Br. J. Pharmac. (1983), **79**, 409–414

The β-adrenoceptor of pig coronary arteries: determination of β_1 and β_2 subtypes by radioligand binding

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1 β-Adrenoceptors of pig coronary arteries were investigated by the use of a new ligand, ^{125}iodocyanopindolol (ICYP) in binding studies.

2 Inhibition of ICYP binding by betaxolol (a selective β_1-antagonist), zinterol (a selective β_2-agonist) and ICI 118551 (a selective β_2-blocking drug) resulted in non-linear Scatchard plots, suggesting that both β-adrenoceptor subtypes are present in pig coronary arteries.

3 Computer analysis of the data gave a $\beta_1 : \beta_2$-adrenoceptor ratio of approximately 65:35.

Introduction

Lands, Arnold, Mc Auliff, Luduena & Brown (1967) introduced the classification of β-adrenoceptors into β_1 and β_2-receptors. Both β_1 and β_2-receptors may be present within the same tissue as shown by Minneman, Hedberg & Molinoff (1979a) for rat lung and by Rugg, Barnett & Nahorski (1978) who provided evidence, for rat and rabbit lung, that the β_1 and β_2-adrenoceptors coexist in different proportions in the two species. For the vascular bed, especially for coronary arteries, this problem was not extensively explored.

In this paper, we attempt to identify the β-adrenoceptors of coronary arteries by radioligand binding studies. Widely used for the study of adrenoceptors in various tissues, this technique was difficult to apply to the vascular bed. However, it had been applied to the study of β-receptors in the rat aorta and inferior vena cava (Limas & Limas, 1979), in rat mesenteric arteries (Woodcok, Olsson & Johnston, 1980), and to the study of α-adrenoceptors in aorta, femoral, renal and mesenteric arteries of dogs (Tsai & Lefkowitz, 1978; Bobik, 1982) and to the aortae of cattle (Carman-Krzan, 1980), and rabbits (Fuder, Nelson, Miller & Patil, 1981). No work has so far been described on coronary β-receptors. The purpose of this paper was to identify the β-adrenoceptors of pig coronary arteries by use of a new ligand ^{125}iodocyanopindolol (ICYP) introduced by Engel, Hoyer, Berthold & Wagner (1981).

This has improved binding properties for β-adrenoceptors. Compared to tritiated compounds ([^{3}H]-dihydroalprenolol, [^{3}H]-DHA), it has a very high specific activity (about 2000 Ci/mmol versus 20 to 60 Ci/mmol for [^{3}H]-DHA) and is more selective than iodohydroxybenzylpindolol (IHYP), for which different investigators have reported an important affinity for α-adrenoceptors (Sporn & Molinoff, 1976) and 5-hydroxytryptamine receptors (Dickinson, Nahorski & Willcocks, 1981). ICYP binds to β_1 and β_2-adrenoceptors with equal affinity (Engel *et al.*, 1981). Analysis of the displacement curves of this ligand by selective β_1 or β_2 agents reveals the presence of one or two types of β-receptors and the relative proportions of each receptor subtype. The coexistence in a tissue of both β_1 and β_2-receptors results in curvilinear modified Scatchard plots. To gain information on the β-receptor type in pig coronary arteries we examined the inhibition of specific ICYP binding by a selective β_1-antagonist (betaxolol) a selective β_2-agonist (zinterol) and a selective β_2-blocking drug (ICI 118551; erythro-DL-1 (7-methylindan-4-yloxy)3 isopropylaminobutan-2-ol).

Methods

Membrane preparation

Pig hearts were taken immediately after slaughter and put on ice. The anterior descending and circum-

flex branches of the left coronary artery were rapidly excised. Adhering adventitial tissue was removed and the arteries were then frozen for at least 24 h. Membranes from the vessels were prepared according to the technique of Tsai & Lefkowitz (1978) to which some modifications were made. Thawed arteries were minced in 10 volumes (w/v) of ice-cold buffer containing 0.25 M sucrose, 1 mM $MgCl_2$ and 5 mM Tris (pH 7.4) and homogenized with a Polytron homogenizer. The homogenate was filtered through a single layer of gauze. The filtrate was centrifuged at 4°C at 3000 *g* for 10 min and the pellet was discarded. The supernatant was diluted by half with 5 mM Tris-HCl buffer (pH 7.4) and centrifuged at 55,000 *g* for 30 min at 4°C. The resulting pellet was resuspended in phosphate buffer (50 mM, pH 7.4) and centrifuged at 39000 *g* for 10 min. The final pellet was homogenized in Tris-HCl buffer (10 mM, pH 7.4) containing 0.154 M NaCl and 1.1 mM ascorbic acid to give a protein content of 300 to 400 μg/ml. Approximately 3 mg protein were obtained from 1 g coronary arteries. Protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall, (1951).

