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Monoamine oxidase activities in dissociated cell fractions from rat skeletal muscle

GEOFFREY A. LYLES* AND DAVID R. ARCHER

Department of Pharmacology and Clinical Pharmacology, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK

Collagenase was used to dissociate rat skeletal muscle (gastrocnemius) into its constituent cells, from which a myocyte fraction enriched in striated muscle cells, and a non-myocyte fraction containing cells of connective tissue and vascular origin, were prepared. The activities of amine oxidase enzymes and alkaline phosphatase (AP) were then assayed in these fractions, and also in homogenates prepared from corresponding samples of non-dissociated tissue. The specific activities of the semicarbazide-sensitive amine oxidase (SSAO) and AP were considerably higher (30 to 35-fold) in non-myocyte than in myocyte fractions. AP is generally considered to be present predominantly in vascular cells of skeletal muscle, with little, if any, in skeletal muscle cells themselves. Thus, the results obtained may indicate a similar localization for SSAO activity. Support for this came from histochemical studies, which showed staining for SSAO primarily over the walls of larger blood vessels in the muscle. Unlike SSAO and AP, were marked differences in MAO-A activity between myocyte and non-myocyte fractions were not found, suggesting that MAO-A is more probably a constituent of cells within both fractions.

The effects of inhibitor drugs upon the in-vitro and in-vivo deamination of various monoamines have provided considerable evidence for the existence of different amine oxidase enzymes in animal tissues. For instance, these studies have revealed that the outer mitochondrial membrane enzyme monoamine oxidase (MAO) exists in two distinct forms called MAO-A and MAO-B, which differ not only in their substrate specificities, but in particular with regard to their relative sensitivities to inhibition by the acetylenic drugs clorgyline and selegiline (deprenyl). These properties and the therapeutic implications for exploiting these differences with selective inhibitors have been reviewed recently by Fowler & Ross (1984).

In addition to these MAO activities, a semicarbazide-sensitive amine oxidase (SSAO) has been described in an increasing number of organs, with particularly high enzyme activity being found in blood vessels (Lewinsohn et al 1978). Its substrates in the rat include 2-phenylethylamine, tyramine, tryptamine and dopamine, but the most actively metabolized, with a low K_m value (around 5 μM) is the synthetic monoamine, benzylamine (Clarke et al 1982). This enzyme is insensitive to the acetylenic MAO inhibitors, but conversely is inhibited by semicarbazide, as well as by other hydrazine derivatives (reviewed by Lyles 1984). Biochemical and histochemical studies of the properties and localization of SSAO have supported the notion that vascular smooth muscle cells are an important source of the SSAO activity found in blood vessels of man and the rat (Lewinsohn 1981, 1984; Ryder et al 1979; Lyles & Singh 1985), although the physiological importance of the enzyme remains unclear.

In our previous investigations into the localization of SSAO activity, the proteolytic enzymes collagenase and elastase have been used to dissociate rat aorta (Lyles & Singh 1985) and rat cardiac ventricular tissue (Lyles et al 1984) into isolated cell fractions, to compare the resultant activities of amine oxidase enzymes between those fractions enriched with different cell types. In the heart, considerably higher specific activity of SSAO was found in non-myocyte fractions (containing connective tissue cells, endothelial and smooth muscle cells) compared with fractions enriched with striated cardiomyocytes. Similar results with alkaline phosphatase, used as an enzyme marker for cells of the coronary vasculature, were consistent with the interpretation that SSAO is associated, at least in part, with blood vessels of this organ, with little, if any, associated with the striated muscle cells.

In this paper, we report the results of similar comparisons between myocyte and non-myocyte fractions prepared from skeletal muscle, another tissue which like the heart contains striated muscle cells interspersed with a dense microcirculatory

* Correspondence.

network. These data, along with the histochemical findings presented here, also indicate that the vasculature, and in particular the smooth muscle of larger blood vessels, is an important source of SSAO activity in the tissue. In contrast to the situation with SSAO, the absence of marked differences in the MAO-A activities between myocyte and non-myocyte fractions, suggests a more heterogeneous distribution of MAO-A activity among the cellular constituents of skeletal muscle.

Preliminary results of these studies have been communicated previously (Archer & Lyles 1984).

