Presynaptic β -adrenoceptors in rat atria: evidence for the presence of stereoselective β_1 -adrenoceptors

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- 1 Presynaptic β -adrenoceptor activity was studied in rat isolated atria, previously loaded with [3 H]-noradrenaline. The stimulation-induced release of 3 H transmitter was measured in the presence of cocaine, and adrenaline was used as a facilitatory β -adrenoceptor agonist.
- 2 Adrenaline (0.1 and 2 nM) increased, by about 50%, the evoked efflux of tritium. With phenoxybenzamine present, the same activity was shown with 10 nM adrenaline.
- 3 The β_2 -selective adrenoceptor blocking drugs: IPS 339 and ICI 118 551 caused a concentration-dependent decrease in the activity of adrenaline. Cardioselective β -blocking drugs: acebutolol, betaxolol, nebivolol and its isomers (R 67 138 and R 67 145) also reduced dose-dependently the agonistic action of adrenaline. The order of potency for nebivolol and its isomers was R 67 138 > nebivolol > R 67 145. The activity of pindolol was not concentration-dependent. The inhibitory effect of acebutolol was also observed in the presence of blockade of α -adrenoceptors.
- 4 The postsynaptic β -adrenoceptor blocking activity of nebivolol and its isomers was studied in pithed rats. They reduced isoprenaline-induced tachycardia without altering hypotensive responses. The order of potency was: R 67 138 > nebivolol > R 67 145.
- 5 It is concluded that in rat isolated atria, presynaptic β_2 - and β_1 -adrenoceptors coexist and that facilitatory β_1 -adrenoceptors are stereospecific.

Introduction

The existence of presynaptic β -adrenoceptors was first proposed by Adler-Graschinsky & Langer (1975), who demonstrated the facilitatory effect of the β -agonist, isoprenaline, on the impulse-evoked release of noradrenaline. Further support for the existence of these receptors was obtained from a number of mainly *in vitro* experiments, in various tissues including guinea-pig and rat atria (Majewski *et al.*, 1980; 1981), rat portal vein (Dahlöf *et al.*, 1978; Westfall *et al.*, 1979), cat spleen (Celuch *et al.*, 1978), and human blood vessels (Stjärne & Brundin, 1976). Their presence was shown by the increase in release of noradrenaline during low frequency nerve stimulation by, most frequently, isoprenaline and adrenaline, with inhibition of neuronal uptake by cocaine or desipramine. The facilitatory mechanism mediated by these receptors was inhibited by β -adrenoceptor blocking drugs.

Attempts have been made to characterize the presynaptic β -adrenoceptors in terms of β_1 or β_2

subtypes, by use of selective agonists and antagonists. The results are somewhat controversial, possibly because the selectivity of drugs used is only relative and/or the facilitatory β -adrenoceptors differ with tissue or species.

Finally, the stereospecific nature of presynaptic β -adrenoceptors has been little studied. Celuch *et al.* (1978) in the cat isolated perfused spleen showed an increase in [3 H]-noradrenaline overflow induced by (–)-isoprenaline but not by the (+)-isomer. Using stereoselective β antagonists, Misu *et al.* (1981, 1983) demonstrated the greater antagonistic activity of (–)-propranolol and (–)-metoprolol on the effects of salbutamol in guinea-pig pulmonary artery. On the other hand, Kalsner (1980) did not find differences between (+)- and (–)-propranolol activities in guinea-pig atria, using isoprenaline as a β -agonist. More recently, Chang *et al.* (1986) demonstrated the stereoselectivity of presynaptic β_1 -adrenoceptors in the rat hypothalamus.

The 3 ml Krebs-Henseleit solution samples were added to 10 ml scintillation fluid (Picofluor). Radioactivity was counted in a Packard Tricarb 4530 liquid scintillation counter.

