THE NATURE OF THE M BAND ENZYME IN RAT VENTRICULAR MUSCLE

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The types of cardiac cholinesterases (1) and the responses of the myocardium to acetylcholine (2, 3) differ in various species and at various sites in the heart. It has not been possible to explain the differing physiological responses in terms of the types of cholinesterases present (1–3). A comparative study of the histochemical localization at the ultrastructural level of cholinesterase activity in cardiac muscle, correlated with biochemical and physiological findings, might shed some light on the

functions of the cholinesterases of cardiac muscle.

Barrnett and Palade (4) applied the thiolacetic acid histochemical method to rat ventricular muscle. Non-specific esterases (5) and specific (acetyl) cholinesterase (6) are known to hydrolyse thiolacetic acid, and the latter may be distinguished by the use of specific inhibitors. Hydrolysis of thiolacetic acid, CH_3COSH, yields acetic acid and hydrogen sulfide: the latter is precipitated as lead sulfide, giving, at the sites of enzyme activity, an electron-opaque deposit.

In rat ventricular muscle, Barrnett and Palade (4) found the most striking activity in the M band. This activity was reported to be inhibited partially by 10^{-5} M eserine and almost completely by 10^{-5} M tetraethylpyrophosphate and by 10^{-4} M diisopropylfluorophosphate. They suggested that this enzyme was possibly a cholinesterase. No other site of cholinesterase activity in ventricular muscle was observed.

We have not been able to confirm these observations on the effects of inhibitors using a modification of the same technique. On the basis of our findings, we conclude that the M band enzyme is not a cholinesterase, but may be rather an organophosphate-resistant non-specific esterase.

MATERIALS AND METHODS

Barrnett and Palade (4) incubated small blocks of fresh tissue in the medium. We found this procedure to be unsatisfactory. As indicated by Barrnett and Palade, the reaction is confined solely to the surface of the block and is spotty in distribution. In our hands, the reaction is not consistently reproducible, rendering evaluation of the effects of inhibitors, and comparative studies, difficult and unreliable. The use of fresh-frozen sections overcame these difficulties. Morphological preservation was relatively good, and not worse than that obtained with tissue blocks.

Fresh frozen sections, 30 μ thick, were cut from a block of left ventricular muscle of the rat. The sections were washed briefly in 0.44 M sucrose, and then were transferred to the medium. The final concentrations of the constituents of the medium were: thiolacetic acid 0.03 M, sodium cacodylate 0.01 M, $MgCl_2$ 0.004 M, $MnCl_2$ 0.004 M, $CaCl_2$ 0.004 M, sucrose 0.44 M, lead nitrate 0.001 M. The pH was adjusted with NaOH before the lead nitrate was added. Incubations were run at pH levels varying from 5.0 to 6.5, most experiments being run at pH 6.0. The period of incubation varied from 10 to 25 minutes. At the end of the incubation the sections were washed three times in 0.44 M sucrose and once in *s*-collidine buffer. Fixation was carried out for 1 hour in 1.33 per cent osmium tetroxide buffered to pH 7.4 with *s*-collidine (7) containing 0.44 M sucrose. The sections were dehydrated and embedded in Vestopal-W by the method of Kurtz (8). Observations were made in a Zeiss electron microscope, EM-9, and photographs were taken at original magnifications of 6,000 to 20,000. Staining of sections with alkaline lead solutions (9) to enhance contrast was shown not to affect the deposits of lead sulfide.

Controls

In order to exclude non-enzymic deposition of lead, sections were incubated variously in lead nitrate alone, in lead nitrate followed by H_2S, and in thiolacetic acid followed by lead nitrate followed by H_2S. Washing between each successive step was done in 0.44 M sucrose. Sections were also preincubated in 0.44 M sucrose at 80°C for 10 minutes.

Inhibitors

Sections were routinely preincubated in aqueous solutions of the inhibitors containing 0.44 M sucrose, at pH 6.0, for 15 minutes. The inhibitors used, and their concentrations, are specified in the next section. Control sections were always incubated in 0.44 M sucrose at the same pH. After preincubation the sections were transferred to the medium containing, when possible, the inhibitor. In those instances where the inhibitor reacted with components of the medium, the sections were preincubated in the inhibitor, washed briefly, and then transferred to the medium from which the inhibitor was omitted. Freshly made solutions of inhibitors were always used. Their efficacy was routinely tested on suitable subjects such as motor end-plates, liver and kidney esterases, etc., using the thiolacetic acid method, as well as standard azo dye, indoxyl, and thiocholine methods (10).

