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The β_1-adrenoceptor antagonist, betaxolol, is not released from the heart of the anaesthetized dog during sympathetic nerve stimulation

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1 We investigated the hypothesis that the β_1-adrenoceptor antagonist, betaxolol, can be accumulated by cardiac sympathetic nerve endings and then released together with noradrenaline during accelerans nerve stimulation.

2 Dogs were chronically treated with betaxolol (1 mg kg^{-1} daily, s.c.) for 7 days. Twenty four hours after the last dose, there was a significant retention of betaxolol in the heart of these dogs treated chronically with the β_1-adrenoceptor antagonist. However, during *in vivo* accelerans nerve stimulation, the concentration of betaxolol in the coronary sinus was not modified, whereas the noradrenaline concentration increased significantly.

3 Chronic betaxolol treatment antagonized the tachycardia induced by electrical stimulation of the cardiac accelerator nerves or by intravenous isoprenaline. However, the tachycardia induced by nerve stimulation was not antagonized to a greater extent than that induced by isoprenaline.

4 These findings are discussed in relation to a similar *in vivo* study in dogs treated with propranolol, in which the drug was found to be released into the coronary circulation during stimulation of the accelerans nerve.

Introduction

The antihypertensive actions of β-adrenoceptor antagonists like propranolol have been shown to persist for longer than would be suggested by the rate of decrease of plasma concentrations, after withdrawal from chronic treatment with the drugs (Pritchard, 1964; Pritchard & Gillam, 1964; Brudin *et al.*, 1976). This phenomenon is not well understood (Amer, 1977; Pritchard, 1978; Buckingham & Hamilton, 1979), although the drug-induced reduction in the vasoconstrictor response evoked by sympathetic nerve stimulation has been reported to persist in several preparations after withdrawal from repeated administration of β-adrenoceptor blocking drugs (Lewis, 1974; Ljung *et al.*, 1975; Russel *et al.*, 1983).

Propranolol accumulates in the tissues, and its subsequent release together with noradrenaline during sympathetic nerve stimulation has been reported *in vivo* in dogs (Daniell *et al.*, 1979; Russel *et al.*, 1983). These authors suggested that propranolol was released from the noradrenergic nerve terminals. Stimulation-evoked release of tritiated propranolol has been observed *in vitro* with rat cortical synaptosomes (Bright *et al.*, 1985) and rat atrial slices (Vidal & Langer, 1986). The selective β_1-adrenoceptor antagonist, betaxolol (Cavero *et al.*, 1983) is also taken up by rat atrial slices and subsequently released together with noradrenaline by electrical stimulation under *in vitro* experimental conditions (Petruzzo *et al.*, 1986). In order to relate this finding to the situation *in vivo*, we have examined whether betaxolol, accumulated in dog heart during chronic treatment, is released at the same time as noradrenaline when postganglionic sympathetic nerves are stimulated.

A preliminary account of this work has been presented at a British Pharmacological Society Meeting (Duval *et al.*, 1986).

Methods

Experiments were conducted on Mongrel dogs (10–20 kg) of either sex, which had been pretreated subcutaneously with betaxolol once a day for 7 days, at a dose of 1 mg kg^{-1}. Twenty four hours after the last

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dose, the animals were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}, i.v. followed by an infusion of 6 mg kg^{-1} h^{-1}, i.v.) and ventilated with room air by a Braun-Melsungen pump.

A polyethylene catheter was placed in a femoral artery, and connected to a Statham pressure transducer (type PD 23). Heart rate changes were derived from the blood pressure signal (Grass tachograph), and both heart rate and blood pressure were recorded on a Grass polygraph.

The dogs were bivagotomized and prepared for postganglionic ansa-subclavia stimulation as previously described (Dubocovich *et al.*, 1980). Two experimental groups were then established.

Group 1: betaxolol and noradrenaline concentrations in blood plasma

Experimental protocol The circumflex coronary artery was dissected free, and a precalibrated electromagnetic flow transducer (Carolina Medical Electronics) was placed around the isolated segment to monitor continuously the coronary blood flow. The coronary sinus was cannulated via a jugular vein in order to obtain blood samples, the tip of the cannula being placed at least 2 cm from the ostium. The femoral artery which was not used for monitoring blood pressure was also cannulated, to enable sampling of arterial blood from the aortic arch.

