Evidence for tonic activation of prejunctional β -adrenoceptors in guinea-pig pulmonary arteries by adrenaline derived from the adrenal medulla

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1 The effects of ($\pm$)-carteolol 10^{-8} M to 10^{-6} M, a non-selective β -antagonist, applied cumulatively, on stimulation-evoked ^3H -release at 1 Hz were studied in pulmonary arteries isolated from guinea-pigs. The guinea-pigs were subjected to either bilateral adrenalectomy, adrenalectomy followed by injections of deoxycorticosterone acetate (DOCA) and hydrocortisone, bilateral adrenodemedullation or a sham operation, and then loaded *in vitro* with [^3H]-noradrenaline.

2 Carteolol inhibited ^3H -output in arteries from sham-operated animals in a concentration-dependent manner. This inhibitory effect was not found in pulmonary arteries from animals subjected to adrenalectomy or adrenodemedullation. However, DOCA and hydrocortisone pretreatment, did not prevent the disappearance of the carteolol-induced inhibition of ^3H -release.

3 Adrenalectomy and adrenodemedullation depleted or markedly reduced the endogenous contents of adrenaline in pulmonary arteries without altering the levels of dopamine and noradrenaline.

4 It is concluded that adrenaline, mainly derived from the adrenal medulla, acts as an endogenous agonist for tonically functioning prejunctional β -adrenoceptors in guinea-pig pulmonary arteries, probably by being taken up and co-released with noradrenaline.

Introduction

It has been proposed that there is a positive feedback regulation of the release of the transmitter noradrenaline via prejunctional β -adrenoceptors (Adler-Graschinsky & Langer, 1975; Langer 1977; Dahlöf, 1981; Majewski, 1983; Misu & Kubo, 1986). These adrenoceptors were thought to be activated by circulating adrenaline (Stjärne & Brundin, 1975) and/or adrenaline taken up and co-released with noradrenaline (Majewski *et al.*, 1980; 1981) rather than by noradrenaline itself released from noradrenergic nerve endings (Adler-Graschinsky & Langer, 1975). However, the endogenous agonist has not been identified. Guimarães *et al.* (1978) showed that in dog saphenous veins propranolol reduced the evoked ^3H -overflow from strips loaded with [^3H]-adrenaline but not from those loaded with [^3H]-noradrenaline. Majewski *et al.* (1980, 1981) found that in guinea-pig atria and rat atria preincubated with unlabelled adrenaline, and then loaded with [^3H]-noradrenaline, propranolol and other β -adrenoceptor antagonists alone decreased the evoked ^3H -release, while this decrease was not seen after preincubation with unlabelled noradrenaline. From these findings, they proposed that adrenaline

is taken up, stored, released as a co-transmitter with noradrenaline, and then activates prejunctional β -adrenoceptors. We have shown that carteolol, a non-selective β -adrenoceptor antagonist (Nakagawa *et al.*, 1974), readily and stereoselectively inhibits the evoked release of noradrenaline from guinea-pig pulmonary arteries in the absence of any agents (Kuwahara *et al.*, 1986). In the present experiments, by use of carteolol as a tool, we have attempted to clarify the identity of the endogenous agonist for prejunctional β -adrenoceptors in pulmonary arteries, from guinea-pigs subjected to adrenalectomy, adrenodemedullation or sham operation.

Methods

Animals and operative procedures

Bilateral adrenalectomy, bilateral adrenodemedullation or sham operations were carried out under ether anaesthesia on male guinea-pigs weighing 200 to 250 g. Some adrenalectomized guinea-pigs were injected s.c. with deoxycorticosterone acetate

Table 1 Effects of ($\pm$)-carteolol (10^{-8} M, 10^{-7} M and 10^{-6} M), applied cumulatively, on stimulus-evoked ^3H -release in pulmonary arteries isolated from guinea-pigs 5 days after either bilateral adrenalectomy or sham operation