Biochemistry

Homogenates of rat ventricular muscle, made as previously described (1), were tested for enzymatic activity using thiolacetic acid, acetyl- and butyrylcholine as substrates. For activity against thiolacetic acid, iodometric (6), and modified titrimetric and manometric techniques were used. For activity against the choline esters, the titrimetric and manometric methods used were those previously described (1).

RESULTS AND DISCUSSION

In contrast to the results obtained with the use of tissue blocks, good activity, evenly distributed throughout the sections, was routinely observed in the M bands of rat ventricular muscle. This activity was abolished by fixation in formaldehyde and other aldehydes, and by heat. The controls showed no non-specific deposits of lead sulfide. The

reaction was considered to be enzymatic in nature. There was no detectable inhibition by the irreversible inhibitors of cholinesterases, 10^{-4} M diisopropylfluorophosphate (DFP) (Figs. 1 and 2), 10^{-5} M diethyl-*p*-nitrophosphate (E-600), nor by the reversible inhibitor, 10^{-4} M or 10^{-3} M eserine sulphate. All these inhibitors were included in both the preincubation and incubation media. It is realised that it is difficult to evaluate the effects of inhibitors, which are active at fixed concentrations in soluble enzyme and homogenate preparations, on local sites of unknown enzyme concentration in intact tissues. The concentrations of inhibitors used here are generally acceptable in histochemical work (10). The concentration of eserine in particular is much higher than that effective in homogenates of heart muscle (1), and that with which Barrnett and Palade obtained partial inhibition. Further, these inhibitors all produced marked inhibition of the reactions in rat ventricular muscle obtained with acetyl or butyryl thiocholine and l-naphthol acetate as substrates.

Following the classification of esterases given by Pearse (10), the enzyme is neither a cholinesterase or an aliesterase (organophosphate-sensitive or type B non-specific esterase), both of which are sensitive to these concentrations of these inhibitors

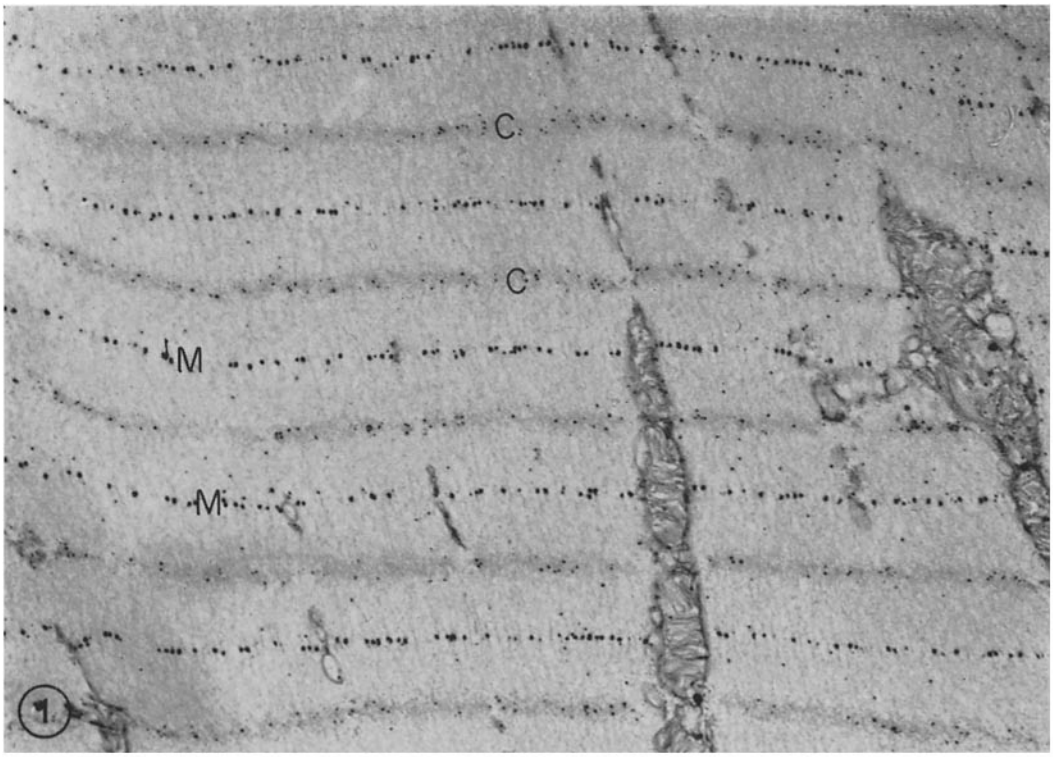
The organophosphate-resistant esterases (non-specific esterases, types A and C) may be further distinguished (with caution) in histochemical systems from each other and from type B esterases, by the use of suitable inhibitors and activators (10). 10^{-4} M *p*-chloromercuribenzoate (inhibits A, activates C) and 0.08 M sodium fluoride (inhibits B) had no clear cut effects on the M band activity. Their inhibitory effects are reversible either by thiols (thiolacetic acid!) or by washing. As they precipitated with the lead they could not be included in the incubation medium, and therefore the results are unreliable. 10^{-3} M β-phenylpropionic acid (inhibits C) did not inhibit M band activity, but it is similarly difficult to evaluate the effect of this competitive inhibitor in the histochemical system.