Figure 2 The effect of the β -adrenoceptor blocking drugs: propranolol (P), acebutolol (Ac), IPS 339 (IPS) and practolol (Pr), on adrenaline (2 nM, Ad)-induced increase in stimulation-evoked efflux of tritium from rat isolated atria (C = control group). There were two periods of stimulation (3 Hz, 15 s). The efflux of tritium in the second period was expressed as a percentage of that in the first (S_2/S_1 %). The vertical bars represent s.e.means. ** $P < 0.01$; *** $P < 0.001$ vs adrenaline alone.

Pithed rats

Three groups of rats were pithed (Shipley & Tilden, 1947) after pentobarbitone anaesthesia. They were artificially respired (Harvard rodent respirator, 1 ml 100 g⁻¹, 50 strokes min⁻¹) and mean blood pressure recorded from the left carotid artery (Narco physiograph MK III). Heart rate was derived from the arterial pulse signal (Narco biotachometer). The pudendal vein was cannulated for intravenous administration of drugs.

The maximal tachycardia and hypotensive effect produced by isoprenaline (1 μ g kg⁻¹) were measured before and 5, 10 and 20 min after the i.v. injection of a β -adrenoceptor antagonist.

Drugs

The following drugs were used: cocaine hydrochloride (Coopération pharmaceutique française), [³H]-noradrenaline (C.E.A., 1480 kBq mmol⁻¹), noradrenaline bitartrate (Aguettant), adrenaline hydrochloride (Sigma), isoprenaline chlorhydrate (Isuprel, Winthrop), phenoxybenzamine (S, K & F), acebutolol hydrochloride (Rhône-Poulenc), *t*-butyl-amino-3-ol-2 propylloximino-9 fluorene hydrochloride (IPS 339) (gift from Dr Wermuth), betaxolol (Synthelabo), pindolol (Sandoz), propranolol hydrochloride, practolol and ($\pm$)-erythro-3-isopropylamino-1-(7-methyl-4-indanyloxy)-2-butanol hydrochloride (ICI 118 551)

(ICI Pharma), nebivolol, R 67 138 and R 67 145 (Janssen).

Statistical analysis

Results are expressed as mean $\pm$ s.e.mean. n is the number of assays. Significance tests were performed by Student's t test.

Results

Rat isolated atria

Stimulation-induced efflux of tritium in control experiments with two or three periods of stimulation In those experiments with two periods of stimulation, the ratio (S_2/S_1) $\times 100$, was 90 ± 5.7 ($n = 11$).

When three successive periods of stimulation were applied, the ratios (S_2/S_1) $\times 100$ and (S_3/S_1) $\times 100$ were 91 ± 4.4 and 97 ± 8 respectively ($n = 7$). As these ratios were not significantly different, the mean value of all the data was used for subsequent calculations: 94 ± 4.3 ($n = 14$).

In control experiments with phenoxybenzamine, the stimulation-induced release of tritium was increased 3.3 fold as compared with the overflow in the absence of α -adrenoceptor blockade. The ratio (S_2/S_1) $\times 100$ was 83 ± 7 ($n = 5$).

Effect of adrenaline on the stimulation-induced efflux of [³H]-noradrenaline In the first series of experiments, 0.05 nM adrenaline did not enhance [³H]-noradrenaline released by field stimulation: (S_2/S_1) $\times 100 = 108 \pm 10.7$ ($n = 6$). By contrast, 0.1 and 2 nM significantly increased it: (S_2/S_1) $\times 100 = 135 \pm 7.6$ ($n = 5$) and 150 ± 6.3 ($n = 6$) respectively ($P < 0.001$ versus control assay).

The same enhancement was observed in the second series of experiments with a lower adrenaline concentration (0.1 nM) and three periods of stimulation. The ratios (S_2/S_1) $\times 100$ and (S_3/S_1) $\times 100$ were 141 ± 5.7 and 154 ± 15 ($n = 7$). These data did not differ in value, thus in further calculations where the effect of a drug was being compared with the adrenaline group, the mean value of the response elicited by the second and the third period of stimulation was used: 147 ± 8 ($n = 14$). It differed significantly from control: 94 ± 4.3 ($P < 0.001$).