Group	n	S_1	S_2 (S_2/S_1)	S_3 (S_3/S_1)	S_4 (d.p.m. mg^{-1}) (S_4/S_1) (%)
Sham-operated					
Control	10	1059.6 $\pm$ 69.6	1092.2 $\pm$ 56.2 (104.6 $\pm$ 3.9)	1079.8 $\pm$ 61.6 (102.7 $\pm$ 2.2)	1021.6 $\pm$ 52.8 (97.7 $\pm$ 3.4)
Carteolol	8	903.0 $\pm$ 109.6	864.6 $\pm$ 99.4 (97.4 $\pm$ 4.6)	825.2 $\pm$ 103.1***† (91.3 $\pm$ 2.2¶¶)	770.3 $\pm$ 99.8***† (84.7 $\pm$ 3.6¶§)
Adrenalectomized					
Control	11	971.8 $\pm$ 111.3	972.3 $\pm$ 100.6 (102.5 $\pm$ 4.0)	970.8 $\pm$ 101.2 (102.3 $\pm$ 3.8)	912.2 $\pm$ 86.8 (97.2 $\pm$ 4.3)
Carteolol	8	837.9 $\pm$ 49.2	839.6 $\pm$ 58.6 (100.2 $\pm$ 3.0)	823.4 $\pm$ 61.3 (98.2 $\pm$ 4.1)	791.6 $\pm$ 51.0* (94.4 $\pm$ 2.5)

Strips were incubated for 1 h in 10^{-7} M [^3H]-noradrenaline, washed, set up and then superfused with Krebs medium. Supramaximal transmural stimuli were applied 4 times at 1 Hz for 100 s, 90, 105, 120 and 135 min after the start of superfusion (S_1 to S_4). Above-mentioned concentrations of carteolol were applied cumulatively immediately after the S_1 , S_2 and S_3 period of stimulation, respectively. Data shown are mean $\pm$ s.e.mean of n absolute values of evoked ^3H -release at S_x and of release ratios, S_x/S_1 expressed as percentage in parentheses. Significantly different: * P < 0.05, ** P < 0.01, compared to S_1 (paired t test); † P < 0.05, compared to corresponding control (Student's t test); ¶ P < 0.05, ¶¶ P < 0.01, compared to corresponding control, and § P < 0.05, compared to carteolol in arteries from adrenalectomized animals (unpaired Wilcoxon's rank-sum test).

(DOCA) 0.2 mg per animal daily plus hydrocortisone 1.0 mg per animal daily for 4 days.

Strips of pulmonary arteries

Guinea-pigs were killed by a blow on the head 5 days post-operatively and spiral preparations of the pulmonary arteries were prepared and incubated at 37°C for 1 h with oxygenated Krebs-bicarbonate solution containing 10^{-7} M [^3H]-noradrenaline (specific activity 38.6 Ci mmol $^{-1}$, The Radiochemical Centre, Amersham/Searle, U.S.A.) and ascorbic acid 100 mg l $^{-1}$ as described previously (Misu *et al.*, 1981; 1983). After washing for 10 min with noradrenaline-free medium, the preparations were mounted vertically between a pair of platinum stimulating electrodes, of 0.5 mm diameter and separated by 2 mm along the whole length of the strips (approximately 15 mm, weighing 5 to 6 mg), and superfused with Krebs solution at a constant rate of 1 ml min $^{-1}$ by means of microtube pumps (MP-3A, Tokyo Rikakikai, Japan). The Krebs solution had the following composition (mM): NaCl 120.7, KCl 5.9, CaCl $_2$ 2.5, NaHCO $_3$ 15.5, NaH $_2$ PO $_4$ 1.2 and glucose 11.5, and was maintained at 37°C, pH 7.2 to 7.4, and bubbled with 95% O $_2$ plus 5% CO $_2$. A 90 min equilibration period was allowed, and then supramaximal transmural field stimulation (2 ms, 10 V at 1 Hz for 100 s) was applied with an electrical stimulator and an isolator (SEN 3201 and SS-201J, Nihon Kohden, Japan) 4 times (S_1 to S_4 periods) at 15 min intervals.

Carteolol 10^{-8} M, 10^{-7} M and 10^{-6} M was applied cumulatively immediately after the S_1 , S_2 and S_3 periods of stimulation, respectively. Superfusate was collected for 2 min intervals before, during and after the period of nerve stimulation, 2 ml of ACS-II solution was added and total ^3H -activities, expressed as disintegration per min (d.p.m.), were determined by a liquid scintillation spectrometer (Packard 2660, U.S.A.). The stimulus-evoked release of ^3H (S) was calculated as the difference between resting efflux before stimulation and total efflux, detected in 3 successive samples during and after stimulation. The resting efflux before stimulation was regarded as spontaneous release (Sp). The effect of each concentration of carteolol on the spontaneous and evoked release was evaluated from absolute values of Sp_x and S_x and also by % ratios of Sp_x/Sp_1 and S_x/S_1 , for the standardization of data obtained. These experiments were done in the absence of cocaine or an α_2 -adrenoceptor antagonist.

