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Innervation of arteriovenous anastomoses in the brood patch of the domestic fowl

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Summary. The innervation of blood vessels in the brood patch (thoracic skin) of the domestic fowl was studied by use of the catecholamine fluorescence technique, acetylcholinesterase staining, and the immunoperoxidase technique for demonstration of vasoactive intestinal polypeptide (VIP). Large arteries and veins were sparsely innervated, whereas arteriovenous anastomoses (AVAs) were densely innervated by adrenergic, acetylcholinesterase-positive, and VIP-immunoreactive nerve fibres. The rich supply of different vasomotor nerves to AVAs emphasizes the importance of these vascular shunts in regulating blood flow and, in turn, the transport of heat to the brood patch. Furthermore, the presence of VIP-immunoreactive nerve fibres in the vasculature of the brood patch suggests that VIP might be the mediator of the previously reported cold-induced vasodilatation.

Key words: Brood patch – Arteriovenous anastomoses – Innervation – Vasoactive intestinal polypeptide (VIP) – Domestic fowl

Arteriovenous anastomoses (AVAs) are medium-sized blood vessels through which blood may be shunted directly from arterioles to venules without passing through the capillaries. AVAs have been described in many tissues; they are particularly abundant in the skin of homeothermic animals (Clara 1956). In birds, they occur in large numbers in the feet, brood patch, and naked skin of the head (Midtgård 1986). By use of radioactive microspheres it has been shown that the AVAs in both mammals and birds form the structural basis for high rates of blood flow and resultant transport of heat from the core of the body to the periphery (Hales 1985; Wolfenson 1983). Thus, AVAs are believed to have a thermoregulatory role in most skin areas, in contrast to the capillaries which serve tissue nutrition.

The innervation of AVAs has been studied in mammals including man by use of histochemical methods, electron microscopy, and immunohistochemistry (e.g., Cauna 1970; Bell et al. 1978; Böck 1980; Molyneux and Harmon 1983). In contrast, there is only very little information about the pattern of innervation of avian AVAs. Molyneux and Harmon (1982), using electron microscopy after chemical sympathectomy, found that the AVAs in the feet of ducks were supplied with adrenergic, cholinergic, and possibly peptidergic nerves. The nature of the peptide has not been determined, but the immunohistochemical studies of mammalian AVAs (Weihe et al. 1981; Molyneux and Harmon 1983) suggest vasoactive intestinal polypeptide (VIP) and/or substance P.

In the present study, the catecholamine fluorescence method, acetylcholinesterase staining, and the immunoperoxidase method for demonstration of VIP were used to investigate the innervation of avian AVAs. The brood patch was selected as the subject of this investigation, firstly because it is a highly vascularized skin area with many AVAs (Midtgård 1985), and secondly because it has been shown that it responds to local cooling by increasing the blood flow (Midtgård et al. 1985), and this cold-induced vasodilatation appears to involve AVAs (Hales and Midtgård, unpublished observations).

Materials and methods

Animals

Four bantam hens (*Gallus gallus domesticus*), which had been incubating eggs for at least 2 weeks, were used in this study. Small pieces of the thoracal part of the brood patch were excised under deep ether anaesthesia and processed as described below.

Acetylcholinesterase staining

Lewis' acetylcholinesterase (AChE) staining method as described by Silver (1974) was used for demonstration of cholinergic nerves. The tissue was fixed in 3% formaldehyde in isotonic Na_2SO_4 for 4 h at 4° C and stored overnight in 20% ethanol. After washing, the tissue was frozen in a cryostat and 20–30 µm-thick sections were cut. The sections were incubated 2–4 h in a medium containing acetylthiocholine iodide and copper glycinate as substrate and ethopromazine (10^{-4} M) as inhibitor of pseudocholinesterase. The reaction product was developed using 2% Na_2S, and the sections were finally washed, dehydrated, and embedded in Dammar xylene.

