THE DISTRIBUTION OF CHOLINESTERASE IN THE RABBIT EAR ARTERY

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Acetylcholinesterase and butyrylcholinesterase activities were demonstrated in transverse sections of the rabbit ear artery using a "direct coloring" thiocholine technique. BW 284C51, iso-OMPA and DFP were used as inhibitors, and acetyl and butyrylthiocholine iodides as substrates. Both enzymes caused stain deposits at the medial-adventitial border of the artery, in a region where sympathetic nerves are present. Sections treated to demonstrate acetylcholinesterase showed staining which was lighter than that for butyrylcholinesterase. Degeneration of the sympathetic nerves following superior cervical gangliectomy virtually abolished stain at the medial-adventitial border. These results provide some support for the evidence of other workers that a cholinergic mechanism may be linked to sympathetic nerve transmission in the rabbit ear artery.

The position of the sympathetic nerves is important in the adrenergic function of the rabbit ear artery (16). These nerves are located at the medial-adventitial border of the artery. The possibility that acetylcholine is important in sympathetic transmission (3), and that it may be involved in vasodilator phenomena in the ear (10), led us to investigate the position of cholinergic structures in the artery wall. In the present study the rabbit ear artery has been examined histochemically for the presence of acetylcholinesterase (AChE) and butyrylcholinesterase (ChE). The study is essentially an extension of that of Grant and Thompson (8). Their work showed that both enzymes were present in homogenates of ear vessels, and that there was a close association in the rabbit ear between the distribution of cholinesterase and the nerves which ramify along the arteries, arterioles and arteriovenous anastomoses in this tissue. The present study deals with the association between cholinesterase and noradrenergic nerves within the artery wall.

MATERIALS AND METHODS

The distribution of cholinesterase activity in the rabbit ear artery was demonstrated by the method of Karnovsky and Roots (11). They proposed as the basis of their method that cholinesterase acted on thiocholine esters in the medium to liberate thiocholine which reduced ferriyanide ions present to ferrocyanide. These combined with Cu²⁺ to form insoluble copper ferrocyanide (Hatchett's brown).

Twenty-eight semi-lop-eared rabbits of either sex, bred at the central animal house, University of Adelaide, were used in the study.

Removal of tissues: Rabbits were anesthetised with ethyl carbamate (2.5-5 g in aqueous solution intraperitoneally). Heparin, 1000 units/kg, was injected into a marginal ear vein. Segments of the proximal part of the central ear artery were dissected free and removed as described by de la Lande, Cannell and Waterson (4) or blocks 3 x 3 cm containing a segment of the artery were taken from the full thickness of the ear.

Pretreatment of tissues: Four rabbits were prepared by unilateral excision of a superior cervical ganglion 14-21 days before use, as described by de la Lande and Rand (5). Two rabbits were pretreated with reserpine (Serpasil, Ciba), 2.5 mg/kg, intraperitoneally 12 and 24 hr before use. Arteries from four rabbits were perfused with Krebs bicarbonate solution as described by de la Lande et al. (4).

Sectioning: Specimens were frozen either in acetone containing Dry Ice or in liquid nitrogen and sectioned immediately or after storage for up to 14 days at -50°C. Transverse sections were cut at 8-20 μ on a cryostat (Cryo-cut, American Optical Company) at -17°C.

Reagents: Sections were fixed in formalin-sucrose-ammonia (13). All other reactions were carried out in 0.1 M sodium hydrogen malate.
Figs. 1-3. Transverse sections of the wall of rabbit ear artery in the region of the medial-adventitial border (arrow). Scale, 50 μ. a, adventitia; m, media; i, intima.

Fig. 1. Butyrylthiocholine incubation, 3 hr. No inhibitor. Stain due to ChE at the medial-adventitial border (arrow).

Fig. 2. Acetylthiocholine incubation, 3 hr. Inhibitor, iso-OMPA 10-5 M, 1 hr. Stain due to AChE at the medial-adventitial border (arrow).

