifiers. On the other hand, it appears interesting that the effects of the phlorobutyrophenones

on the two systems are not dependent on exactly the same molecular configuration and that the introduction of a methoxy group into the compound alters the toxic effects on mitochondria and myosin in opposite directions. It must also be pointed out that the effect of phlorobutyrophenone derivatives on myosin ATPase differs from that of DNP with respect to its dependence on ionic conditions. This indicates the presence of differences in the modes of action of these substances.

As to the possible significance of these results for an understanding of the toxic and anthelmintic effects of the substances tested, the following points can be made.

The results presented confirm and supplement earlier reports on the inhibitory effect of flavaspidic acid on myosin ATPase.[1] It appears difficult to decide whether electron-transport-coupled phosphorylation in mitochondria or actomyosin ATPase is the more likely point of action of flavaspidic acid in the living cell, since the two preparations are affected at about the same concentrations. Desaspidin, on the other hand, is both a more potent anthelmintic and more toxic to mice than flavaspidic acid[1,5] and the fact that desaspidin is at the same time more toxic to mitochondria and less toxic to actomyosin apparently rules out the possibility that the increased toxicity of desaspidin as compared with flavaspidic acid could be due to an effect exerted via actomyosin.

The suggestion that uncoupling of electron-transport-coupled phosphorylation is an important mode of action of desaspidin, while flavaspidic acid has also other points of action is in good accord with the fact that desaspidin has a marked stimulating effect on both respiration in human amniotic cells and the glycolysis of the worm,[4] while the stimulating effect of flavaspidic acid on both respiration and glycolysis is weak (unpublished observation). The final evaluation of the effect of flavaspidic acid on actomyosin must also wait until data are available concerning the penetration of the substance into muscle cells.

REFERENCES

1. T. K. MARKKANEN, M. PULKKINEN and H. SALMI, *Ann. Med. Exp. Fenn.* **38**, 76 (1960).
2. L. RUNEBERG, *Biochem. Pharmacol.* **11**, 237 (1962).
3. L. RUNEBERG, *Biochem. biophys. Res. Commun.* 14, 58 (1963), *Soc. Scient. Fennica, Comm. Biol.* XXVI. 7 (1963).
4. L. RUNEBERG, *Ann. Med. Exp. Fenn.* **40**, 213 (1962).
5. W. F. H. M. MOMMAERTS, in *Methods in Medical Research VII* (Ed. J. V. WARREN), p. 3, The Year Book Publishers Inc., Chicago (1958).
6. L. ERNSTER, R. ZETTERSTRÖM and O. LINDBERG, *Acta chem. scand.* **4**, 942 (1950).
7. J. J. BLUM, *Arch. Biochem. Biophys.* **97**, 321 (1962).
8. G. D. GREVILLE and D. NEEDHAM, *Biochim. Biophys. Acta*, **16**, 284 (1955).
9. J. B. CHAPPELL and S. V. PERRY, *Biochim. Biophys. Acta*, **16**, 285 (1955).
10. D. GILMOUR and M. GRIFFITHS, *Arch. Biochem. Biophys.* **72**, 302 (1957).
11. H. M. LEVY, N. SHARON, E. M. RYAN and D. KOSHLAND, *Biochim. Biophys. Acta*, **56**, 118 (1962)
12. E. T. FRIESS, *Arch. Biochem. Biophys.* **51**, 17 (1954).
13. A. SZENT-GYÖRGYI, *Chemistry of Muscular Contraction*, 2nd ed., Academic Press, London (1951).
14. D. M. NEEDHAM, in *Structure and Function of Muscle II* (Ed. G. H. BOURNE), p. 55, Academic Press, London (1960).
15. G. ÖSTLING, *Nord. Med.* **67**, 443 (1962).
16. M. BARANY and K. BARANY, *Biochim. Biophys. Acta*, **41**, 204 (1961).