Measurement of catecholamine content of pulmonary arteries

In another series of experiments, the pulmonary arteries were isolated 5 days after the operation, homogenized in 2 ml of 0.1 N HClO $_4$, centrifuged and total aliquots of the supernatant transferred to polypropylene test tubes (10 ml) containing activated alumina 10 mg, disodium EDTA 10 mg and dihydroxybenzylamine 50 pmol as an internal standard. To the test tubes, 1 ml of 1.5 M Tris-HCl buffer (pH 8.6) was added and the preparations were placed

Table 2 Endogenous catecholamine contents of pulmonary arteries isolated from guinea-pigs 5 days after bilateral adrenalectomy, bilateral adrenodemedullation or sham operation

Group	n	Dopamine (pmol mg ⁻¹)	Noradrenaline (pmol mg ⁻¹)	Adrenaline (fmol mg ⁻¹)
Sham operation	9	0.32 $\pm$ 0.06	13.0 $\pm$ 1.5	65.3 $\pm$ 9.7
Adrenalectomy	7	0.25 $\pm$ 0.04	14.3 $\pm$ 2.5	6.4 $\pm$ 1.1**
Adrenodemedullation	7	0.44 $\pm$ 0.03	13.7 $\pm$ 1.2	16.8 $\pm$ 8.9**

Data shown are mean $\pm$ s.e.mean of *n* values. Significantly different: ***P* < 0.01, compared to values from sham-operated animals (Student's *t* test).

in a shaker for 30 min. The supernatant was discarded and the alumina washed three times with diluted water. Adsorbed catecholamines were eluted with 60 μ l of 0.1 N HCl and endogenous catecholamines were measured by high performance liquid-chromatography with electrochemical detection (Yanaco, Japan). Details of the chromatographic data were as follows (Goshima *et al.*, 1985); column, Cosmosil $_5$ C₁₈ (4.6 $\times$ 150 mm, particle size 5 μ m); mobile phase, 0.1 M phosphate-potassium buffer (pH 5.8) containing 10% methanol, 0.02% (–)-heptanesulphonate sodium and 1 mM disodium EDTA; applied potential, 600 mV vs Ag AgCl⁻¹; and flow rate, 1.5 ml min⁻¹.

Drugs used

($\pm$)-Carteolol hydrochloride (Otsuka, Japan) was dissolved in distilled water. DOCA (Nakarai, Japan) and hydrocortisone (Sigma, U.S.A.) was suspended in olive oil.

Analysis

Experiments were performed according to a paired design, whenever possible. Data shown are mean $\pm$ s.e.mean. Data significance was calculated by paired *t* test, Student's *t* test and unpaired Wilcoxon's rank-sum test.

Results

In control pulmonary arteries isolated from sham-operated guinea-pigs, then loaded with [³H]-noradrenaline, the spontaneous ³H-release and the stimulation-evoked ³H-release at 1 Hz were 394.7 $\pm$ 15.7 d.p.m. mg⁻¹ 2 min⁻¹ and 990.0 $\pm$ 63.1 d.p.m. mg⁻¹ (*n* = 18), respectively, 90 min after the start of superfusion. There was no decline of the evoked release in response to the S₁ to S₄ stimulations at 15 min intervals (Table 1). Carteolol 10⁻⁸ M, 10⁻⁷ M and 10⁻⁶ M, applied cumulatively, inhibited the evoked ³H-release in a concentration-

Table 3 Effects of ($\pm$)-carteolol (10⁻⁸ M, 10⁻⁷ M and 10⁻⁶ M), applied cumulatively, on the stimulus-evoked ³H-release from pulmonary arteries isolated from guinea-pigs 5 days after either bilateral adrenalectomy, followed by injections of DOCA plus hydrocortisone, or after bilateral adrenodemedullation