Membranes from guinea-pig left ventricle and from rat cerebellum were prepared according to the method of Minneman *et al.*, (1979a).

Preparation of ICYP

^{125}-Iodocyanopindolol was synthesized according to the method described by Engel *et al.* (1981).

Binding assay

Binding assay was carried out as described by Engel *et al.* (1981): 150 μl of the membrane suspension (derived from coronary arteries, ventricle or cerebellum) containing 50 μg protein, 50 μl of ICYP (30–40,000 ct min^{-1}) and 50 μl of the competing drug at various concentrations were incubated for 55 min at 37°C in 10 mM Tris-HCl, pH 7.4, containing 0.154 M NaCl and 1.1 mM ascorbic acid. Bound and free ligand were separated by rapid filtration through Whatman GF/B filters. Each filter was rapidly washed with an additional volume of 15 ml of the same buffer solution. The radioactivity of the filters was measured in an Autogamma Packard counter at 70% counting efficiency. Specific binding of the ligand was defined as the amount of the label bound in the absence of competing ligand minus the amount bound in the presence of (±)-propranolol 10^{-5} M. Specific binding was never less than 90% of total binding. For determination of the dissociation constant (K_D) and of the maximal number of binding

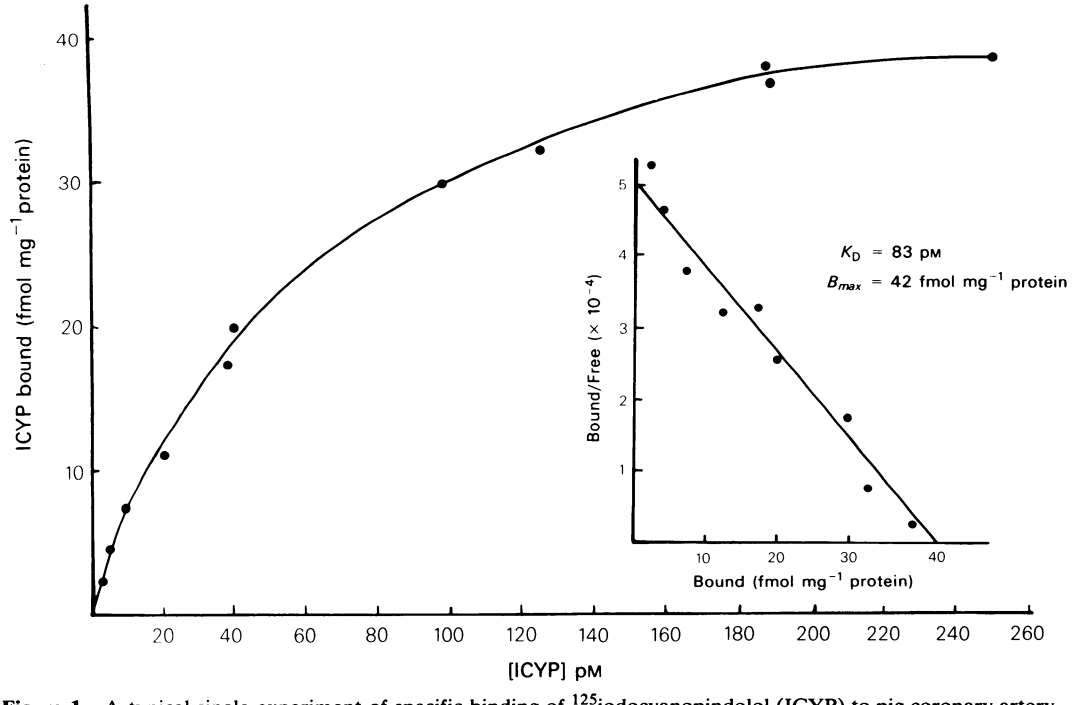

Figure 1 A typical single experiment of specific binding of ^{125}iodocyanopindolol (ICYP) to pig coronary artery membranes. The inset shows a Scatchard-plot. Mean K_D and B_{max} values for six experiments were respectively 90 ± 11 pM and 47 ± 6 fmol mg^{-1} protein.

sites (B_{max}), saturation experiments were performed by incubating 150 μl of membrane preparation with 50 μl of increasing concentrations (4 to 250 pM) of ICYP with and without (±) propranolol 10^{-5} M. Assay conditions were as described above.