Blood samples were taken simultaneously from the coronary sinus and aortic arch, 30 min and 2 min before postganglionic cardiac accelerator nerve stimulation (3 min, 4 Hz, supramaximal voltage: 12–15 V). Further samples were taken 1, 2 and 3 min after beginning the stimulation, and 15 min after stimulation had ceased.

Betaxolol and noradrenaline determinations Betaxolol levels in plasma from the coronary venous and aortic blood samples were determined by gas chromatography-mass spectrometry. The compound was extracted and converted to the trimethylsilyl derivative as previously described (Hermann *et al.*, 1984). Mass spectrometry was performed in the electron impact mode at a resolution of 3000 (10% valley), the ions monitored being m/z 72 (betaxolol) and m/z 74 (deuterated internal standard). This method gives a lower limit of useful measurement than the earlier one (Hermann *et al.*, 1984), where chemical ionization was used. Despite the use of low-mass ion fragments for selected ion monitoring, the specificity was found to be adequate (Lee, Coste & Allen, unpublished). Plasma noradrenaline was measured by h.p.l.c. with electrochemical detection.

At the end of some experiments, the right atrium and left ventricle were excised, in order to measure the concentration of betaxolol remaining in these tissues.

Group 2: positive chronotropic effects of accelerans nerve stimulation and isoprenaline injections

In untreated dogs, the postganglionic cardiac accelerator nerves were stimulated every 3 min, for 1 min periods, at 0.25, 0.5, 1, 2 and 4 Hz (1 ms, supramaximal voltage 10–15 V). Isoprenaline was injected at 0.03, 0.1, 0.3 and 1 ng kg^{-1} i.v.

In chronically treated dogs that had received betaxolol (1 mg kg^{-1} daily, s.c. for 7 days), the same protocol was applied. In this case, accelerans nerve stimulation was carried out at additional frequencies of 8, 16 and 32 Hz. Isoprenaline was injected at the additional doses of 3 and 10 μg kg^{-1}, i.v.

In a further series of experiments, the tachycardic effect induced either by cardiac accelerator nerve stimulation or isoprenaline was determined in untreated dogs, before and after acute cumulative doses of betaxolol at 0.03, 0.1 and 0.3 mg kg^{-1}, i.v.

In another experimental series, dogs chronically treated with betaxolol (1 mg kg^{-1}, s.c. daily for 7 days) received an additional acute dose of 0.3 mg kg^{-1}, i.v. of betaxolol just before accelerans nerve stimulation.

Drugs used

Betaxolol (synthesized by the Chemistry Department of L.E.R.S.) and (−)-isoprenaline bitartrate (Sigma) were used.

Results

Effect of chronic treatment with betaxolol on noradrenaline and betaxolol released during accelerans nerve stimulation

Plasma betaxolol concentrations, in dogs pretreated for 7 days with 1 mg kg^{-1} daily s.c., were 9.53 ± 2.1 ng ml^{-1} (range 0.67–21.19 ng ml^{-1}, $n = 10$). Tissue: plasma ratios were 32 for the right atria, and 39 for the left ventricle ($n = 4$). These results are in agreement with previous data from our laboratories (Ferrandes *et al.*, 1983).

The concentrations of betaxolol in plasma obtained from the coronary sinus of these chronically treated dogs were not significantly affected by accelerans nerve stimulation (Friedman's test). Concentrations of betaxolol in the aortic plasma samples were likewise unaffected (Table 1). In contrast, the concentrations of noradrenaline mea-

Table 1 Effects of accelerans nerve stimulation (4 Hz, 3 min) on the concentrations of betaxolol (BTXL) and noradrenaline (NA) in blood from coronary sinus and aorta in dogs treated chronically with betaxolol