Group	n	S ₁	S ₂ (S ₂ /S ₁)	S ₃ (S ₃ /S ₁)	S ₄ (d.p.m. mg ⁻¹) (S ₄ /S ₁) (%)
Adrenalectomized plus DOCA and hydrocortisone treatment					
Control	4	924.3 $\pm$ 182.3	897.8 $\pm$ 184.4 (97.3 $\pm$ 11.4)	848.1 $\pm$ 174.9 (92.4 $\pm$ 11.5)	798.6 $\pm$ 172.1 (86.4 $\pm$ 12.3)
Carteolol	5	778.3 $\pm$ 234.4	789.0 $\pm$ 207.9 (105.4 $\pm$ 4.8)	717.1 $\pm$ 170.2 (99.3 $\pm$ 7.4)	662.4 $\pm$ 179.7 (88.0 $\pm$ 7.5)
Adrenodemedullated					
Control	12	1151.4 $\pm$ 124.4	1053.2 $\pm$ 124.9* (90.6 $\pm$ 3.0)	1000.8 $\pm$ 114.1** (86.6 $\pm$ 2.7)	965.9 $\pm$ 107.9** (83.6 $\pm$ 3.2)
Carteolol	11	1095.0 $\pm$ 94.1	1018.0 $\pm$ 87.1* (93.3 $\pm$ 2.4)	922.5 $\pm$ 76.8** (85.9 $\pm$ 2.8)	837.7 $\pm$ 67.2** (78.0 $\pm$ 2.1)

Deoxycorticosterone acetate (DOCA) 0.2 mg per animal daily and hydrocortisone 1.0 mg per animal daily were injected s.c. for 4 days. Significantly different: **P* < 0.05, ***P* < 0.01, compared to S₁ (paired *t* test). Other details are as in Table 1. Note the absence of a significant difference between the carteolol-treated groups and their equivalent, time-matched controls.

Table 4 Effects of ($\pm$)-carteolol (10^{-8} M, 10^{-7} M and 10^{-6} M), applied cumulatively, on the spontaneous ^3H -release (Sp) in pulmonary arteries isolated from guinea-pigs 5 days after either bilateral adrenalectomy, adrenalectomy followed by injections of DOCA plus hydrocortisone, bilateral adrenomedullation or sham operation

Group	n	Sp_1	Sp_2 (Sp_2/Sp_1)	Sp_3 (Sp_3/Sp_1)	Sp_4 (d.p.m. mg^{-1}) (Sp_4/Sp_1) (%)
Sham-operated					
Control	10	415.8 ± 14.9	$353.2 \pm 14.7^{**}$ (85.0 ± 2.1)	$320.8 \pm 10.0^{**}$ (77.4 ± 1.4)	$309.0 \pm 14.5^{**}$ (74.2 ± 1.8)
Carteolol	8	368.3 ± 28.4	$340.3 \pm 29.1^{**}$ (92.2 ± 1.9)	$327.9 \pm 30.5^{**}$ ($88.6 \pm 3.4^{\P}$)	$313.5 \pm 30.1^{**}$ ($84.7 \pm 4.6^{\P}$)
Adrenalectomized					
Control	11	402.5 ± 29.6	$356.6 \pm 26.8^{**}$ (88.8 ± 2.2)	$319.1 \pm 23.3^{**}$ (79.6 ± 1.8)	$302.6 \pm 22.9^{**}$ (75.6 ± 2.1)
Carteolol	8	429.9 ± 64.3	389.7 ± 45.7 (93.1 ± 3.5)	$348.7 \pm 42.9^*$ (83.1 ± 2.8)	$330.4 \pm 41.9^{**}$ (78.5 ± 2.5)
Adrenalectomized plus DOCA and hydrocortisone treatment					
Control	4	373.8 ± 65.9	304.3 ± 25.0 (84.7 ± 6.5)	275.3 ± 20.2 (77.2 ± 7.5)	256.6 ± 19.6 (71.6 ± 5.9)
Carteolol	5	338.5 ± 41.2	287.9 ± 22.9 (81.2 ± 4.0)	$261.3 \pm 30.4^*$ (77.6 ± 4.3)	$250.1 \pm 26.9^*$ (74.6 ± 3.8)
Adrenomedullated					
Control	12	$337.1 \pm 25.3^{\dagger}$	321.0 ± 22.6 (95.9 ± 2.2)	$285.4 \pm 18.3^{**}$ (85.3 ± 1.9)	$267.8 \pm 16.5^{**}$ (80.3 ± 1.9)
Carteolol	11	331.2 ± 21.6	304.7 ± 16.7 (93.0 ± 3.4)	$286.2 \pm 15.8^{**}$ (87.4 ± 3.4)	$277.5 \pm 15.5^{**}$ (84.9 ± 3.4)

