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CHOLINE ACETYLTRANSFERASE ACTIVITY IN THE SYMPATHETIC NERVES OF THE RABBIT EAR ARTERY

VERENA M. FLORENCE, W.R. HUME & MARSHA L. MATSUNAGA

Department of Pharmacology, School of Medicine and Section of Oral Biology, School of Dentistry, University of California, Los Angeles, U.S.A.

1 No statistically significant difference in the activity of choline acetyltransferase (ChAT) was detected between sympathetically denervated and control rabbit ear artery (REA) tissue. This was interpreted as evidence against the hypothesis that endogenous acetylcholine plays an obligatory role in sympathetic postganglionic neurotransmission.

2 The values obtained for ChAT activity in the extraneuronal REA tissue were very low, but were greater than the boiled blank values.

3 Treatment with a specific inhibitor of ChAT did not reduce the REA values, while it did for rabbit iris. Addition of acetylcholinesterase to the REA assay reduced the activity of the collectable product to a markedly lesser degree than was observed with other tissues.

4 The specificity of the enzyme assay at the very low yield levels observed in the extraneuronal REA tissue was therefore questioned.

Introduction

The hypothesis that acetylcholine (ACh) plays a role in sympathetic postganglionic neurotransmission (Burn & Rand, 1962; Burn, 1971) led to many equivocal pharmacological studies in sympathetically innervated tissues, and the matter has yet to be resolved. In his early review of the evidence related to the hypothesis Ferry (1966) emphasized that the demonstration of choline acetyltransferase (ChAT) activity would be necessary to prove the existence of an endogenous cholinergic mechanism in sympathetic fibres.

The rabbit ear artery (REA) is densely innervated by sympathetic postganglionic axons only (Hume & Waterson, 1978). If endogenous ACh were part of the neurotransmission process in the REA, it would require local synthesis in the nerve terminal rather than delivery by axoplasmic flow, since the adrenergic neurotransmission system in the REA remains functional *in vitro* for many hours after removal from the host animal and therefore from the nerve cell bodies (de la Lande & Rand, 1965; de la Lande, 1975). For these reasons the nerve tissue of the REA is well suited for the study of the hypothesis.

We have examined the sympathetic nerves of the REA for the presence of ChAT by comparing sympathetically-denervated with control arteries. Using both a sensitive assay and a new inhibitor for the enzyme, we have shown that the vessel's nerves have no detectable capacity to synthesize ACh.

Methods

Eleven New Zealand White rabbits of either sex were prepared by unilateral excision of a superior cervical ganglion as described by de la Lande & Rand (1965). Intramuscular ketamine 20 mg/kg and xylazine 4 mg/kg were used as anaesthetic agents for the surgical procedure. The rabbits were killed by stunning and exsanguination 21 days after denervation. The proximal 30 mm of the left and right central ear arteries, the left and right irides, and a segment of ileum were rapidly removed and dissected over ice in Krebs bicarbonate solution.

To confirm denervation, segments of artery distal to the assayed tissue were treated for catecholamine fluorescence by the technique of Lindvall & Bjorklund (1974).

The composition of the Krebs solution, and the subsequent assay for ChAT using a technique based on that of Fonnum (1975), were as described by Florence & Bevan (1979). Briefly, the 60 μl incubation mixture consisted of 1.25–1.75 mg homogenized tissue; [1-^{14}C]-acetyl-coenzyme A (58.0 mCi/mm, New England Nuclear, Boston, MA) diluted to 0.2 mM with unlabelled acetyl-coenzyme A (Sigma Chemical Co., St. Louis, Mo), NaCl 300 mM, sodium phosphate buffer 50 mM (pH 7.4), choline chloride 8 mM, disodium edetate (EDTA) 10 mM and physostigmine sulphate 0.1 mM. After 15 min incu-

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Table 1 Mean choline acetyltransferase activity of rabbit tissues (nmol ACh g^{-1} wet wt. h^{-1})