Fluorescence microscopy

Adrenergic nerves were demonstrated by either the sucrose phosphate glyoxylic acid (SPG) method (Torre de la and Surgeon 1976) or by formaline-induced fluorescence. Fresh tissue was immediately frozen in a cryostate and 20–30 µm-thick sections were cut. The sections were dipped in the SPG solution, air dried, and heated to 80° C for 5 min. The sections were mounted in paraffin oil and examined

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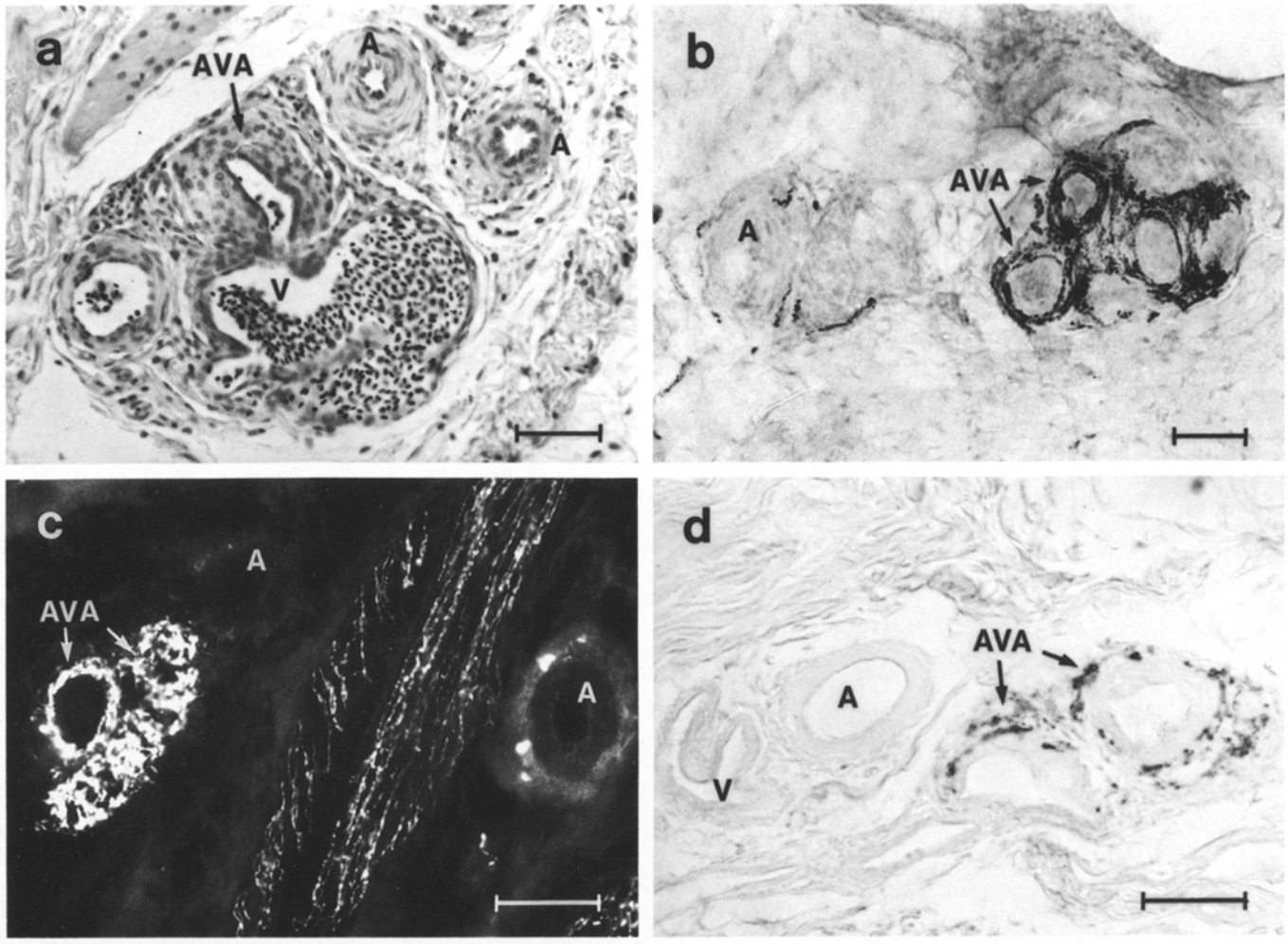