Three adrenaline concentrations were investigated in the presence of α -adrenoceptor blockade: 0.1 nM, 1 nM and 10 nM. In these conditions the ratios (S_2/S_1) $\times 100$ were respectively: 85 ± 8 ($n = 7$), 91 ± 3.8 ($n = 5$) and 121 ± 7.8 ($n = 5$). This last increase was significant ($P < 0.01$ versus control assay: 83 ± 7).

Effect of β -adrenoceptor blocking drugs on adrenaline-induced enhancement of [³H]-noradrenaline

efflux In the first series of experiments, the following compounds were added to the Krebs solution as previously described: propranolol (10 nM), acebutolol (1, 3 and 30 nM), IPS 339 (1 and 10 nM), practolol (3 and 30 nM). Adrenaline concentration was 2 nM.

Each of the β -blocking drugs induced a reduction in the stimulating effect of adrenaline (Figure 2). A dose-dependent activity was observed with both the selective β_1 -blocking drug, acebutolol, and the β_2 -selective blocking agent IPS 339. A 100% inhibition of the action of adrenaline was achieved with acebutolol, 3 nM, and IPS 339, 1 nM. By contrast, 3 and 30 nM practolol caused the same antagonistic activity, exceeding 100%.

In the second series of experiments, six β -adrenoceptor blocking drugs sharing different specificities were assayed: pindolol (0.03, 0.1, and 3 nM), ICI 118 551 (0.1, 0.3, 1, 3 and 10 nM), betaxolol (0.3, 1, 3 and 10 nM), nebivolol and its isomers, R 67 138 and R 67 145 (0.5, 2 and 10 nM). As shown in Figure 3 each drug tested reduced the enhancement by adrenaline of tritium efflux. This inhibitory effect was dose-dependent except for that of pindolol.

Pindolol and ICI 118 551 exhibited significant activity from 0.1 nM (85 and 50% inhibition respectively) and betaxolol from 1 nM (70% inhibition). The order of potency for nebivolol and isomers was R 67 138 > nebivolol > R 67 145. R 67 138 caused significant inhibition from 0.5 nM (69%). With 10 nM the reduction in tritium efflux was 100%, 74% and 54% respectively for the three compounds.

Effect of acebutolol on adrenaline-induced enhancement of [3 H]-noradrenaline efflux in the presence of

phenoxybenzamine The selective β_1 -blocking drug, acebutolol (3 nM) reduced the facilitatory effect of adrenaline on 3 H efflux by about 20% ($n = 3$, data not shown). A higher concentration of acebutolol (30 nM) almost abolished it: $(S_2/S_1) \times 100 = 94 \pm 3.1$ ($n = 6$) ($P < 0.05$ versus 10 nM adrenaline group).

Pithed rats

The initial hypotensive responses to isoprenaline were similar in the three groups of rats (-11 ± 1 mmHg) as were the associated tachycardias (144 ± 6 ; 143 ± 2 and 132 ± 5 beats min).

Nebivolol and its isomers ($125 \mu\text{g kg}^{-1}$) did not significantly reduce heart rate during 20 min following administration. During the same time, blood pressure was not significantly modified by nebivolol and R 67 138 but it was slightly enhanced by R 67 145 ($+10$ mmHg).

As shown in Figure 4, none of the three β -adrenoceptor blocking drugs inhibited the hypotensive effect of isoprenaline. By contrast, inhibition of tachycardia appeared 5 min after injection and developed throughout the following 20 min. The order of potency was R 67 138 > nebivolol > R 67 145. Twenty min after injection the inhibition reached 77%, 65% and 50%, respectively.