Tissue	*Control*	*Denervated*
Iris	1166.8 ± 99.9 (8)	1153.3 ± 87.4 (8)
Ear artery	31.8 ± 5.5 (8)	33.0 ± 5.2 (8)
Ileum	326.4 ± 10.1 (5)	

Values are mean ± s.e.mean. Figures in parentheses are numbers of animals.

bation at 37°C the reaction was stopped with 5 ml of 10 mM sodium phosphate buffer (pH 7.4) and 1 ml of 3-heptanone containing 15 mg sodium tetraphenyl boron (Sigma Chemical Co.). The ^{14}C-acetylated product was counted in Bray's solution (Bray, 1960) with a Beckman LS-1000 scintillation counter at an efficiency of 80%.

In addition, tissues from 3 animals were treated with a ChAT inhibitor, (2-benzoylethyl) trimethylammonium chloride (BETA) (gift from Dr D.J. Jenden) in the concentration range of 10^{-7}M to 10^{-3}M during the assay procedure. Tissues from 2 other animals were treated with 5 units of acetylcholinesterase (AChE) (*Electropherus electricus* 3.1.1.7) in the absence of physostigmine. Boiled tissue assays were also carried out.

The ChAT activity of each sample was measured in duplicates. All values were corrected for tissue weight and for tissue-free blank background counts. Comparisons between ChAT activities of denervated and control tissues were made by the *t* test for paired observations (Dixon & Massey, 1969).

Results

Histochemical data

Histochemical examination of REA segments distal to the assayed tissue showed substantial reduction in fluorescence on the experimental side in 8 of the 11 animals, indicating success of the denervation procedure. The remaining 3 animals, where denervation was less certain, were not included in the subsequent analysis.

Comparison of denervated versus control tissue

In Table 1, the mean values expressed as ACh nmol g^{-1} wet wt. h^{-1} ± s.e. are listed for the three tissues studied. The iris and ileum were included to standardize the assay with tissues known to be cholinergically innervated. Enzymatic activity for the denervated REA segments was 33.0 + 5.2 and for the control REA 31.8 + 5.5. The values for the irides were 1153 ± 87.4 and 1166 ± 99.9 for the denervated and control side tissues, respectively. No significant difference could be detected between the two states for either tissue, as determined by the paired *t* test.

Effects of acetylcholinesterase and tissue boiling on ^{14}C-product formation

By treating the incubations with AChE in the absence of physostigmine, the product formation for the iris and ileum was substantially decreased (Table 2). However, the reduction of ^{14}C-product in REA was only 32–38%. Again, no difference was detectable between the innervated and control tissues.

Boiling the tissues before incubation virtually eliminated any counts above background with all three tissue types (Table 2).

Table 2 Percentage decrease of acetylated product formed in tissues boiled before, or treated with acetylcholinesterase (AChE) during, choline acetyltransferase assay

Tissue	*AChE*	*Boiled*
Iris-control	97.6	99.9
Iris-denervated	98.4	
Ileum	94.5	99.4
Ear artery-control	32.0	99.2
Ear artery-denervated	38.2	

Values are results of experiments on tissues from two animals.

Effects of (2-benzoylethyl) trimethylammonium chloride on rabbit ear artery and iris

The results plotted as percentage of control versus BETA concentration are shown in Figure 1. BETA was effective in decreasing ChAT activity of the iris in the concentration range of 10^{-7} to 10^{-3}M. The corresponding concentrations had no consistent effect on decreasing enzymatic activity in REA.