Fig. 1 a–d. Structure and innervation of arteriovenous anastomoses in the brood patch of the domestic fowl. *A* artery; *AVA* arteriovenous anastomosis; *V* vein. Scale bars = 50 μm. **a** Paraffin section of a glomus organ with an AVA which has been cut at its inlet to the vein (haematoxylin-eosin stain). **b** Thick cryostat section showing high acetylcholinesterase activity around blood vessels in a glomus organ. The AVA, which has been cut twice, is completely encircled by acetylcholinesterase-positive nerve fibres (haematoxylin counterstain). **c** Adrenergic innervation visualized with the glyoxylic acid technique. Note the dense innervation of the AVA compared to the arteries. (The varicose fibres in the middle portion innervate feather muscles.) **d** VIP-immunoreactive nerve fibres around an AVA

with a Leitz Ortholux microscope with excitation filter BP 350–460 and suppression filter LP 515. In some cases where fluorescence was induced by 1-h exposure to 37° C formaldehyde vapour the sections were afterwards processed for demonstration of AChE.

Immunoperoxidase staining

Vasoactive intestinal polypeptide (VIP) was demonstrated by immunohistochemistry using the indirect peroxidase-antiperoxidase (PAP) method (Sternberger 1979). Briefly, the tissue was fixed in a buffered mixture of formaldehyde and saturated aqueous picric acid (Stefanini et al. 1967) for 15 min, washed, dehydrated, and embedded in paraffin. Sections, 10 μm thick, were mounted on gelatine-coated slides. After rehydration, the sections were preincubated for 30 min in normal porcine serum diluted 1:10 with a HCl-hydroxymethyl aminomethane buffer (TRIS). The sections were then incubated ~72 h at 4° C with a highly purified rabbit antiserum against chicken VIP in dilutions of 1:200 to 1:3,200. The VIP antiserum (code 3615-4), which was kindly supplied by Prof. J. Fahrenkrug, Bispebjerg Hospital, Copenhagen, has previously been documented (Korf and Fahrenkrug 1984). Subsequently, sections were incubated for 30 min with porcine IgG to rabbit IgG (Dakopatts, code z 196) diluted 1:10 in TRIS. Finally, the sections were incubated with the PAP complex (Dakopatts, code z 113) diluted 1:80 in TRIS, and the peroxidase activity was visualized by exposure to 0.03% diaminobenzidine containing 0.01% H_2O_2. (All incubations were followed by rinsing in TRIS.)

Control for specific staining was performed by omission of the primary, secondary, or tertiary antibody layer on some slides or by absorption of the primary antibody with purified chicken VIP (10 nmol/l). Immunoperoxidase staining was absent in all these controls.

Some paraffin sections were stained with haematoxylin and eosin for routine histologic examinations.

Results

Acetylcholinesterase-positive nerves

The medium-sized subcutaneous arteries and veins in the brood patch were usually surrounded by several AChE-positive nerve fibres at the junction of the tunica adventitia

and tunica media. The glomus organs, which consist of AVAs and their associated vessels (Fig. 1a), were heavily innervated with AChE-positive nerves. The nerve fibres often encircled the blood vessels completely and appeared to be varicose (Fig. 1b).

Adrenergic nerves

The AVAs were surrounded by a dense network of adrenergic nerves, in contrast to their arteries of origin which displayed only few fluorescent nerves (Fig. 1c). The veins generally lacked adrenergic nerves, except for some of the larger subcutaneous veins. Sections processed for both demonstration of adrenergic nerves and AChE staining showed only little correlation in distribution of the two types of nerves.