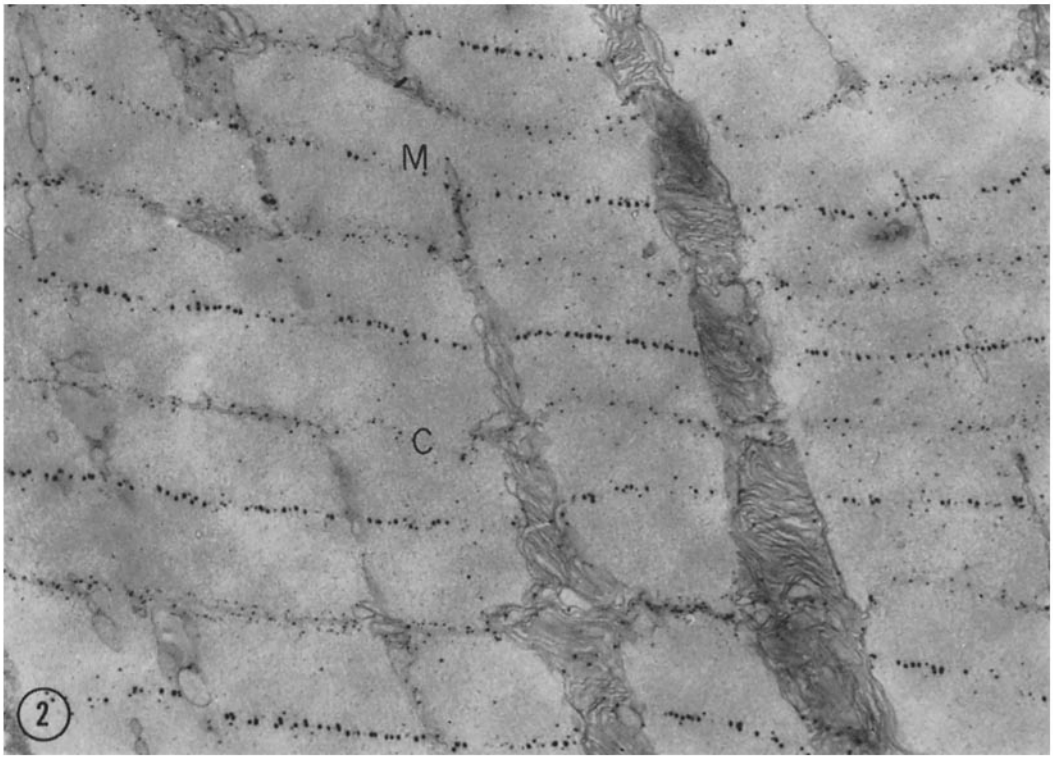
On the other hand, 5×10^{-3} M atoxyl (sodium arsanilate) inhibited completely, even though it was necessary to omit it from the incubation medium. Both atoxyl and sodium fluoride may be used to distinguish type B (organophosphate-sensitive) from organophosphate-resistant esterases (11), the former being more sensitive, but the differences are relative rather than qualitative (11). Atoxyl-inhibition is apparently less readily reversed by washing than is that of sodium fluoride (11).