Discussion

The present experiments have demonstrated a marked facilitation, in rat isolated atria, of the evoked release of [3 H]-noradrenaline by adrenaline when used in very low concentrations: 2 nM and

Figure 3 The effect of the β -adrenoceptor blocking drugs: nebivolol (N), R 67 138, R 67 145, betaxolol (B), pindolol (Pin) and ICI 118 551 (ICI) on adrenaline (0.1 nM, Ad)-induced increase in stimulation-evoked efflux of tritium from rat isolated atria (C = control group). There were three periods of stimulation (3 Hz, 15 s): S_1 as a control, S_2 and S_3 with drugs added. Adrenaline resulted in identical S_2/S_1 and S_3/S_1 ratios, so the effects of various drugs added in S_2 or in S_3 were plotted (S_D). The vertical bars represent s.e.means. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs adrenaline alone.

Figure 4 The effect of nebivolol and its isomers R 67138 and R 67145 ($125 \mu\text{g kg}^{-1}$ i.v.) on (a) the hypotension and (b) the tachycardia induced by isoprenaline (Iso) ($1 \mu\text{g kg}^{-1}$) in pithed rats. The vertical bars represent s.e.means. $**P < 0.01$; $***P < 0.001$ vs isoprenaline alone.

0.1 nM. Both concentrations induced about the same effect, the enhancement attaining 50% to 60% of the initial value. A lower concentration: 0.05 nM has been found to be inactive.

Similar investigations on rat atria have shown an enhancement of the stimulation-induced efflux of radioactivity with a higher concentration of adrenaline (10 nM) (Majewski *et al.*, 1981; 1982). These authors demonstrated that, depending on concentration, adrenaline had either no effect on (1 nM), or inhibited ($1 \mu\text{M}$), noradrenaline release. The latter higher dose of adrenaline enhanced efflux of radioactivity in the presence of phenoxybenzamine, suggesting it could also activate prejunctional α -adrenoceptors.

We, successfully, used lower concentrations of adrenaline to increase the evoked release of [^3H]-noradrenaline. Moreover, in our control groups the ratio S_2/S_1 (efflux of radioactivity in the second period of stimulation as a percentage of that in the first) was nearly 100% while it was about 50% in the other studies cited. This may be due to our conditions of stimulation: the size of electrodes, their distance and the potency of the resultant field. In any case, our experimental conditions allowed very low adrenaline concentrations to activate presynaptic β -adrenoceptors, and these results afford support for the hypothesis that circulating adrenaline may be the natural agonist for these receptors (Langer & Armstrong, 1984).

With phenoxybenzamine present, the large increase observed in evoked [^3H]-noradrenaline overflow confirms previous *in vitro* findings (Dahlöf *et al.*, 1978) and results of a recent *in vivo* study (Remie *et al.*, 1988). However, the activation of facili-

tatory prejunctional β -adrenoceptors cannot be achieved using the same weak concentrations as in the absence of α -blockade.

When α - and β -adrenoceptor presynaptic feedback is operative, the adrenaline-induced increase in noradrenaline overflow quickly reaches a maximum. With larger concentrations of adrenaline, α -adrenoceptor-mediated inhibition occurs and this overrides the facilitation of noradrenaline release. However, when α -adrenoceptors are blocked, the physiological balance between the two mechanisms is broken, resulting in a strongly elevated concentration of noradrenaline in the junctional cleft, which could compete with adrenaline for presynaptic β -adrenoceptors. This hypothesis is in agreement with our findings that presynaptic β_1 -adrenoceptors are effective in the preparation we used.

In these conditions, all the β -adrenoceptor blocking drugs we studied reduced the effect of adrenaline on tritium efflux. These drugs differ by their selectivity: acebutolol and betaxolol are known as cardioselective β -blockers, nebivolol is a new compound still under investigation with a high affinity for the β_1 -adrenoceptor, as demonstrated by binding assays (affinity $\beta_1/\beta_2 = 47$) and *in vitro* studies (IC_{50} in guinea-pig isolated trachea is 288 times higher than in atria) (Janssen preclinical report). Two selective β_2 adrenoceptor blocking agents were assayed: IPS 339 (Imbs *et al.*, 1977) and ICI 118 551 (O'Donnell & Wanstall, 1980) and finally, two β -adrenoceptor blocking drugs possessing partial agonist activity, practolol and pindolol, were also studied.

