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Autoradiographic analysis of the distribution of β-adrenoceptors in the dog splenic vasculature

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1 The technique of *in vitro* labelling and autoradiography has been used to localize β-adrenoceptors in sections of the splenic vascular bundle of the dog.

2 Binding of (−)-[^{125}I]-cyanopindolol (Cyp) to sections of splenic vascular bundle equilibrated within 150 min and slowly dissociated after addition of (−)-propranolol. The process was saturable with a dissociation constant (K_D) of 40.3 ± 4.4 pM and B_{max} of 18.9 ± 1.7 fM (in 6 sections).

3 Binding to sections was stereoselective, the (−)-isomer of propranolol being 90 times more effective than the (+)-isomer in competing for (−)-[^{125}I]-Cyp binding.

4 Delineation of β-adrenoceptor subtypes using the selective antagonists betaxolol (β_1) and ICI 118,551 (β_2) indicated that the receptors present were almost exclusively of the β_2-subtype.

5 Autoradiographic studies under the conditions evaluated in the biochemical experiments showed that β-adrenoceptors are unevenly distributed in the dog splenic vein, artery and associated nerve bundles. High concentrations of receptors are associated with the splenic nerves and lower but still significant concentrations in the vasculature.

6 Higher resolution studies with nuclear emulsion coated coverslips revealed concentrations of β-adrenoceptors over cells adjacent to the lumen in veins. In arteries most β-adrenoceptors were found associated with the medial layer with fewer receptors towards the intima or adventitia. Serial sections of either artery or vein incubated with (−)-[^{125}I]-Cyp in the presence of (−)-propranolol showed low levels of non-localized binding.

Introduction

Vascular β-adrenoceptors are stimulated by noradrenaline released from nerve endings and circulating adrenaline released from the adrenal medulla. These receptors are located on smooth muscle cells where they cause relaxation (Somlyo & Somlyo, 1979; Bolton, 1979) and on noradrenergic nerve terminals where they facilitate transmitter release (Rand *et al.*, 1980; Majewski, 1983). Radioligand binding studies on homogenates of vascular tissues have allowed the direct measurement of the density and characteristics of the receptors (Woodcock *et al.*, 1980; Schwartz & Velly, 1983) but do not allow the cellular localization of these receptors to be determined. The subclassification of β-adrenoceptors into β_1- and β_2-subtypes (Lands *et al.*, 1967) is now widely accepted and the proportion of each subtype in vascular tissues varies. Functional studies have indicated β_1-adrenoceptors in feline cerebral arteries (Edvinsson & Owman, 1974) and in coronary arteries (Bohr, 1967), a mixture of subtypes in dog saphenous vein and renal vascular bed, and a predominance of β_2-adrenoceptors in dog femoral and mesenteric blood vessels (Taira *et al.*, 1977). Although these functional studies establish the presence of β-adrenoceptors in many types of blood vessel little is known about the distribution of these receptors within the vasculature. In this study the characteristics of (−)-[^{125}I]-cyanopindolol (Cyp) binding to slide-mounted tissue sections have been examined together with the localization of these sites in a vascular preparation consisting of dog splenic vein, artery and the associated nerve bundles.

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Methods

Preparation of tissues

Greyhound (20–30 kg) or mongrel dogs (18–25 kg) were injected intravenously with heparin and a lethal dose of pentobarbitone. The upper abdomen was opened and the splenic artery, vein and nerve bundles dissected free of fat and omentum. The blood vessels

were flushed slowly with Krebs solution (composition in mM; NaCl 119, KCl 4.8, $MgSO_4$ 1.2, NaH_2PO_4 10.0, $CaCl_2$ 1.27, pH 7.6) to remove blood, blotted dry and rapidly frozen in isopentane cooled in liquid N_2. Tissues were mounted in O.C.T. embedding medium on cold cryostat chucks, 10 μm serial sections were cut with a Jung Cryocut E cryostat and were thaw-mounted onto gelatinized microscope slides. Sections were dried at room temperature and stored at −20°C overnight.