MATERIALS AND METHODS

Materials

[Methylene-^{14}C]benzylamine hydrochloride and [G-^{3}H]5-hydroxytryptamine (5-HT) creatinine sulphate were obtained from Amersham International PLC (Amersham, UK).

The following reagents were purchased from Sigma London (Poole, UK): collagenase (type 1A), horseradish peroxidase (type II), bovine serum albumin (Fraction V), diethanolamine, *p*-nitrophenol, *p*-nitrophenyl phosphate (diTris salt), semicarbazide hydrochloride, hydroxylamine hydrochloride, benzylamine hydrochloride and 3-amino-9-ethylcarbazole. Clorgyline hydrochloride (M & B 9302) was a gift from May and Baker Ltd (Dagenham, UK).

Female Wistar rats (250–400 g) were supplied from our Departmental breeding colony, Animal Services Unit, University of Dundee.

Methods

Preparation of cell fractions and homogenates. These techniques were modified from those previously used to dissociate cardiac ventricular tissue into various cell fractions (Lyles et al 1984). Animals were killed by stunning followed by cervical dislocation. The medial head of the gastrocnemius muscle was dissected and pooled from the two legs of each animal, and then washed in isolation buffer (g litre^{-1}: NaCl 6·8, KCl 0·4, glucose 0·9, Na_2HPO_4 0·21, NaH_2PO_4. H_2O 0·06, pH 7·4). The muscles were finely sliced longitudinally with a scalpel blade. Most was then used for dissociation into cell fractions, but a small sample (approx. 0·2 g) was stored intact at −20 °C for eventual homogenization as undissociated tissue.

For preparation of cell fractions, tissue was incubated with shaking at 37 °C in 3·5 ml isolation buffer containing 1 mg ml^{-1} collagenase. After 30–40 min, 6·5 ml isolation buffer was added and the tissue suspension was passed through a nylon filter (mesh size approx. 0·2 mm). This initial filtrate contained a considerable proportion of damaged cells and smaller tissue debris dissociated from the original cut surfaces of the muscle slices, and was consequently discarded. The undissociated tissue retained on the mesh was resuspended in a further 3·5 ml collagenase-buffer and incubation was continued for another 2–3 h before addition of 6·5 ml buffer and filtration as above. During this period, a large amount of the intact tissue was dissociated by the collagenase treatment, and examination of the filtrate by light microscopy revealed the presence of long sections of striated muscle cells (hereafter called myocytes). Occasionally some myocytes retained an adherent small section of intact capillary. Other constituents of the filtrate included short sections of blood vessels, as well as single cells of connective tissue and vascular origin. These included fibroblasts as well as endothelial and smooth muscle cells.

In order to separate a myocyte from a non-myocyte fraction, the filtrate was centrifuged at 3*g* for 5 min. The supernatant was removed and used as a source for isolating the non-myocyte fraction (see below), whereas the myocyte-enriched pellet was resuspended in 2 ml isolation buffer and layered carefully onto a cushion of isolation buffer containing 4% (w/v) bovine serum albumin (BSA). The myocytes were further enriched by allowing them to sediment under gravity through this BSA solution. The resulting myocyte pellet was resuspended in 4 ml 1 mM potassium phosphate buffer pH 7·8, centrifuged at 800*g* for 10 min, and then this resuspension/centrifugation step was repeated. This procedure removes contaminating BSA from the fraction, which would interfere with protein assays in the eventual cell homogenates. The final myocyte pellet was resuspended and stored at −20 °C in 2 ml 1 mM potassium phosphate, pH 7·8 (the eventual homogenization buffer).

The non-myocyte fraction was isolated from the supernatant resulting from the first centrifugation of the filtrate (see above). This supernatant was recentrifuged at 3*g* for 10 min to remove any residual myocytes, and the new supernatant was then removed and centrifuged at 800*g* for 10 min. The cell pellet was resuspended in 2 ml isolation buffer and layered on 8 ml BSA-containing buffer as above, and centrifuged at 50*g* for 10 min. The resulting pellet, confirmed by light microscopy to consist predominantly of non-myocyte cells, was resuspended in 4 ml 1 mM potassium phosphate buffer pH 7·8, and finally washed, centrifuged and stored as described above for the myocyte pellet.

Thawed cell suspensions and undissociated tissue samples were homogenized in 1 mM potassium phosphate buffer, pH 7·8 using a ground glass hand-held homogenizer (tissue (g) : buffer (ml) ratio of 1 : 20 for the undissociated tissue samples) and the homogenized suspensions were decanted from undisrupted particulate matter to provide the enzyme sources for assays.