		Control values		*% of control*			
		30 min before stim.	*Before stim.* 2 min	*During stim. (4 Hz)* 1 min	2 min	15 min	*After stim.*
BTXL concentrations ($ng\,ml^{-1}$)	Coronary sinus	9.36 ± 2.2	97.5 ± 1.4	96.4 ± 1.6	100.5 ± 3	102.8 ± 3.4	91.8 ± 2.6
	Aorta	9.53 ± 2.1	97.5 ± 1.5	101.7 ± 2.1	97.2 ± 2.6	101.4 ± 3.6	98.2 ± 3.9
NA concentrations ($ng\,ml^{-1}$)	Coronary sinus	0.45 ± 0.1	89.3 ± 7.5	348.3 ± 74*	882 ± 296**	354.4 ± 87*	230 ± 58
	Aorta	0.40 ± 0.1	132.7 ± 19	262 ± 81*	377 ± 84*	295 ± 70*	138 ± 23
Coronary blood flow $ml\,min^{-1}$		22.1 ± 3	100 ± 3	138 ± 5	128 ± 4	116 ± 4	106 ± 4

The concentrations determined 30 min before stimulation were taken as control values. Betaxolol was administered daily for 7 days ($1\,mg\,kg^{-1}$, s.c.) and the experiment was carried out 24 h after the last injection. Values are mean ± s.e.mean. $n = 10$.
Statistical comparisons were performed using Friedman's test: ** $P < 0.001$; * $P < 0.05$.

sured in coronary sinus plasma increased about 7 fold during nerve stimulation. Noradrenaline concentrations were also increased in aortic plasma, but to a lesser extent (about 3 fold; Table 1).

Effect of betaxolol pretreatment on the tachycardia induced by electrical stimulation and by intravenous isoprenaline

Following chronic betaxolol treatment ($1\,mg\,kg^{-1}$ daily, s.c., 7 days), mean arterial blood pressure was slightly but significantly ($P < 0.05$) lower than that of a control series of anaesthetized dogs (102 ± 6 mmHg, $n = 10$, 122 ± 3 mmHg, $n = 18$, respectively). However, heart rate was either not modified or only very slightly increased by chronic betaxolol treatment.

Following the administration of betaxolol ($1\,mg\,kg^{-1}$, s.c. daily for 7 days), the β-adrenoceptor antagonist effects were the same for the tachycardia induced by accelerans nerve stimulation as for the tachycardia induced by exogenous isoprenaline (Figure 1). Likewise, the dose-response curves to isoprenaline and the frequency-response curves to accelerans nerve stimulation were antagonized to the same extent in dogs treated acutely with 0.03, 0.1 and $0.3\,mg\,kg^{-1}$ of betaxolol given intravenously (Figure 2). The degree of β-adrenoceptor blockade produced by chronic treatment, was similar to that produced by the acute dose of $0.03\,mg\,kg^{-1}$, i.v. of betaxolol (Figures 1 and 2).

A single dose of $0.3\,mg\,kg^{-1}$ betaxolol, given intravenously to dogs chronically pretreated with betaxolol produced no additional blockade of the tachycardic response (induced either by electrical stimulation or by isoprenaline injections), when compared with untreated dogs (results not shown).

Discussion

The present results indicate that following withdrawal from chronic betaxolol treatment, the drug is readily detectable in the heart of the dog. However, betaxolol was not released by sympathetic nerve stimulation in our experiments and this is in contrast

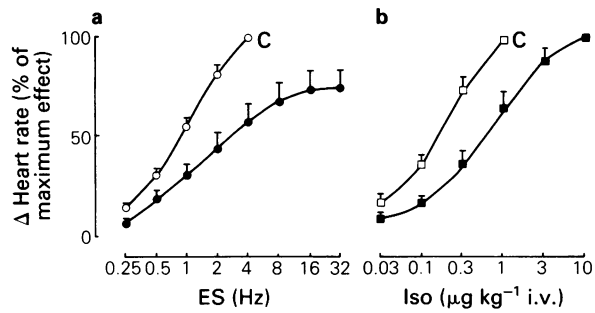

Figure 1 Effect of chronic treatment with betaxolol ($1\,mg\,kg^{-1}$, daily, s.c. for 7 days) on the tachycardia induced by electrical stimulation (ES), and by isoprenaline (Iso) in anaesthetized dogs. Response curves for the increase in heart rate were obtained: (a) with postganglionic stimulation in untreated dogs (○, $n = 18$) and in betaxolol-treated dogs (●, $n = 9$); (b) i.v. injections of isoprenaline in untreated dogs (□, $n = 14$) and in betaxolol-treated dogs (■, $n = 9$). Ordinates: increase in heart rate expressed as % of maximum response evoked at 4 Hz (111 ± 4 beats min^{-1}, a) and $1\,\mu g\,kg^{-1}$, i.v. isoprenaline (102 ± 4 beats min^{-1}, b) in untreated animals. Abscissae: frequency of electrical stimulation (ES, Hz, a) doses of isoprenaline (Iso $\mu g\,kg^{-1}$, i.v., b). Values shown are means with s.e.mean shown by vertical lines.