Analysis of data

The experimental data given in the paper are means±s.e.mean. The equilibrium dissociation constant (K_D) and the maximal number of binding sites (B_{max}) were calculated from plots according to Scatchard. For the identification of the coronary receptors, the concentration-inhibition curves were transformed into modified Scatchard plots, i.e. by plotting percentage inhibition of binding versus percentage inhibition divided by the concentration of the competing agent. By analysing competition curves with a computer modelling technique (program SAAM; Berman & Weiss, 1967; Berman 1968) the proportions of β_1 and β_2-adrenoceptors can be determined. This technique enables an accurate estimate of the proportions of the two subtypes of receptors but involves one important assumption, namely that there are only two β-adrenoceptor subtypes.

Results

ICYP binding to pig coronary arteries

Specific ICYP binding to membranes from pig coronary arteries increased linearly with increasing membrane concentrations ranging from 10 to 100 μg protein per assay.

Binding assays were all performed at a concentration of 50 μg protein per assay. Specific ICYP binding rose with increasing ICYP concentrations ranging from 4 to 200 pM. A typical binding experiment is shown in Figure 1. Scatchard analysis of these data gave linear plots suggesting a single class of binding sites. Mean K_D value, calculated by non-linear regression analysis, was 90±11 pM ($n=6$) and the number of binding sites (B_{max}) 47±6 fmol mg^{-1} protein.

Inhibition of ICYP binding by drugs acting selectively on β_1- and β_2-adrenoceptors

Figure 2 shows the inhibition curves of ICYP binding by betaxolol (β_1-selective), zinterol and ICI 118551 (β_2-selective) and the transformation of these curves into modified Scatchard plots. Scatchard plots can be best described by two distinct straight lines for each agent, indicating the presence of two binding sites, one of low affinity and the other of high affinity. For betaxolol, the high affinity component is related to β_1-adrenoceptors. For zinterol and ICI 118551 the high affinity component is related to the presence of β_2-adrenoceptors. K_d values of each competitive agent are calculated from the slopes of each component. Computer modelling of these curves according to a two-class model, yields to the percentage distribution of β_1 versus β_2-adrenoceptors. The percentage distribution was 62:38 with betaxolol, 67:33 with zinterol and 63:37 with ICI 118551.

Since previous workers (Minneman, Hegstrand & Molinoff, 1979b) have shown that agonists can bind to a single type of receptor existing in low and high affinity states, we carried out binding assays for zinterol in which GTP was added, as described by Minneman *et al.* (1979b). Under these conditions too, the inhibition of ICYP binding by zinterol resulted in non-linear Scatchard plot with two components.

Table 1 gives results obtained on pig coronary arteries with betaxolol, zinterol and ICI 118551 compared with those obtained with the same agents on guinea-pig left ventricle, which is considered to contain only β_1-adrenoceptors, and on mature rat cerebellum, which is considered to contain only β_2-adrenoceptors (unpublished results).

Analysis of these data reveals a few discrepancies, especially for betaxolol. Selectivity calculated from affinities observed with tissues containing only one β-receptor subtype is more pronounced than that observed with pig coronary arteries where β_1- and β_2-adrenoceptors coexist. It should also be noted that the β_2-selectivity we observed for ICI 118551 in binding assays was only 9, whereas O'Donnell & Wanstall (1980) evaluating β_2-selectivity from pA_2 values obtained on guinea-pig trachea and atria found a value of 54 whilst Bilski, Dorries, Fitzgerald, Jessup, Tucker & Wale (1979), assessing β_2-selectivity from pA_2 values obtained on guinea-pig uterus and atria, found a selectivity of 123.