Labelling of tissues

Labelling of slide-mounted tissue sections was carried out with (−)-[^{125}I]-Cyp prepared as previously described (Lew & Summers, 1985). Slides, each containing 6 sections were brought up to room temperature in a closed slide box and then incubated at 25°C for 150 min with (−)-[^{125}I]-Cyp in 170 mM Tris, pH 7.6, 10 μM phenylmethylsulphonylflouride (PMSF) and 0.01% ascorbate. After incubation, sections were washed for two periods of 15 min in Tris buffer at 37°C, rapidly rinsed in distilled water and either wiped from the slide with Whatman GF/B glass fibre filters (biochemical experiments) or dried in a current of cold dry air (autoradiographic experiments). Radioactivity in the sections was estimated by conventional gamma counting techniques. Non-specific binding was determined in adjacent sections labelled in the presence of (−)-propranolol (1 μM). Concentrations of (−)-[^{125}I]-Cyp used in biochemical experiments were 10-250 pM (saturation studies) and 20 pM (autoradiographic studies).

Autoradiographic procedures

^{3}H-Ultrofilm autoradiography was carried out as previously described (Lew & Summers, 1985). Autoradiography with nuclear emulsion coated coverslips was carried out as described by Young & Kuhar (1979) except that after photographic development and fixation the sections were rinsed in Krebs buffer, histologically fixed in acetone/McIlwain's buffer (3:2) and stained with 1% pyronin Y.

Analysis of results

Estimation of the dissociation constant and maximal number of binding sites in saturation studies and K_i values in competition studies was performed using the computer programme 'EBDA' running on the University of Melbourne VAX/VMS system (McPherson, 1983).

Drugs and chemicals

The following were used: Na^{125}I (Amersham International); (−)-cyanopindolol (Sandoz, Basel); (−)

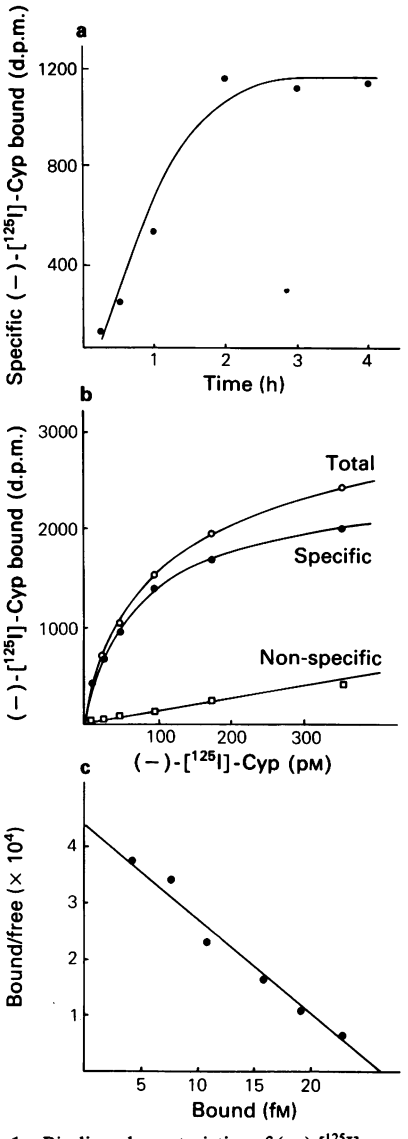

Figure 1 Binding characteristics of (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-Cyp) to sections of dog splenic artery, vein and nerve. (a) Association of (−)-[^{125}I]-Cyp to slide-mounted sections of dog splenic vasculature showing equilibration of binding within 150 min. (b) An example of a saturation curve showing total (○), specific (●) and nonspecific binding (□) defined by 1 μM (−)-propranolol with increasing concentrations of (−)-[^{125}I]-Cyp. (c) Scatchard analysis was performed using computer assisted iterative curve fitting (Munson & Rodbard, 1980; McPherson, 1983). The typical example above shows a linear relationship with a K_D of 56.4 pM and B_{max} of 0.17 fmol/section. Each point is the mean of triplicate determinations from a single experiment. Scafit analysis and pooled data from 7 experiments gave a K_D value of 40.3 ± 4.4 pM and B_{max} of 18.9 ± 1.7 fM (n = 7).