Significantly different: $*P < 0.05$, $**P < 0.01$, compared to Sp_1 (paired t test); $\P P < 0.05$, compared to corresponding control (unpaired Wilcoxon's rank-sum test); $^{\dagger}P < 0.05$, compared to control in arteries from sham-operated animals (Student's t test). Other details are as in Tables 1 and 3. Note the absence of a significant difference between the carteolol-treated groups and their equivalent, time-matched controls, except for the sham-operated groups.

dependent manner, compared to both the release before its application in the same preparations and the corresponding, time-matched control.

Bilateral adrenalectomy depleted the endogenous content of adrenaline in the pulmonary arteries, compared to that in the arteries of sham-operated animals (Table 2). However, there were no changes in the equivalent noradrenaline and dopamine values. Adrenalectomy produced no changes in either the spontaneous ^3H -release (414.0 ± 31.2 d.p.m. mg^{-1} 2 min^{-1}) or the stimulation-evoked ^3H -release (915.4 ± 68.0 d.p.m. mg^{-1}) at S_1 ($n = 19$), compared to the respective values of arteries from sham-operated animals. Any tonic action of endogenous adrenaline on the pulmonary arteries was not reflected in the absolute values of the spontaneous and evoked release. There was also no decline of the ^3H -release evoked by repetitive stimulation in arteries from adrenalectomized guinea-pigs (Table 1). Carteolol 10^{-6} M inhibited the evoked release, compared to the release before its application. However, this inhibition was not significantly different from the corresponding time-matched control in terms of

either absolute value or release ratio. Furthermore, in arteries from guinea-pigs subjected to adrenalectomy there was no carteolol-induced inhibition of evoked ^3H -release (as occurred in sham-operated animals).

Injections of DOCA 0.2 mg per animal day^{-1} plus hydrocortisone 1.0 mg per animal day^{-1} for 4 days to adrenalectomized guinea-pigs, before the isolation of pulmonary arteries, did not modify the spontaneous and evoked ^3H -release at S_1 , compared to the equivalent release obtained in arteries from adrenalectomized animals (Tables 1, 3 and 4). There was no decline of the control ^3H -release evoked by repetitive nerve stimulation (Table 3). Hence, DOCA and hydrocortisone pretreatment did not prevent the loss of the carteolol-induced inhibition of evoked ^3H -release.

Bilateral adrenomedullation also markedly reduced the endogenous content of adrenaline without affecting the noradrenaline and dopamine content (Table 2). Adrenomedullation did not affect the evoked ^3H -release at S_1 , compared to the equivalent value for release in arteries from sham-

operated controls (Tables 1 and 3). In this series of experiments, a progressive decline in the ^3H -release evoked by repetitive nerve stimulation was seen even in preparations not exposed to carteolol (compare S_2 to S_4 with S_1 , Table 3). Although this decline was also seen in carteolol-treated preparations, there were no significant differences between the carteolol-treated groups and the corresponding, time-matched controls in terms of either absolute values or release ratios (Table 3). Thus, adrenomedullation appeared to prevent the carteolol-induced inhibition of the evoked release. On the other hand, the spontaneous ^3H -release at S_1 in arteries from adrenomedullated animals was decreased, compared to the corresponding release in arteries from sham-operated animals (Table 4). This decrease might be due to some differences arising from both groups of experiments, which were not done in a paired design.

The spontaneous ^3H -release gradually declined with time in almost every series of experiments (Table 4). Carteolol 10^{-8} M to 10^{-6} M , applied cumulatively, appeared to prevent the fall in the spontaneous release ratios, compared to the corresponding control, in arteries from sham-operated animals. However, carteolol had no consistent effect in the other series of experiments.