VIP-immunoreactive nerves

VIP-immunoreactive nerves were practically absent from the large arteries and veins. Occasionally, two or three nerves with very weak immunoreactivity were observed at the junction of the tunica adventita and tunica media. In contrast, the AVAs were well supplied with VIP-immunoreactive nerves (Fig. 1d).

Discussion

The present study supports previous observations in the feet of ducks (Molyneux and Harmon 1982) that the AVAs are densely innervated with adrenergic and cholinesterase-positive nerves. In addition, it was found that the AVAs in the brood patch are well supplied with VIP-immunoreactive nerves, and in this respect the avian AVAs resemble those in the fingers of humans (Weihe et al. 1981). While the catecholamine fluorescence and the immunoperoxidase techniques visualize the neurotransmitters directly, the AChE staining method demonstrates only the enzyme which hydrolyzes the transmitter, and AChE is apparently not restricted to true cholinergic nerves. It has been shown that AChE is also associated with catecholamine-containing nerves (Eränkö et al. 1970) and with VIP-immunoreactive nerves around blood vessels (Lundberg et al. 1979). Therefore, it cannot be concluded at present whether the neurotransmitters noradrenaline, acetylcholine and VIP belong to the same or three different populations of nerves.

The effect of different vasoactive drugs on blood flow through AVAs has been studied by direct observation of the microcirculation (review, Sherman 1963) and by use of microspheres. Adrenaline and noradrenaline constrict the AVAs, whereas acetylcholine acts as a dilator (Grant 1930; Hales et al. 1982). There is at present no information about the effect of VIP on AVA blood flow, but since VIP has been reported to be a potent vasodilator in all vascular preparations so far studied, this substance probably also dilates AVAs. Apparently, the AVAs are innervated by both vasoconstrictor and vasodilatory nerves. This differential innervation reflects a high degree of vasomotor control suggesting that the AVAs are important sites for regulation of the cutaneous circulation.

Circulation in the brood patch is considered important for regulation of heat flow from the incubating bird to the eggs (Drent 1975). Recent experiments have shown that cold eggs increase brood patch blood flow in incubating hens (Midtgård et al. 1985) and that this cold-induced vasodilatation (CIVD) may involve AVAs (Hales and Midtgård, unpublished). Although the CIVD of the brood patch was originally thought to be independent of the nerve supply, the present finding of vasodilatory nerves in the region of AVAs clearly suggests that the CIVD is a neurogenic mechanism, and the transmitter released may be acetylcholine or VIP or a combination of both.

In birds, CIVD has been most extensively studied in the feet of the Giant petrel (*Macronectes giganteus*) in the Antarctic (Johansen and Millard 1974; Murrish and Guard 1977). The CIVD consists of an initial short phase, which appears to be cholinergic, and a long-lasting phase, which is atropine resistant. The atropine-resistant active vasodilatation was considered by Murrish and Guard (1977) to be beta-adrenergic, but this could not be confirmed in a comparable study of domestic ducks and chickens (McGregor 1979). Hillman et al. (1982) found that adenosine triphosphate selectively increased AVA blood flow in the feet of chickens, and consequently they suggested that purinergic nerves were responsible for the non-adrenergic, non-cholinergic active vasodilatation. This view is supported by the morphologic observation that some nerve terminals around AVAs contain a high proportion of large dense-cored vesicles (Böck 1980; Molyneux and Harmon 1982), which is characteristic of purinergic nerves (Burnstock 1972). However, VIP and other neuropeptides are also stored in large, dense-cored vesicles (Larsson 1977; Hökfelt et al. 1980), and there is sufficient experimental evidence to believe that VIP plays an important role in neurogenic vasodilatation in mammals (Fahrenkrug et al. 1978; Lundberg et al. 1982; Hellstrand et al. 1985). Therefore, it seems reasonable to conclude from the present findings of numerous VIP-immunoreactive nerves around AVAs and the previous physiological experiments that VIP is the mediator (i) of active vasodilatation in the feet and (ii) of CIVD in the brood patch of birds.

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