The selective β_1 - or β_2 -adrenoceptor blockers exhibited an inhibitory effect which was concentration-dependent. The selective β_1 -blocking drug acebutolol has been assayed both in the absence and in the presence of a α -adrenoceptor blockade. Its antagonistic effect was shown in both cases, but was larger when presynaptic α -adrenoceptors were effective. This was probably due to the lower active concentration of the β -agonist, adrenaline, in the first experiments.

IPS 339, usually considered to be a selective β_2 -adrenoceptor blocking drug, at 10 nM was able to reduce S_2/S_1 to levels significantly less than control. It should be noted that a similar finding was not observed with the other β_2 -adrenoceptor blocking drug ICI 118 551. This unexpected finding could result from an effect of the compound on another kind of receptor. This compound has not been extensively studied and its selectivity and affinity for neuronal receptors is not known. It is possible that at a concentration of 10 nM it activates prejunctional inhibitory receptors. The inhibitory effect induced by practolol was identical for the two concentrations studied, both perhaps being too high and resulting in a maximal effect. By contrast, the inhibitory effect of

pindolol was not dose-related. The largest concentration used (3 nM) did not result in a 100% inhibition. This can be attributed to the important intrinsic sympathomimetic activity of this drug. It has been demonstrated that the stimulant effects of pindolol differ considerably from one tissue to another (Clark *et al.*, 1982), depending on the subtype of β -adrenoceptor involved. More recently, Aellig & Clark (1987) suggested that although pindolol is a non-selective β -adrenoceptor blocking agent, it selectively stimulates β_2 -adrenoceptors. Thus, in the present experiments, it is possible that pindolol had some agonist activity on presynaptic β_2 -adrenoceptors.

As discussed above, nebivolol is a highly selective β_1 -adrenoceptor blocking agent. In the pithed rat, we further observed its antagonistic action on the tachycardia produced by isoprenaline, with no modification of its vascular effects. The β -adrenoceptor blocking activities of its isomers were qualitatively similar, i.e. cardioselective, but their potencies differed quantitatively, R 67 138 was the most active and R 67 145 the least active. It is interesting to note that the relative potencies of the three drugs were the same on presynaptic and postsynaptic receptors.

A number of authors have demonstrated the existence of β_2 -adrenoceptors on presynaptic sym-

thetic fibres (Westfall *et al.*, 1979; Langer, 1980; Dahlöf, 1981; Majewski, 1983; Göthert, 1986; Misu & Kubo, 1986). Moreover, most earlier workers concluded that presynaptic β -adrenoceptors are exclusively of the β_2 subtype, due to the failure of various β_1 -agonists and antagonists to stimulate or inhibit [3 H]-noradrenaline release in *in vitro* experiments (Westfall *et al.*, 1979; Dahlöf *et al.*, 1980). In contrast, our findings support the hypothesis of the presence of presynaptic β_1 - as well as β_2 -adrenoceptors, in the rat atria. Their activity could be shown by the use of more physiological conditions, i.e. low adrenaline concentration as β -agonist, in the absence of α -adrenoceptor blockade. Moreover, the present study demonstrates their stereoselectivity. A similar observation has been made by Chang *et al.* (1986) in rat hypothalamic slices, using (+)- and (-)-acebutolol. Finally, our results support the hypothesis of Misu & Kubo (1986), that presynaptic β_1 - and β_2 -adrenoceptors coexist in several tissues and therefore the antihypertensive action of β -antagonists may, in part, be due to blockade of presynaptic β -adrenoceptors.

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