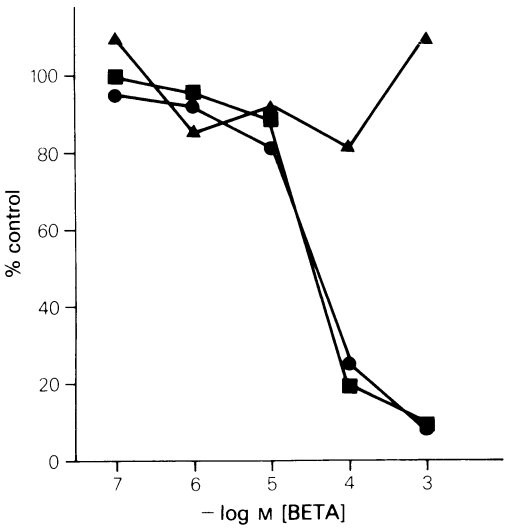

Figure 1 Choline acetyltransferase (ChAT) activity of samples of rabbit iris and ear artery (REA) in the presence of (2-benzoylethyl) trimethylammonium chloride (BETA), expressed on the mean percentage of three determinations compared with BETA-free control. Symbols: (▲) REA; (●) iris; (■) iris:REA, 1:1.

To test the possibility that the REA contained either an inhibitor of ChAT activity or an antagonist to BETA, mixed homogenates of equal parts of REA and iris were prepared. They were assayed in the presence of a range of concentrations of BETA. The resulting inhibition curve was identical to that of iris alone (Figure 1), indicating that the REA had neither of these characteristics.

Discussion

There is substantial evidence from other studies for the modulation of sympathetic neurotransmitter release by exogenous ACh in the REA and other tissues (see Vanhoutte, 1974). The presence of AChE related to the sympathetic postganglionic nerves of the REA has been demonstrated histochemically (Waterson, Hume & de la Lande, 1970; Hume & Waterson, 1978). Yet the present work showed that there was no capacity for endogenous ACh production by the nerve fibres of the REA. The lack of significant difference between the assay counts from denervated versus control REA tissue demonstrated that the capacity of the REA to synthesize the small amount of collectable, acetylated product was due to the extraneuronal tissue. The work therefore failed to support the hypothesis that endogenous ACh plays an obligatory role in sympathetic postganglionic neurotransmission.

Consolo, Garattini, Ladinsky & Thoenen (1972) compared tissues from normal versus 6-hydroxydopamine-treated (chemically sympathectomized) cats. They found no significant difference in choline or ACh content of iris tissues between the two types of cat, which is consistent with the present observation of lack of significant difference in ChAT activity between control side and denervated side iris tissue. It is therefore apparent that ACh synthesis is not related to the sympathetic nerves of the iris. As was observed in the present study of the REA, Consolo *et al.* noted very low assay counts above blank for both normal and sympathectomized cat spleen tissue. The authors concluded that the sympathetic nerves of the spleen did not contain ChAT, and that the slight activity of the homogenates, 'may be due to unknown non-enzymatic factors'.

It was of interest to investigate further the nature of the low counts in the extraneuronal REA tissue. The mean REA count was 27% above the mean tissue-free blank value, and was in the order of 10% of the value for rabbit ileum, which is known to contain cholinergic nerves (Boyd, Gillespie & MacKenna, 1962) and which may be assumed to have a sparse innervation, relative to tissue mass.

Boiling the homogenate of REA before incubation reduced radioactivity in the organic phase, which indicated that undenatured protein was required for the formation of the acetylated, extractable product. However, the potent and selective ChAT inhibitor BETA (Rowell, Chaturvedi & Rama Sastry, 1978), which reduced the radioactivity derived from rabbit iris homogenates in a dose-dependent manner, had no corresponding effect on the REA counts. This showed that the transfer of labelled acetyl groups to the molecules collected from the REA tissue homogenates occurred through different processes from those which functioned in the iris. Treatment with AChE reduced the labelled yield from iris and ileum substantially, but reduced the yield from REA to a lesser degree, suggesting that much of the labelled product from this tissue was not ACh. It is therefore reasonable to propose that the major part of the very low counts obtained from the non-neural, REA tissue were not due to the action of the enzyme which has the function of choline acetylation elsewhere, and that one or more non-ACh acetylated products were trapped to a small but detectable degree by the extraction procedure.

We concluded that there was no evidence for

ChAT activity in the sympathetic nerves of the REA, and that the low counts obtained in the ChAT assay of extraneuronal REA tissue were due primarily to some other protein-dependent system.

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