Discussion

Data presented here enable us to conclude that β_1 and β_2-adrenoceptors coexist in the large coronary arteries of the pig. Previous studies using classical pharmacological techniques have given conflicting results. Johannsen, Mark, Breen & Marcus (1978) and Yoshimoto, Fuchs, Ertl, Geiger, Frenken & Ruchholtz (1980) showed the existence of β_2-adrenoceptors in the canine coronary vessels *in vivo*. However, studies on intact animals are open to criticism since changes in cardiac work lead to simultaneous changes in the coronary flow (Parratt, 1971). In theory, at least, *in vitro* studies eliminate these difficulties. Baron, Speden & Bohr (1972), Johansson (1973) and Drew & Levy (1972), using dog or pig coronary arteries, concluded that the β-

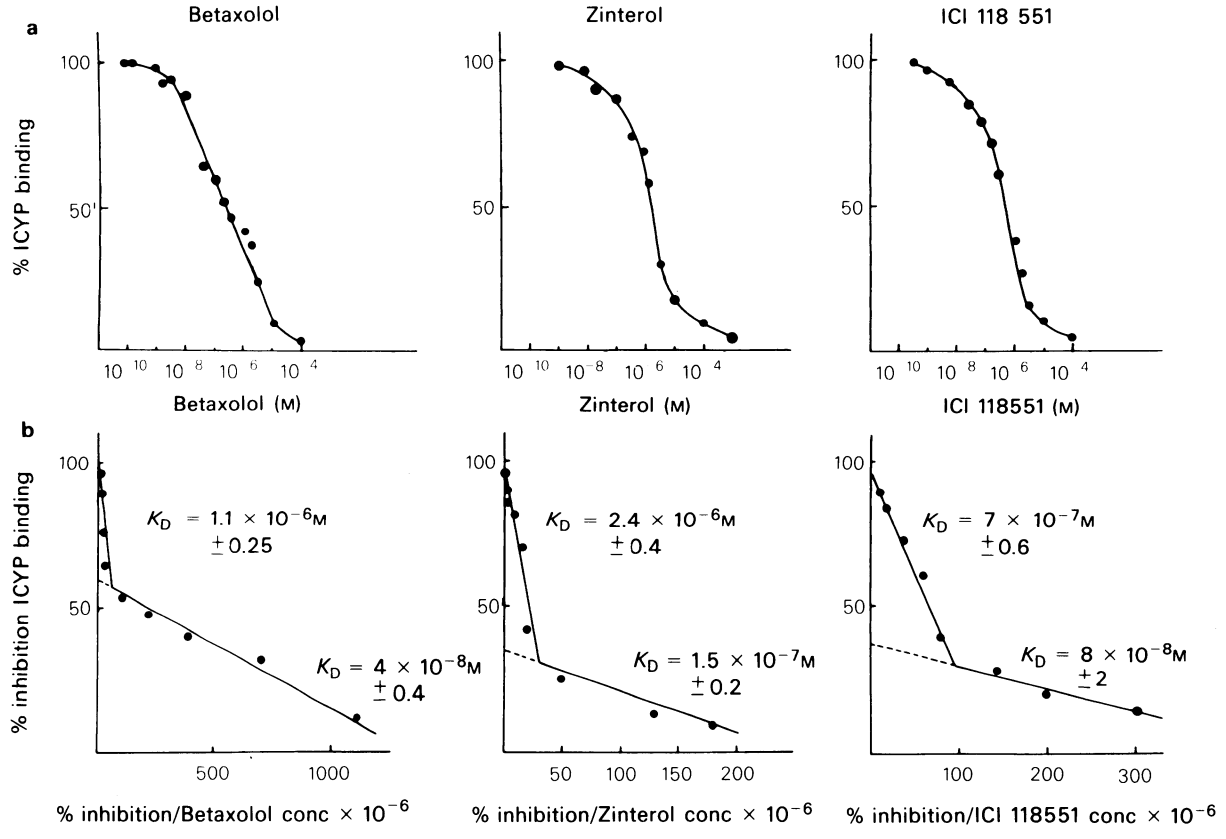

Figure 2 (a) Inhibition curves of ^{125}iodocyanopindolol (ICYP) binding by betaxolol, zinterol and ICI 118551. Each point is the mean of five experiments done in triplicate. (b) Modified Scatchard-plots. K_D values are means ± s.e.mean of five experiments.