and (+)-propranolol, ICI 118,551 (ICI Ltd.); betaxolol (Synthelabo); pyronin Y (Sigma); NTB3 nuclear emulsion, Rapid Fix, D19, Dektol (Kodak). ^{3}H-Ultrofilm (LKB Bromma). All other chemicals were of analytical grade.

Results

Biochemical characterization of (−)-[^{125}I]-cyanopindolol binding to slide-mounted sections of dog splenic vascular bundle

Biochemical studies showed that binding of (−)-[^{125}I]-Cyp to slide-mounted sections of dog splenic blood vessel preparations had the characteristics required for binding to β-adrenoceptors. Specific binding equilibrated within 150 min, was saturable and of high affinity (Figure 1 a,b,c). The mean dissociation constant (K_D) obtained by computer assisted iterative curve fitting (McPherson, 1983) was 40.3 ± 4.4 pM

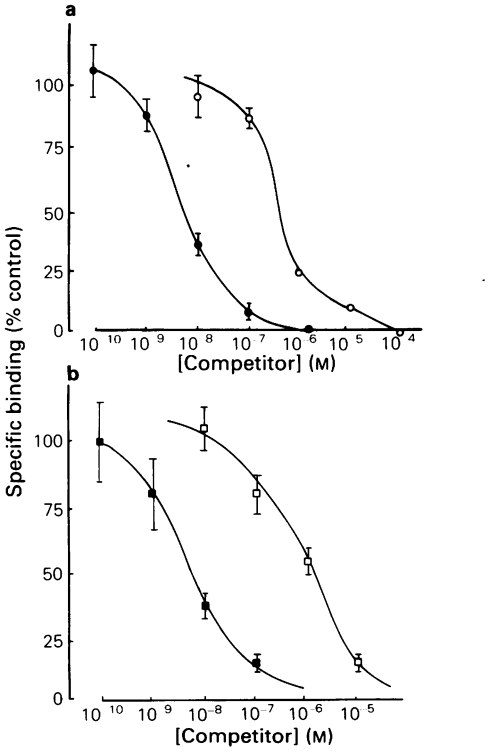

Figure 2 Characteristics of (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-Cyp) binding to slide-mounted sections of dog splenic vascular bundle: stereoselectivity and delineation of receptor subtypes. (a) Inhibition of specific binding with increasing concentrations of (−)-propranolol (●) and (+)-propranolol (○). Each point is the mean of 3 and 4 experiments for the (−)- and (+)-isomers of propranolol, respectively, each performed in triplicate, vertical lines show s.e.mean. (b) Inhibition of specific binding with increasing concentrations of ICI 118,551 (■) and betaxolol (□). Each point is the mean of 3 experiments each performed in triplicate, for both ICI 118,551 and betaxolol; vertical lines show s.e.mean.