Discussion

($\pm$)-Carteolol 10^{-8} M to 10^{-6} M inhibited the evoked release of noradrenaline in pulmonary arteries from sham-operated guinea-pigs in a concentration-dependent manner. The carteolol-induced inhibition is consistent with our previous findings in the same arteries from intact animals (Kuwahara *et al.*, 1986). Furthermore, we demonstrated that the same concentrations of (+)-carteolol produced no inhibition of the evoked release of noradrenaline. These results indicate that prejunctional β -adrenoceptors function tonically and some endogenous agonist is released and activates these adrenoceptors to facilitate the further release of noradrenaline.

Bilateral adrenalectomy and adrenomedullation prevented the occurrence of the carteolol-induced inhibition of the evoked release of noradrenaline and depleted or markedly reduced the endogenous concentration of adrenaline in the pulmonary arteries, without affecting that of noradrenaline and dopamine, compared to sham-operated preparations. Furthermore, DOCA and hydrocortisone pretreatment did not prevent the loss of the carteolol-induced inhibition of release in arteries from adrenalectomized animals. These results indicate that adrenaline within the noradrenergic nerve endings is mainly derived from the adrenal medulla and suggest that adrenaline is taken up and co-exists

with noradrenaline. Thus, released adrenaline, but not noradrenaline and dopamine, appears to act as an endogenous agonist for prejunctional β -adrenoceptors. This idea is consistent with the characterization of prejunctional β -adrenoceptors as the β_2 subtype (Misu *et al.*, 1983; Majewski, 1983; Misu & Kubo, 1986). Adrenomedullation-induced modifications of the catecholamine content of guinea-pig pulmonary arteries are consistent with those in rat portal veins (Dahlöf *et al.*, 1979). Both [^3H]-noradrenaline and [^3H]-adrenaline are bound in dense core vesicles in the noradrenergic nerve endings (Wolfe & Potter, 1963) and are localized in small particles associated with the microsomes of various noradrenergic tissues in rats (Potter & Axelrod, 1963). [^3H]-adrenaline taken up is released by noradrenergic nerve stimulation (Rosell *et al.*, 1964; Guimarães *et al.*, 1978; Majewski *et al.*, 1980; 1981). The present findings support the adrenaline-mediated positive feedback loop hypothesis proposed by Majewski *et al.* (1980, 1981), i.e. adrenaline is taken up, stored and released as a co-transmitter with noradrenaline and then activates prejunctional β -adrenoceptors.

The spontaneous release in almost every group of experiments and even the evoked release in some experiments gradually declined with time. Such a decline is, to some extent, unavoidable as [^3H]-noradrenaline pooled within noradrenergic nerve endings and available for release is limited in its amount in preload experiments such as these. This is one of the reasons for doing the experiment in a paired design whenever possible. It is not clear at present why there was a decline of the evoked ^3H -release only in the adrenomedullation group of experiments. However, the extent of the decline in the carteolol-applied preparations was similar to that in control preparations. From these findings, adrenomedullation appears to prevent the carteolol-induced inhibition of the evoked release just as in the case of adrenalectomy.

Carteolol (10^{-8} M to 10^{-6} M) slightly increased the spontaneous release in some pulmonary arteries from sham-operated guinea-pigs, but not in those from animals with either adrenalectomy, adrenalectomy followed by the injections of DOCA plus hydrocortisone or adrenomedullation. However, this increase was not seen in intact guinea-pig pulmonary arteries (Kuwahara *et al.*, 1986), or in renal and mesenteric arteries from young and adult spontaneously hypertensive rats and age-matched Wistar Kyoto rats (Misu *et al.*, 1987). Higher concentrations of carteolol (10^{-5} M and 10^{-4} M) increase the spontaneous ^3H -release in guinea-pig pulmonary arteries and this increase is partially inhibited by the combined application of cocaine ($5 \times 10^{-5}\text{ M}$) and 3-O-methylnoradrenaline (10^{-5} M) (unpublished data). It

is possible that carteolol may have a slight indirect sympathomimetic action and/or a blocking effect on neuronal or extraneuronal uptake.

In conclusion, the endogenous agonist for tonically functioning prejunctional β -adrenoceptors is adrenaline, which is mainly derived from the adrenal

medulla and is probably taken up and co-released with noradrenaline in guinea-pig pulmonary arteries.

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