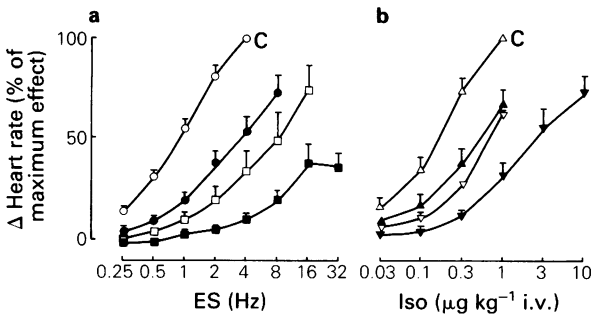

Figure 2 Effect of acute administration of betaxolol on the tachycardia induced by electrical stimulation and by isoprenaline in anaesthetized dogs. Response curves for the increase in heart rate were obtained: (a) with post-ganglionic nerve stimulation in untreated dogs (○, $n = 18$) and following the acute administration of betaxolol, 0.03 (●), 0.1 (□) and 0.3 (■) mg kg^{-1}, i.v. ($n = 5$); (b) i.v. injections of isoprenaline in untreated dogs (△, $n = 14$) and following the acute administration of betaxolol, 0.03 (▲), 0.1 (▽) and 0.3 (▼) mg kg^{-1}, i.v. ($n = 5$). Ordinates: increase in heart rate expressed as % of maximum response evoked at 4 Hz (111 ± 4 beats min^{-1}, a) or 1 μg kg^{-1}, i.v. isoprenaline (102 ± 4 beats min^{-1}, b) in untreated animals. Abscissae: frequency of electrical stimulation (ES, Hz, a) doses of isoprenaline (Iso μg kg^{-1}, i.v., b). Values shown are means with s.e.mean indicated by vertical lines.

with the findings of Daniell *et al.* (1979), who demonstrated a release of propranolol under similar experimental conditions. The release of noradrenaline elicited by accelerans nerve stimulation was present both in our experiments and in those by Daniell *et al.* (1979). One difference between the studies concerns the concentrations of the drugs in the plasma. Daniell *et al.* (1979) used a dosage schedule for propranolol (120 mg per dog every 6 h, p.o.) that gave a mean plasma concentration of 47 ng ml^{-1} (181 nmol l^{-1}), although the authors do not indicate the time interval between the last dose and the measurement of plasma propranolol concentrations. We used a low dose of betaxolol (1 mg kg^{-1} daily, s.c.) giving a mean plasma concentration of 9.5 ng ml^{-1}(31 nmol l^{-1}) measured 24 h after the administration of the last dose, in order to simulate the conditions following acute withdrawal from β-adrenoceptor blockade after chronic treatment. However, under both experimental conditions, the plasma concentrations were higher than the pA_2 values for β_1-receptor-mediated antagonism of isoprenaline-induced tachycardia in guinea-pig atria (pA_2 of 8.60 and 8.57 for propranolol and betaxolol respectively; Cavero *et al.*, 1983).

Separate experiments in which a higher dose (3 mg kg^{-1} daily) of betaxolol was used gave a plasma concentration of 56 ± 5 ($n = 4$) ng ml^{-1} (182 nmol l^{-1}), but again there was no release of betaxolol during accelerans nerve stimulation (data not shown). These concentrations of the β_1-selective antagonist betaxolol were still too low for antagonism at β_2-receptors (Cavero *et al.*, 1983), while propranolol is a non-selective antagonist. However, there is no evidence to indicate that the stimulation-evoked release of propranolol is associated with an effect involving blockade of β_2-receptors.