Enzyme assays. Amine oxidase activities were assayed by the method of Callingham & Laverty (1973) as described fully by Lyles & Callingham (1982), using 1 mM [^{3}H]5-HT (sp. act. 2 μCi μmol^{-1}) or 1 μM [^{14}C]benzylamine (sp. act. 10 μCi μmol^{-1}) as substrates for MAO-A and SSAO, respectively. Confirmation of the specificity of these substrates for the enzymes is shown in Results. For inhibitor studies, appropriate aqueous solutions of clorgyline were preincubated with homogenate samples for 20 min at 37 °C before addition of radioactive substrate. Assay times were 20 min (benzylamine) and 60 min (5-HT), chosen after preliminary studies to ensure linear metabolite production with time.

Alkaline phosphatase activities of homogenates were assayed as previously described (Lyles et al 1984) on the same day as the corresponding amine oxidase assays.

Protein concentrations of homogenates were estimated by the method of Lowry et al (1951) with bovine serum albumin as standard.

Statistical significance was tested by the non-parametric Wilcoxon rank-sum method (two-tailed).

Histochemistry. Cryostat-cut sections of rat gastrocnemius muscle (20 μm) were stained for SSAO activity by the coupled peroxidatic oxidation method described by Ryder et al (1979), as modified and reported in detail by Lyles & Singh (1985).

RESULTS

Inhibition of 5-HT and benzylamine deamination by clorgyline in gastrocnemius muscle homogenates

In preliminary studies, homogenates prepared from undissociated gastrocnemius samples were preincubated with increasing concentrations of clorgyline (10^{-10} to 10^{-3} M) and then the remaining deaminating activity towards 1 mM 5-HT or 1 μM benzylamine was measured. Inhibition plots from these data (Fig. 1) showed that 5-HT metabolism was completely inhibited by 10^{-7} M clorgyline, whereas clorgyline concentrations up to 10^{-3} M had no significant inhibitory effect on benzylamine metabolism. These results indicate that at the substrate concentrations used, 5-HT and benzylamine are metabolized predominantly, if not exclusively, by MAO-A and SSAO, respectively.

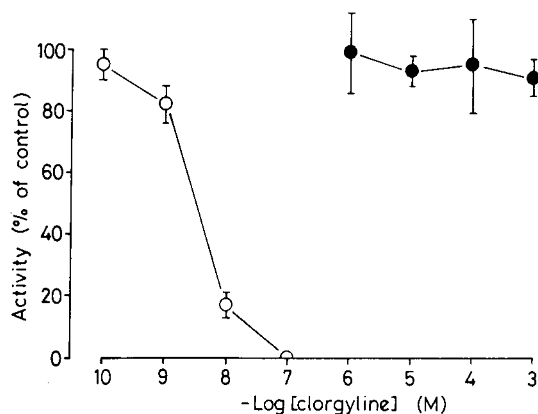

FIG. 1. Inhibitory effects of clorgyline on the metabolism of 1 mM 5-HT (○) and 1 μM benzylamine (●) by homogenates of rat gastrocnemius muscle. Each point represents the mean ± s.e. of the ratio (shown when exceeding symbol size) for triplicate determinations on separate homogenates from 3 rats.

In this series of experiments, attempts were also made to study metabolism of 1 mM [^{14}C]benzylamine (sp. act. 1 μCi μmol^{-1}) by muscle homogenates. However, even after prolonged incubation periods (1 h), metabolite production barely exceeded blank values and was insufficient for accurate and reliable determination of 1 mM benzylamine deamination, or its sensitivity to inhibition by clorgyline. Consequently, no further study with this benzylamine concentration was carried out.

Enzyme activities in homogenates of cell fractions dissociated from gastrocnemius muscle

The specific activities of MAO-A, SSAO and alkaline phosphatase were assayed in homogenates of the corresponding myocyte and non-myocyte fractions prepared from gastrocnemius muscle of individual rats. For comparison, these assays were concurrently performed on homogenates prepared from corresponding undissociated muscle samples. These results are summarized in Table 1.