Both atoxyl and cacodylate are pentavalent arsenicals, and we found that preincubation in 0.05 M cacodylate, pH 6.0, also abolishes the M band reaction. Barrnett and Palade found that the use of cacodylate buffer stabilizes the medium, preventing the formation and precipitation of lead thiolacetate. Pentavalent arsenicals react with thiols (including thiolacetic acid) to form trivalent arsenicals, and in the presence of excess thiol, arylthioarsenites (12). Whether or not cacodylate reacts similarly, the possible inhibitory effects of arsenicals on esterases (13) should be kept in mind when applying the thiolacetic acid–cacodylate medium to sites of low activity. In our experiments the concentration of cacodylate was therefore kept as low as that of Barrnett and Palade (0.01 M). Substitutions of other buffers were not satisfactory, although the medium made with 0.05 M Tris-HCl was fairly stable. The reactions and effects of inhibitors using the Tris-buffered medium were identical with those obtained with the cacodylate medium, and no additional sites of activity were detected.

Biochemical estimation of activity of homogenates against thiolacetic acid revealed no measurable activity. The same preparations showed high activity against acetylcholine. Fixed and unfixed frozen sections showed good activities against l-naphthol acetate, acetyl- and butyrylthiocholine, with marked or complete inhibition by low concentrations of DFP and eserine, yet the same material in the thiolacetic acid method showed no activity attributable to cholinesterase. Rat ventricular cholinesterase is firmly bound to tissue components (14), and loss into the washes or medium is not considered significant. It is unlikely that the freezing and thawing involved in the preparation of sections rendered the enzyme insensitive to specific inhibitors; repeated freezing and thawing of homogenates has no such effect.

One can but conclude that thiolacetic acid is a poor substrate for rat ventricular cholinesterase, which has been shown to be largely of the non-specific (butyryl) type (1, 15). It is of interest that in the pancreas, which has a high activity of butyryl cholinesterase, Wachstein (5), using the thiolacetic acid method, did not find activity attributable to cholinesterase. The question as to whether thiolacetic acid is hydrolysed only by some types of cholinesterase requires further