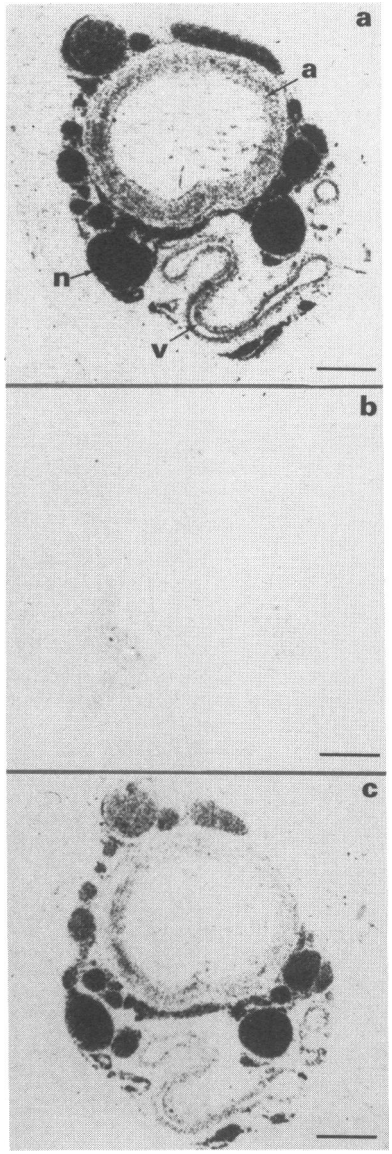

Figure 3 Autoradiographic localization of β-adrenoceptors in dog splenic artery (a), vein (v) and nerve bundle (n). Sections were labelled with 20 pM (−)-[^{125}I]-Cyp in the absence of an antagonist (a) or in the presence of 50 nM ICI 118,551 (b), or 500 nM betaxolol (c). Bar represents 1 mm.

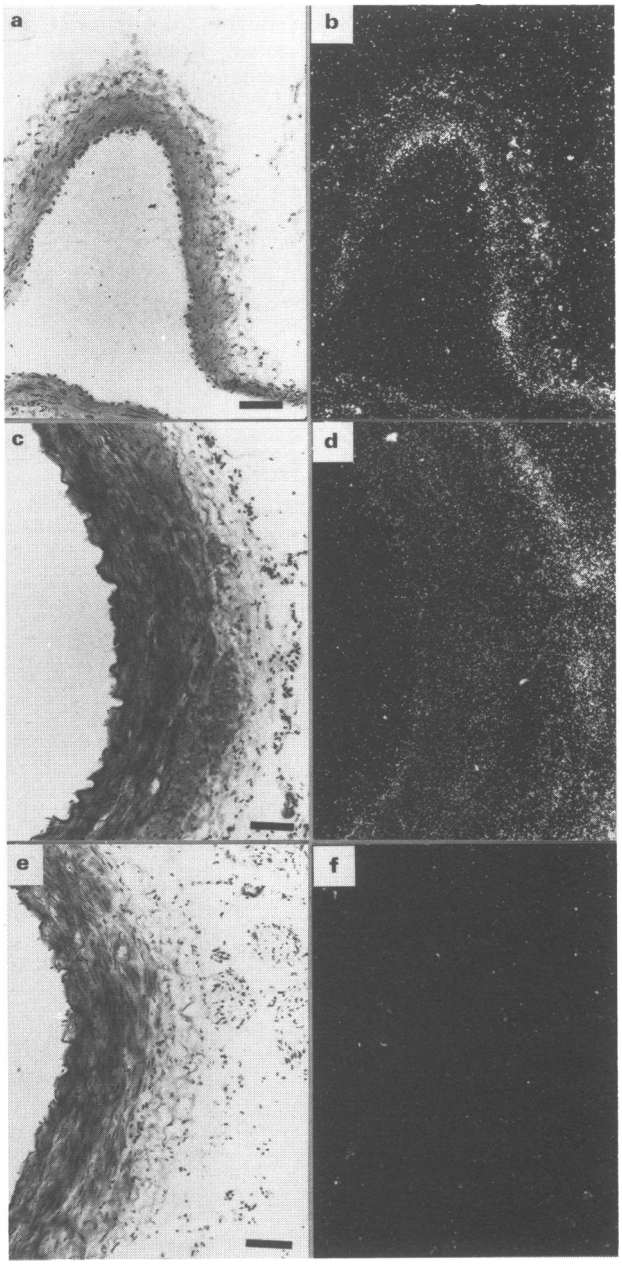

Figure 4 Distribution of β-adrenoceptors in the dog splenic vasculature incubated with (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-Cyp). (a) Splenic vein, histologically fixed and stained to reveal tissue structure; (b) Dark-field photomicrograph of the same section showing a concentration of silver grains over the intima; (c) and (d) bright and dark field photomicrographs of splenic artery; (e) and (f) bright and dark field views of sections incubated with (−)-[^{125}I]-Cyp in the presence of 1 μM (−)-propranolol. Note the reduced grain density in (f) compared with (d) and the even distribution of grains over the tissue and non-tissue areas. Bar represents 100 μm.