5-HT metabolism in non-myocytes was significantly (approx. 2-fold) greater than that in myocytes, which in turn was similar, although slightly higher than that in undissociated tissue homogenates. During the experiments, samples of these homogenates were also routinely assayed after preincubation with 10^{-6} M clorgyline. Complete inhibition was obtained in each case, confirming that 5-HT metabolism can

Table 1. Amine oxidase and alkaline phosphatase activities of homogenates of dissociated cell fractions and undissociated tissue from rat gastrocnemius muscle. MAO-A was assayed with 1 mM 5-HT and SSAO with 1 μM benzylamine. Enzyme activities, assayed in triplicate, are mean values ± s.e.m. of corresponding individual samples obtained from either 5 (for MAO-A) or 7 rats (for SSAO and alkaline phosphatase). Statistical comparisons between non-myocyte and myocyte fractions. $^{*}P < 0{\cdot}05$, $^{**}P < 0{\cdot}001$.

Enzyme activity (nmol h^{-1} (mg prot)$^{-1}$)	Myocyte	Non-myocyte	Undissociated tissue
MAO-A	16·50 ± 2·7*	36·5 ± 8·5	12·90 ± 2·5
SSAO (no clorgyline present)	0·11 ± 0·04**	3·8 ± 1·1	0·26 ± 0·06
SSAO (10^{-3}M clorgyline present)	0·12 ± 0·03**	4·5 ± 1·3	0·24 ± 0·07
Alkaline phosphatase (μmol h^{-1} (mg prot.)$^{-1}$)	0·24 ± 0·04**	7·1 ± 1·7	1·50 ± 0·29

be ascribed to MAO-A activity in all homogenates assayed.

Benzylamine (1 μM) metabolism was also assayed with and without preincubation in the presence of 10^{-3} M clorgyline. The lack of any significant inhibitory effect of clorgyline upon the specific activity measured, again justifies the conclusion that SSAO activity was being assessed. The results in Table 1 indicated a striking difference in the specific activity of SSAO between the myocyte and non-myocyte fractions, with the latter being approximately 35-fold greater than the former. An intermediate activity of SSAO was found in undissociated tissue homogenates, although it was much closer to that of the myocyte fractions.

The activity of alkaline phosphatase showed a similar relative distribution to that of SSAO, being approximately 30-fold greater in non-myocyte than in myocyte fractions.

Histochemistry

Sections of rat gastrocnemius muscle were stained for SSAO activity with 50 μM benzylamine as substrate in the incubation medium. Fig. 2 shows a typical photomicrograph in which strong staining is found over the walls of the large blood vessels lying within the connective tissue matrix of the perimysium. The surrounding striated muscle tissue was unstained, suggesting that if any SSAO activity is associated with skeletal muscle cells or their adjacent capillaries, it is undetectable under these staining conditions, and must be considerably lower, if present, than the activity found associated with the larger blood vessels in the tissue.

In other tissue sections (not shown), the histochemical staining of these blood vessels in the skeletal muscle was not observed if the substrate benzylamine was omitted from the incubation medium, and was also prevented by treatment of tissue sections with the SSAO inhibitors hydroxylamine (1 μM) or semicarbazide (1 mM). In contrast, staining was not prevented by treatment with the MAO inhibitor clorgyline (1 mM).

FIG. 2. Photomicrograph showing a cryostat-cut section of rat gastrocnemius muscle stained for SSAO activity. Staining is found in the walls of the blood vessels, appearing here in transverse section within the connective tissue of the perimysium which runs from middle left to right. No significant staining of surrounding striated muscle tissue was found (Mag. 80×).

DISCUSSION

The present results, comparing the activities of MAO-A, SSAO and alkaline phosphatase (AP) in cell fractions from rat skeletal muscle, were similar to our previous studies with the rat heart, another tissue containing a large striated muscle content. For example, comparative assays of SSAO activity revealed a markedly higher specific enzyme activity associated with non-myocyte than with myocyte fractions. This 35-fold difference observed in the current results with skeletal muscle was more striking than the approximately 6-fold difference seen previously in the heart (Lyles et al 1984) and suggests even more strongly that SSAO may be predominantly associated with some cell type(s) enriched in the non-myocyte fraction. Further support for this supposition comes from the similarly large ratio of AP-specific activities between the corresponding fractions. The rationale for including AP assays in these studies was based on previous evidence, mainly histochemical, that AP is a useful marker enzyme for the vasculature within heart and skeletal muscle, with little if any enzyme activity being present on the striated muscle cells (Romanul & Bannister 1962; Beckett & Bourne 1972). Although the latter reports have localized AP activity mainly to the endothelium of the microvasculature within these tissues, evidence for the association of AP with vascular smooth muscle and fibroblasts has also been described (Gardner & Laing 1965; Twietmeyer et al