Fig. 3. Control incubation, 3 hr. Medium contained no substrate. No stain at the medial-adventitial border (arrow).
FIGS. 4 AND 5. Transverse sections of control (Fig. 4) and experimental (Fig. 5) rabbit ear arteries from animal which had undergone unilateral superior cervical ganglionectomy 21 days before sacrifice. Butyrylthiocholine incubation, 2 hr. No inhibitors. Medial-adventitial border arrowed. Scale, 100 μm. a, adventitia; m, media; i, intima. In Figure 5 a few discrete areas of stain remain in the adventitia. This was a consistent finding after ganglionectomy.
medium (pH 6.0). Stock solutions as described by Karnovsky and Roots (11) were kept for up to 30 days at 4°C. The cholinesterase inhibitors used were:
1. Iso-OMPA (tetraisopropyl pyrophosphoramide, Koch-Light).
2. BW 284C51 (1,5-bis-(N-allyl-N,N-dimethyl-4-ammoniumphenyl)pentan-3-one dibromide, Burroughs Wellcome).
3. DFP (diisopropyl fluorophosphoramide, Koch-Light).

The inhibitors iso-OMPA and BW 284C51 were prepared by serial dilution in malate buffer, and DFP was prepared from a stock solution of 10⁻¹ M in propylene glycol by dilution in malate buffer.

**Stain procedure:**

**Fixation:** Sections, on glass, were placed in fixative for 15-30 min, at room temperature, and then rinsed briefly in two changes of buffer.

**Preincubation:** Sections were either exposed to one or two of the cholinesterase inhibitors in buffer for 1 hr at 37°C or held in buffer under the same conditions without inhibitors. Concentrations of inhibitors used were: iso-OMPA, 10⁻⁴ M or 3 X 10⁻⁵ M; BW 284C51, 3 X 10⁻⁵ M or 10⁻⁴ M; DFP, 10⁻⁴ M. At these concentrations the inhibitors are selective against, respectively, ChE, AChE and both enzymes (1).

**Incubation:** After a brief buffer rinse, sections were placed in the incubation solution as described by Karnovsky and Roots, containing either acetylthiocholine iodide or butyrylthiocholine iodide (Koch-Light), both at a concentration of 500 μg/ml. For those sections preincubated in BW 284C51, this inhibitor was included in the incubation medium at the same concentration. The substrate was omitted in treating control sections. An incubation period of 2-3 hr gave optimal definition of stain in this tissue. Sections were dehydrated and mounted with Xam (Gurr).

**Photomicrographs:** Sections were examined using a Zeiss S.V. microscope at calculated magnification of 100X or 250X, using illumination from a tungsten lamp. Photomicrographs were made with a Zeiss Ikon automatic camera and Ilford Pan F film.

**RESULTS**

**No inhibitors:** Following incubation with both acetylthiocholine and butyrylthiocholine, discrete heavy deposits of brown stain were observed near the medial-adventitial border of the ear artery (Fig. 1). In addition, the acetylthiocholine-incubated sections often showed staining of red blood cells in the lumen of the artery.

**Inhibitor preincubation:**

**BW 284C51, 3 X 10⁻⁴ M or 10⁻⁴ M:** The distribution and density of staining near the medial-adventitial border were indistinguishable from that found with “no inhibitors.” In acetylthiocholine-incubated sections, the red blood cells did not stain.

**Iso-OMPA, 10⁻⁴ M or 3 X 10⁻⁵ M:** In butyrylthiocholine-incubated sections staining was not evident. Acetylthiocholine-incubated sections showed faint but definite staining near the medial-adventitial border (Fig. 2). Staining of red blood cells was comparable to that observed in sections not exposed to inhibitor.

**Iso-OMPA and BW 284C51:** No stain was evident in sections treated simultaneously with iso-OMPA and BW 284C51.

**DFP, 10⁻⁴ M:** No brown stain was evident in sections treated with DFP.

**Control incubation:** Control sections incubated in a medium not containing substrate showed no staining (Fig. 3).

**After chronic sympathetic denervation:** In the denervated arteries only a few discrete areas of stain were seen close to the media (Fig. 5). Control arteries from the contralateral ears showed staining as described in “no inhibitors” and “inhibitor preincubation” above (Fig. 4).

**Reserpine pretreatment:** Arteries taken from reserpine-pretreated rabbits showed a distribution and density of stain similar to that described in “no inhibitors” and “inhibitor preincubation” and the control arteries in “after chronic sympathetic denervation.”