($n = 7$) and the maximal density of binding sites (B_{max}) was 18.9 ± 1.7 fM (for 6 sections). The Hill coefficient in the saturation experiments was 0.97 ± 0.05 indicating a lack of co-operativity in binding.

Binding of (−)-[^{125}I]-Cyp to slide-mounted sections of dog splenic vascular bundle was stereoselective with regard to the isomers of propranolol. Competition curves obtained with (−)- and (+)-propranolol were similar in profile but the K_i value for (−)-propranolol was 1.6 ± 0.1 nM ($n = 3$) and that for (+)-propranolol 144 ± 23 nM ($n = 4$) (Figure 2a).

Competition experiments using the subtype selective β_2-adrenoceptor antagonist ICI 118,551 (Bilski *et al.*, 1980; Dickinson *et al.*, 1981) and the β_1-subtype selective antagonist betaxolol (Boudot *et al.*, 1979; Dickinson *et al.*, 1981) showed that both competitors produced sigmoid competition curves and that ICI 118,551 was much more effective at competing for (−)-[^{125}I]-Cyp binding. The K_i values for ICI 118,551 and betaxolol were, respectively, 3.1 ± 0.3 nM ($n = 3$) and 1050.1 ± 230 nM ($n = 3$) (Figure 2b). These K_i values are similar to those obtained for these antagonists competing at β_2-adrenoceptors in rat and guinea-pig kidney sections with similar techniques (Summers *et al.*, 1985; Lew & Summers, 1985).

Autoradiographic localization of β-adrenoceptors in splenic vascular bundles

Autoradiography using ^{3}H-Ultrofilm showed that β-adrenoceptors are unevenly distributed in the dog splenic vein, artery and the associated nerve bundles (Figure 3a). The major concentration of β-adrenoceptors was associated with the splenic nerves, with fewer receptors associated with the splenic vein and artery. The localization of β-adrenoceptor subtypes was studied by use of ICI 118,551 and betaxolol at concentrations shown in the biochemical studies to displace (−)-[^{125}I]-Cyp binding selectively from β_2- and β_1-subtypes respectively. The β_2-selective antagonist ICI 118,551 almost completely inhibited binding in all areas of the vascular sections (Figure 3b), whereas betaxolol had no appreciable effect (Figure 3c).

Higher resolution studies with nuclear emulsion coated coverslips confirmed the concentration of receptors associated with nerves. In the veins (Figure 4 a,b), the receptor population was concentrated towards the intimal surface. The main receptor concentration in arteries was associated with medial smooth muscle cells with lower concentrations towards the adventitia and few at the intimal surface (Figure 4 c,d). Serial sections incubated with (−)-[^{125}I]-Cyp in the presence of 1 μM (−)-propranolol showed low levels of non-localized binding to areas in vascular tissues (Figure 4 e,f), indicating that the binding sites are predominantly β-adrenoceptors.