1979). Thus, the much higher specific activity of AP present in our non-myocyte fractions which contained predominantly those cells of vascular and connective tissue origin, is consistent with these histochemical conclusions of other workers. It was impossible to isolate the myocyte fraction without some contamination with larger segments of blood vessel which co-sedimented with the myocytes. In addition, some myocytes occasionally retained an adherent strip of capillary. Thus, the smaller activity of AP (and SSAO) found in the myocyte fractions may be a consequence of this vascular contamination, or may reflect enzyme activity which is truly associated with the striated muscle itself.

Further support for the blood vessels providing at least one major source of the SSAO activity within skeletal muscle homogenates was obtained in the histochemical results described here. We have previously used this technique to demonstrate the association of SSAO with vascular smooth muscle in the rat aorta (Lyles & Singh 1985), and in the present study, staining again occurred predominantly and most intensively over the walls of larger blood vessels within the gastrocnemius sections. No evidence for significant staining of striated muscle cells or their interspersed capillary network was obtained, although it is possible that any weaker enzymatic activity at these sites may not have been detected under the conditions employed. It would thus appear likely that the vascular smooth muscle cells would therefore have made an important contribution to the SSAO activity found in the non-myocyte fractions assayed here.

Although the specific activity of MAO-A was significantly higher in non-myocyte than myocyte fractions, the ratio of activities in these respective fractions was considerably smaller than that obtained with SSAO and AP. Thus, it seems more likely that MAO-A is present in both striated muscle cells as well as in some, or all, of those cells comprising the non-myocyte fraction from skeletal muscle. This accords with our earlier conclusions obtained after collagenase-dissociation of rat cardiac tissue (Lyles et al 1984).

Others have previously reported that the predominant MAO activity associated with rat skeletal muscle is the A-form of the enzyme, while MAO-B activity (towards benzylamine) was either very low or undetectable by radiochemical assay in several skeletal muscles examined, including gastrocnemius (Meltzer & Arora 1979). In the adult rat heart, where MAO-A is also the predominant activity, a significant contribution of MAO-A to metabolism of 1 mM benzylamine is found, despite an unfavourably lower rate constant for turnover of benzylamine by MAO-A compared with MAO-B (Lyles & Callingham 1979; Parkinson et al 1980). In contrast, it would seem that the MAO-A content of gastrocnemius muscle, and the apparent absence of MAO-B, were such that no measureable metabolism of 1 mM benzylamine above blank values occurred in our experiments. In addition, the high substrate inhibition of SSAO which is encountered at benzylamine concentrations far above its K_m (approx. 5 μM) would also considerably counteract a contribution by this enzyme (Lyles & Callingham 1975; Clarke et al 1982).

Despite the apparent absence of MAO-B in many rat skeletal muscles, some detectable activity towards benzylamine of this enzyme form has been reported for thigh and intercostal muscle, although MAO-A activity still remains the major form present (Arora & Meltzer 1977a; Meltzer & Arora 1979; Kwatra & Sourkes 1980). Our preliminary studies using benzylamine with homogenates from these particular muscles are in agreement with these findings (unpublished results). It is of interest that in contrast to the rat, human skeletal muscle (pectoral and peroneus brevis) contains predominantly MAO-B, and this activity has been reported to be significantly decreased in muscle biopsies from schizophrenic patients compared with controls (Arora & Meltzer 1977b; Meltzer & Arora 1980).

In conclusion, these experiments have provided evidence for some relative differences in the cellular distribution of the MAO-A and SSAO activities which are found in rat gastrocnemius muscle. In particular, the considerably greater specific activity of SSAO found in non-myocyte fractions, considered in conjunction with the histochemical evidence presented here, suggests strongly that the vascularity, and especially its smooth muscle, contributes greatly to the SSAO activity which can be measured in this and probably various other tissue homogenates. However, at present the significance of a vascular localization of SSAO remains unclear and the possible consequences of specific inhibition of this enzyme by various agents requires further investigation.

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