**Perfusion:** Perfusion of the arteries with Krebs bicarbonate solution for up to 6 hr produced no detectable changes in stain density or distribution.

**DISCUSSION**

The results of this study indicate that ChE and AChE are located very close to the medial-adventitial border of the artery, in a region known to contain a noradrenergic nerve terminal network (6). In the present study, where tissue sections were treated, a more detailed examination of the terminal distribution of the enzyme was possible than in the earlier study of Grant and Thompson (8), who examined whole mounts of rabbit ear and rabbit ear artery for cholinesterase using the method of Gomori (7).

Evidence for the presence of the two enzymes, ChE and AChE at the medial-adventitial border, is based on the effects of different combinations of substrate and inhibitor.

1. Butyrylthiocholine, a substrate for ChE (7), produced heavy stain. Inhibition with iso-OMPA, which is effective against ChE (1), pre-
vented staining, thereby confirming the presence of ChE in this artery.

2. Acetylthiocholine, a substrate for both ChE and AChE (12), also produced a heavy stain. Iso-OMPA inhibition in this case markedly reduced stain formation, presumably because of ChE inhibition, but there still remained a small but definite amount of staining in the region of the medial-adventitial border. Presumably this stain was due to AChE.

3. Direct evidence for the presence of AChE is provided by use of the inhibitor BW 284C51, which is highly effective against AChE (1). The stain attributed to AChE activity in the paragraph above was prevented by the addition of BW 284C51 to the incubating medium. In this situation both enzymes were inhibited (ChE by iso-OMPA, and AChE by BW 284C51), and no staining occurred. The histochemical findings of this study relating to the identity of the two cholinesterases are consistent with the results of manometric estimations made by Thompson and Tickner (15) and Grant and Thompson (8). These workers estimated cholinesterase activity in minced whole rabbit ear arteries, and concluded that the arteries contained both enzymes, butyrylcholinesterase predominating.

The concentration of the two enzymes near the sympathetic nerve terminals in the artery wall and the virtual disappearance of the enzymes after chronic sympathetic denervation suggest a close relationship between an intact sympathetic innervation and the presence of the enzymes. However, cholinesterase staining (due to both ChE and AChE) was unaffected by removal of noradrenaline from the nerve endings with reserpine, suggesting that the effects of sympathetic denervation are in fact due to degeneration of the nerves and not solely to the disappearance of noradrenaline which is known to follow sympathetic denervation.

Burn and Rand (3) proposed that acetylcholine may be a mediator in the release of noradrenaline in sympathetic postganglionic nerves. Their findings were based on modification of responses to sympathetic nerve stimulation in a variety of tissues, including the vessels of the rabbit ear. Thompson and Tickner (15) had previously reported that rabbit ear vessels contained acetylcholine and choline acetylase in addition to cholinesterase, while de la Lande and Rand (5) found that exogenous acetylcholine dilated the perfused rabbit ear artery. Schenk and El Badawi (14) demonstrated a close relationship between AChE and adrenergic nerves in a variety of blood vessels from dogs and cats. They suggested that AChE may be in sensory or in autonomic nerves, but provided morphologic evidence for a generalized dual adrenergic and cholinergic innervation of arteries and arterioles. Bell (2) demonstrated a dense nerve plexus associated with high AChE activity near the medial-adventitial border of the main uterine artery of the guinea-pig, and postulated dual adrenergic-cholinergic innervation of the artery. These observations are consistent with the conclusions of Hamberger, Norberg and Sjöqvist (9) that sympathetic ganglia contain two separate cell populations, a larger adrenergic and a smaller cholinergic.

The present findings support either the concept of dual innervation of the artery wall or a separate system in which a cholinergic element is contained within the post ganglionic sympathetic neural pathway, as proposed by Burn and Rand (3). Whichever system is operative, the present findings suggest that there is a close relationship between the cholinergic element and the noradrenergic fibers which pass through the superior cervical ganglion, and that destruction of the sympathetic nerves supplying the vascular smooth muscle may be accompanied by a simultaneous loss of both noradrenergic and of cholinergic control, if the latter exists.

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