Table 1 Comparison of dissociation constants of betaxolol, zinterol and ICI 118551 obtained from pig coronary arteries, from guinea-pig left ventricle and from mature rat cerebellum

	Pig coronary arteries						
Compound	*Receptor subtypes* (%) β_1/β_2	*Dissociation constant* (M) *from Scatchard-plots* β_1	β_2	*Selectivity* β_1/β_2	*Guinea-pig left ventricle dissociation constant* (M)*	*Mature rat cerebellum dissociation constant* (M)*	*Selectivity* β_1/β_2
Betaxolol	62/38	4.10^{-8} ±0,4	$1,1.10^{-6}$ ±0,25	27,5	$0,6.10^{-8}$ ±0,04	$1,2.10^{-6}$ ±0,2	200
Zinterol	67/33	$2,4.10^{-6}$ ±0,4	$1,5.10^{-7}$ ±0,2	0,062	$0,9.10^{-6}$ ±0,07	$0,2.10^{-7}$ ±0,03	0,02
ICI 118551	63/37	7.10^{-7} ±0,6	8.10^{-8} ±2	0,11	8.10^{-8} ±0,9	$0,9.10^{-8}$ ±0,2	0,11

*Unpublished results: values are means ± s.e.mean of five experiments done in triplicate. Membranes were prepared according to Minneman *et al.*, 1979a. Binding assays were performed by the method described for coronary arteries.

adrenoceptors were of the β_1-type. On the other hand, Bayer, Mentz & Förster (1974) working with pig isolated coronary arteries and Henry, Yokoyama & Fisher (1978) with dog arteries suggested that the β-adrenoceptors of the large coronary vessels are different from those of the myocardium and belong to the β_2-type. It appears that even studies on isolated arteries can give conflicting results. Moreover, this method can only be applied to the proximal parts of the large vessels taken from the epicardial region. Of course, this criticism also applies to the binding studies used in this paper. Nevertheless, ICYP is an ideal ligand for this kind of study; it binds to β_1 and β_2-adrenoceptors with equally high affinity and has a very low non-specific binding. With betaxolol, the proportion of high affinity sites (β_1) is 62%; zinterol and ICI 118551 produce an exact 'mirror image', the proportions of high affinity sites (β_2) being respectively 33% and 37%. The use of three different drugs with different affinities and selectivities yielded essentially constant percentages of β_1 and β_2-adrenoceptors; this is consistent with the assumption that there are only two receptor subtypes.

In contrast, the β_1 and β_2 affinities observed with tissues containing only one subtype of β-receptor do not completely tally with affinities observed with tissues containing both subtypes. It can be assumed that the tissue distribution of agents with different physicochemical properties could vary substantially from organ to organ and from species to species. The coexistence of two β-receptor subtypes in the coronary arteries does not enable conclusions to be drawn regarding a physiological function of these receptors. However, the belief that large coronary vessels are regulated by β_1-adrenoceptor-mediated increases in myocardial metabolic demands and by β_2-adrenoceptor-mediated vasodilatation (Vatner & Macho, 1981), may no longer be valid.

Reprint requests to J.V., please.

References

BARON, G.D., SPEDEN, R.N. & BOHR, D.F. (1972). Beta-adrenergic receptors in coronary and skeletal muscle arteries. *Am. J. Physiol.*, **23,** 878–881.

BAYER, B.L., MENTZ, P. & FORSTER, W. (1974). Characterization of the adrenoceptors in coronary arteries of pigs. *Eur. J. Pharmac.*, **29,** 58–65.

BERMAN, M. (1968). The application of multicomportmental analysis to problems of clinical medicine. *Ann. intern. Med.*, **68,** 423–448.

BERMAN, M. & WEISS, M.F. (1967). *SAAM Manual*-NIH PHS Publication 1703, Bethesda, Maryland, U.S.A.

BILSKI, A., DORRIES, S., FITZGERALD, J.D., JESSUP, R., TUCKER, H. & WALE, J. (1979). ICI 118551, a potent beta$_2$-adrenoceptor antagonist. *Br. J. Pharm.*, **69,** 292P–293P.

BOBIK, A. (1982). Identification of alpha-adrenoceptor subtypes in dog arteries by ^{3}H-yohimbine and ^{3}H-prazosin. *Life Sci.*, **30,** 219–228.

CARMAN-KRZAN, M. (1980). Alpha-adrenergic receptors in beef aortic membranes characterised by [^{3}H]dihydroergocryptine binding. Archs *int. Pharmacodyn.*, **246,** 237–250.

DICKINSON, K.E.J., NAHORSKI, S.R. & WILLCOCKS, A.L. (1981). Serotonin recognition sites are labelled in cerebral cortex by the beta-adrenoceptor antagonist [^{125}I]-hydroxybenzylpindolol. *Br. J. Pharmac.*, **72,** 165P.