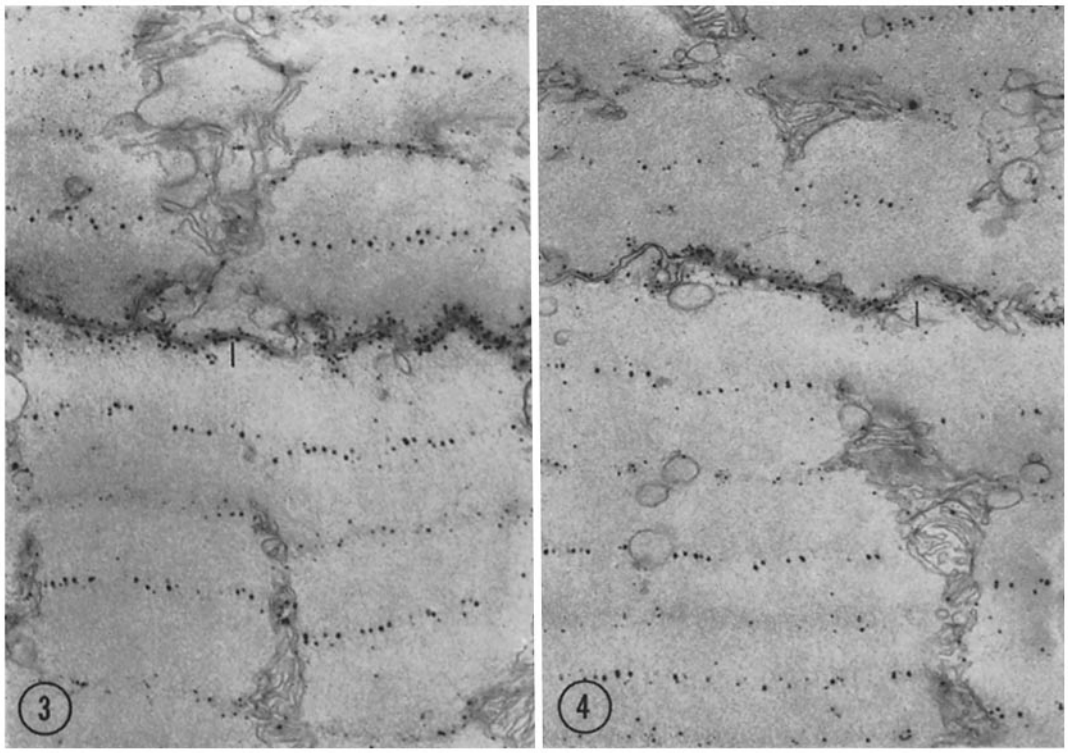


FIGURE 3 Control. Marked reaction in region of intercalated disc.

FIGURE 4 DFP, 10^{-4} M. No inhibition of reaction at intercalated disc.

investigation, for it is known that some acetylcholinesterases split thiolacetic acid (4, 6).

Deposits of lead were also seen at the level of the Z band, in the mitochondria, at the intercalated discs, (Fig. 3), and in elements of the endoplasmic reticulum. The reaction in the latter site, as with Barrnett and Palade (4), was too slight for the effects of inhibitors to be assessed with confidence. No evidence was obtained to suggest that the reactions at the other sites were due to cholinesterases (Fig. 4). In agreement with Barrnett and Palade (4), the mitochondrial reaction was insensitive to cholinesterase inhibitors. Cholinesterase activity at the intercalated disc has been claimed, with thiolacetic acid as substrate, but only after the tissues were preincubated in hyaluronidase. This activity was sensitive to 10^{-3} M eserine, but insensitive to 5×10^{-5} M DFP (16). Hyaluronidase pretreatment did not affect our results. The reaction at the Z band may be non-enzymatic: it persists and is augmented after heating the tissues.

All illustrations are of rat ventricular muscle. The controls were preincubated in 0.44 M sucrose for 15 minutes, and the test sections in DFP 10^{-4} M for 15 minutes. Sections were then incubated in the thiolacetic acid medium, pH 6.0, for 15 minutes, with and without DFP 10^{-4} M present. All magnifications 24,000. *M*, M band; *C*, contraction band, superimposed on Z band; *I*, intercalated disc.

FIGURE 1 Control. Marked deposition of lead in the M band. Small, sparser deposits in the Z (contraction) band.

FIGURE 2 DFP, 10^{-4} M. No inhibition of M band reaction.

Our observations suggest that the so called M band enzyme of rat ventricular muscle is an organophosphate-resistant esterase, and not, as previously has been suggested (4), a cholinesterase. Its role in cardiac metabolism and physiology, and the reasons for its precise localization at the level of the M band, are at present a mystery. It may well be involved, as Barrnett and Palade point out for esterases hydrolysing thiolacetic acid, in acyl transfer reactions. However, the inhibition by atoxyl and by formalin are unusual for organophosphate-resistant esterases (11). It is possible that the M band reaction is, in fact, non-enzymatic in nature, and simply delineates a site which is readily acetylated by thiolacetic acid, a site which is destroyed or blocked by heat, formalin, and atoxyl. Further investigation is necessary. The true localization of cholinesterase in cardiac muscle remains to be established.

Note added in press: Since this paper was submitted for publication, we have shown, by means of a modified thiocholine technique suitable for electron microscopy, that cholinesterases of rat ventricular muscle are localized in elements of the sarcoplasmic reticulum, and possibly in the A band as well. In confirmation of this paper, no activity was detected in the M band. Details will be published at a later date.

This investigation was supported by grants from the Emil Barell Foundation, from Le Fonds National Suisse pour la Recherche Scientifique (No. 2364), by grant A 5431 and training grant 2G-113 from the National Institutes of Health, United States Public Health Service.

The senior author expresses his appreciation to Professor Ch. Rouiller and Professor J. M. Posternak for the hospitality of their laboratories, and to Dr. Fritz Baumann and Dr. J. R. Scherrer for help with the biochemical techniques.

Received for publication, April 4, 1963.

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