Discussion

There is abundant pharmacological evidence for the presence of β-adrenoceptors in blood vessels. The predominant β-adrenoceptor in vascular tissue is the β_2-subtype, although both β_1- and β_2-adrenoceptor subtypes may coexist in some blood vessels. Thus dog femoral and superior mesenteric vessels contain only β_2-adrenoceptors (Taira *et al.*, 1977) whereas dog saphenous vein (Guimares & Paiva, 1981) and the renal vascular bed contain both β-adrenoceptor subtypes (Taira *et al.*, 1977). β_1-Adrenoceptors predominate in cat cerebral arteries (Edvinsson & Owman, 1974) and small coronary arteries of several species (Bohr, 1967). The autoradiographic findings reported here indicate a predominance of β_2-adrenoceptors in the dog splenic artery and splenic vein. This agrees with previous functional studies in which the β_2-adrenoceptor agonist, fenoterol was found to be a potent vasodilator in dog splenic artery (Reuwer *et al.*, 1983). It has been suggested that β_1-adrenoceptors are activated by noradrenaline released from nerve terminals, whereas β_2-adrenoceptors are hormonal receptors activated by circulating adrenaline (Zaagsma *et al.*, 1979; Bryan *et al.*, 1981; Ariens & Simonis, 1983). The results obtained here show that β_2-adrenoceptors are concentrated towards the intimal surface of veins, where they are ideally situated to respond to circulating catecholamines including adrenaline. It is likely that these represent β-adrenoceptors associated with the vascular endothelium and the nuclei of the cells concerned can clearly be seen in Figure 4a. Recent studies (Stephenson & Summers in preparation) show that the concentration of grains over the intimal surface is not seen in blood vessels which have had the endothelium remvoved by gentle rubbing. In functional studies in dog coronary arteries the relaxation produced by β-adrenoceptor agonists is impaired but not abolished by endothelium removal suggesting that there are two mechanisms involved, a direct action on vascular smooth muscle and an indirect action mediated through the endothelium (Rubanyi & Vanhoutee, 1985). In the arteries there were β-adrenoceptors clearly located over smooth muscle and the density of these receptors is less towards the adventitia. There was no evidence in arteries for receptors clustered at the intimal surface indicating that there is variation between blood vessels with regard to the presence of β-adrenoceptors associated with the vascular endothelium.

An additional finding reported here is that the splenic nerves contain high concentrations of β-adrenoceptors, even higher than in the associated vasculature. Indirect evidence has demonstrated the existence of presynaptic β-adrenoceptors located on terminal varicosities which are involved in the regulation of transmitter release (Rand *et al.*, 1980; Majewski,

1983). This study has demonstrated that high concentrations of β-adrenoceptors are also associated with dog splenic nerve fibres. These adrenoceptors may be undergoing axonal transport to the nerve terminal or returning to the cell body although the possibility cannot be ruled out in view of the high concentration of receptors present that they subserve a function in the axon. In this regard, increases in cyclic AMP content of segments of bovine splenic artery are observed following stimulation with β-adrenoceptor agonists and these effects are blocked by the β-adrenoceptor antagonist, propranolol (Merican & Nott, 1983). Axonal transport of β_1-adrenoceptors has been reported in rat brain (Levin, 1982), while β_2-adrenoceptors are transported in the rat sciatic nerve (Zarbin *et al.*, 1983). The β_2-adrenoceptors that are transported anterogradely are reported to bind agonists with higher affinity than the β_2-adrenoceptors transported retrogradely. In the study described here it was not possible to say whether there are β-adrenoceptors associated with the terminal regions of the adrenergic nerves in the adventitia.

Although species variability in the innervation of spleen occurs there is general agreement that the dog spleen is innervated almost entirely by postganglionic sympathetic nerves (Davies & Withrington, 1973). Muscarinic receptors are known to be transported in these sympathetic nerves (Laduron, 1984). It appears therefore that the necessary mechanisms for anterograde and retrograde transport exist in the dog splenic nerves. The β-adrenoceptors labelled autoradiographically in this tissue are possibly undergoing axonal transport, but under the conditions of the present experiments using an antagonist ligand, receptors in the high and low affinity states cannot be distinguished.

In conclusion, these studies demonstrate the localization of β-adrenoceptors in a vascular preparation. The receptors are predominantly of the β_2-subtype and are associated with the sympathetic nerve bundles, the smooth muscle cells of the artery and the intimal surface of the vein.

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