DREW, G.M. & LEVY, G.P. (1972). Characterization of the coronary vascular beta-adrenoceptor in the pig. *Br. J. Pharmac.*, **46,** 348–350.

ENGEL, G., HOYER, D., BERTHOLD, R. & WAGNER, H. (1981). (±) ^{125}Iodocyanopindolol, a new ligand for beta-adrenoceptors: identification and quantification of subclasses of beta-adrenoceptors in guinea-pig. *Naunyn-Schmiedebergs Arch. Pharmac.*, **317,** 277–285.

FUDER, H., NELSON, W.L., MILLER, D.D. & PATIL, P.N. (1981). Alpha-adrenoceptors of rabbit aorta and stomach fundus. *J. Pharmac. exp. Ther.*, **217,** 1–9.

HENRY, P.D., YOKOYAMA, M. & FISHER, S. (1978). Hypoxic coronary contraction mediated by beta$_2$-adrenergic receptors. *Am. J. Cardiol.*, **41,** 357–360.

JOHANNSEN, U.J., MARK, A.L., BREEN, D.R. & MARCUS, M.L. (1978). Effects of stimulation of coronary beta$_2$-receptors on transmural myocardial blood flow. *Fedn. Proc.*, **37,** 236.

JOHANSSON, B. (1973). The beta-adrenoceptors in the smooth muscle of pig coronary arteries. *Eur. J. Pharmac.*, **24,** 218–224.

LANDS, A.M., ARNOLD, A., MC AULIFF, J.P., LUDUENA, F.P. & BROWN, T.G. (1967). Differentiation of receptor systems activated by sympathomimetic amines. *Nature (Lond.)*, **214,** 597–598.

LIMAS, C.J. & LIMAS, C. (1979). Decreased number of beta-adrenergic receptors in hypertensive vessels. *Biochim. biophys. Acta*, **582,** 533–536.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193,** 265–275.

MINNEMAN, K.P., HEDBERG, A. & MOLINOFF, P.B. (1979a). Comparison of beta-adrenergic receptor subtypes in mammalian tissues. *J. Pharmac. exp. Ther.*, **211,** 502–508.

MINNEMAN, K.P., HEGSTRAND, L.R. & MOLINOFF, P.B. (1979b). Simultaneous determination of beta$_1$ and beta$_2$-adrenergic receptors in tissues containing both receptor subtypes. *Mol. Pharmac.*, **16,** 34–46.

O'DONNELL, S.R. & WANSTALL, J.C. (1980). Evidence that ICI 118551 is a potent, highly beta$_2$-selective adrenoceptor antagonist and can be used to characterize beta-adrenoceptor populations in tissues. *Life Sci.*, **27,** 671–677.

PARRATT, J.R. (1971). Pharmacological aspects of the coronary circulation. *Prog. med. Chem.*, **6,** 11–66.

RUGG, E.L., BARNETT, D.B. & NAHORSKI, S.R. (1978). Coexistence of beta$_1$ and beta$_2$-adrenoceptors in mam-

malian lung: evidence from direct binding studies. *Mol. Pharmac.*, **14,** 996–1005.

SPORN, J.R. & MOLINOFF, P.B. (1976). Beta-adrenoceptors in rat brain. *J. cycl. Nucleotide Res.*, **2,** 149–161.

TSAI, B.S. & LEFKOWITZ, R.J. (1978). ^{3}H-dihydroergocriptine binding to alpha-adrenergic receptors in canine aortic membranes. *J. Pharmac. exp. Ther.*, **204,** 606–614.

VATNER, S.F. & MACHO, P. (1981). Regulation of large coronary vessels by adrenergic mechanisms in conscious dogs. *Basic Res. Cardiol.*, **76,** 508–517.

WOODCOCK, E.A., OLSSON, C.A. & JOHNSTON, C.I. (1980). Reduced vascular beta-adrenergic receptors in deoxycorticosterone salt hypertensive rats. *Biochem. Pharmac.* **29,** 1465–1468.

YOSHIMOTO, N., FUCHS, M., ERTL, G., GEIGER, M., FRENKEN, M. & RUCHHOLTZ, E.R. (1980). Effects of propranolol on the arterial and arteriolar segments of the canine coronary system. *Arch. Pharmac.*, **313,** suppl. R48.

(*Received September 15, 1982. Revised January 5, 1983.*)