The *in vitro* release of [^{3}H]-betaxolol by electrical depolarization from superfused atrial slices appears to be restricted to this tissue because we have not observed this effect with slices of ventricle, although tritiated noradrenaline is released from slices of both atrial as well as ventricular tissues (Arbilla *et al.*, 1986). Tritiated propranolol gave essentially the same results as tritiated betaxolol in this model (Arbilla *et al.*, 1986), suggesting that the effects observed *in vitro* are not critically dependent on the physico-chemical properties or the β_1-adrenoceptor selectivity of the drug being used.

It is difficult to support the view of Daniell *et al.* (1979) that β-adrenoceptor antagonists are released from storage sites in sympathetic nerve endings, since except in atrial slices there is no release from other noradrenergically innervated tissues such as the vas deferens (Lewis, 1977) or the ventricles (Arbilla *et al.*, 1986). A unique feature of atrial tissue is the presence of secretory granules that contain the atrial natriuretic peptides (ANP; see Sonnenberg, 1986). While there is no direct supporting evidence, it is tempting to speculate that these granules may be the site of release of the drugs. Basic lipophilic drugs such as propranolol, which readily enter the tissues, are taken up by acidic (negatively charged) intracellular organelles such as the lysosomes of alveolar macrophages (see for example Vestal *et al.*, 1980). Since endocrine secretory granules have an acidic interior (Orci *et al.*, 1987), lipophilic β-adrenoceptor antagonists are likely to be accumulated by the atrial granules, from which they would be released.

While there is some controversy concerning the physiological stimuli for the release of ANP (Kuchel *et al.*, 1987), peripheral sympathetic nervous activity is likely to be one of the factors involved. Isolated rat atria released ANP in response to superfusion with adrenoceptor agonists, (Schiebinger *et al.*, 1987) and the release was mediated predominantly by β-adrenoceptors, and inhibited by cholinoceptor agonists. It is not known whether the electrical stimulation applied to atrial slices during our release studies *in vitro* (Arbilla *et al.*, 1986; Petruzzo *et al.*, 1986) would evoke the concomitant release of ANP. In the anaesthetized dog, ANP was not released in response to sympathetic stimulation (Ledsome *et al.*, 1986). This negative result might have been due to

the effects of anaesthesia or surgery (Schiebinger *et al.*, 1987). In fact, there are technical difficulties associated with the study *in vivo* of the release of ANP, or other substances which may be stored in the ANP-containing granules.

If the results obtained with [^{3}H]-betaxolol in tissue slices (Petruzzo *et al.*, 1986; Arbilla *et al.*, 1986) can be extrapolated to the situation *in vivo*, stimulation-evoked release of a reasonable quantity of betaxolol from the atria in the dog would still be insufficient to cause a measurable rise in the concentration of betaxolol in the coronary sinus. Our negative results with betaxolol in the dog *in vivo* are consistent with the suggestion that coronary sinus blood represents mainly the venous drainage of the ventricles. It should be noted that although Daniell *et al.* (1979) reported that propranolol concentrations in the coronary sinus were increased during stimulation, they compared the means of successive measurements using Student's *t* test, in the evaluation of a difference of marginal statistical significance.

Our pharmacological results indicate that even if betaxolol had been released during sympathetic nerve stimultion, the amounts were insufficient to produce a selective enhancement of β-adrenoceptor blockade because the positive chronotropic response curve elicited by accelerans nerve stimulation was not antagonized significantly more than the dose-response curve elicited by isoprenaline. One possible explanation for this result is that the concentrations of noradrenaline released during stimulation are probably high enough to compete with the additional amount of β-adrenoceptor antagonist.

The mechanism of action of the antihypertensive effects of β-adrenoceptor antagonists is not yet clearly established. In addition the residual effects after drug withdrawal are well documented (Brudin *et al.*, 1976) but remain to be explained. Recently, Wellstein *et al.* (1985) showed that the effect of propranolol on exercise-induced tachycardia in man is determined simply by competition of extracellular noradrenaline and propranolol for the receptors, and that it is not necessary to invoke the presence of 'deep pools' of the β-adrenoceptor antagonist.

In summary, our study demonstrates that following one week administration, the β_1-selective antagonist betaxolol is accumulated in the dog heart but it is not released in response to accelerans nerve stimulation.

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