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Conjugated catecholamines and pressor responses to angiotensin, luteinizing hormone-releasing hormone and prazosin in conscious toads

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1 Synthetic angiotensin II (Ang II), mammalian luteinizing hormone-releasing hormone (LHRH) and salmon LHRH (sLHRH) were injected intravenously into conscious, adult toads (*Bufo marinus*) to elucidate the cardiovascular actions of the hormones. The maximal increases in pulse pressure elicited by the three peptides did not differ from each other but only Ang II increased cardiac frequency. The maximal increases in mean arterial blood pressure (MAP) caused by LHRH and sLHRH were identical, while Ang II caused a 100% greater maximal effect. The median effective doses (ED_{50}) for both Ang II and LHRH were approximately 0.1 nmol kg^{-1} whereas the potency of sLHRH was 10 fold less. Pressor responses to LHRH and sLHRH were blocked completely by (D-pGlu[1], D-Phe[2], D-Trp[3,6])-LHRH but this antagonist did not inhibit Ang II.

2 Significant proportions of circulating, endogenous dopamine, noradrenaline (NA) and adrenaline (Ad) were found to be sulphoconjugated. Arterial plasma concentration of free NA increased simultaneously with the rise in blood pressure following Ang II injection. The magnitude of the free NA response increased with increasing Ang II dose but even a high dose failed to augment the plasma level of conjugated NA. Ang II did not alter concentrations of free or conjugated dopamine and Ad.

3 Intraarterial injection of an α-adrenoceptor antagonist, prazosin, caused sustained elevation of arterial pressure and free Ad. Subsequently Ang II lowered plasma Ad concentration. Prazosin inhibited the NA response to Ang II yet the pressor effects of the α-adrenoceptor antagonist and Ang II were additive. Administration of a β-adrenoceptor antagonist, propranolol, largely reversed the cardiovascular sequelae of α-adrenoceptor blockade.

4 It is concluded, firstly, that the cardiovascular actions of Ang II and LHRH are mediated through different receptors. Secondly, although it had been shown that α- and β-adrenoceptor mechanisms mediate the pressor effect of LHRH, the present experiments showed that mobilization of catecholamines cannot account for the pressor response to Ang II. Thirdly, both free and conjugated catecholamines circulate in toads; however the extent of conjugation can be dissociated from the changes in free NA and Ad induced by Ang II and prazosin.

Introduction

Systemic injection of mammalian luteinizing hormone-releasing hormone (LHRH; also named gonadotropin-releasing hormone) produces large, catecholamine-dependent increases in the arterial blood pressure of conscious toads (*Bufo marinus*, Wilson *et al.*, 1984). Amphibian brain contains endogenous LHRH but the LHRH-like peptide extracted from amphibian sympathetic ganglia and adrenal glands is chromatographically and immunologically distinct from the brain hormone (Eiden *et al.*, 1982). Both LHRH and salmon LHRH (sLHRH) are potent depolarizing agents when applied directly to amphibian sympathetic ganglia, and it has been suggested that sLHRH may resemble an amphibian sympathetic transmitter (Jan & Jan, 1983). Angiotensin II (Ang II) also resembles LHRH, both in structure (Capponi & Catt, 1979) and in modulating the neural membrane potential of toad isolated spinal cord (Phillis & Kirkpatrick, 1979). However, Ang II reportedly caused little or no elevation of arterial pressure when injected systemically into *B. marinus* (Grill *et al.*, 1972). One aim of the present studies, therefore, was to compare the effects of LHRH, sLHRH and Ang II on blood pressure in *B. marinus*. We observed that all

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three hormones acutely raised pulse pressure and mean arterial pressure (MAP).

Ang II and LHRH react with a common receptor in rat adrenal and uterine membranes (Capponi & Catt, 1979). Ang II may mimic LHRH in rat brain, where both peptides stimulate luteinizing hormone secretion (Steele *et al.*, 1983). Therefore we used a competitive antagonist to test whether Ang II, LHRH and sLHRH acted through a common receptor to augment toad blood pressure. Further, because the pressor action of LHRH had been shown to result from stimulation of the sympathoadrenal system (Wilson *et al.*, 1984), we monitored plasma catecholamines during the acute pressor response to Ang II. Our observations suggested a unique role for Ang II in catecholamine and cardiovascular regulation.

Conjugates formed with sulphate or glucuronide comprise major fractions of the endogenous catecholamine levels in mammals and birds (Unger *et al.*, 1980; Wang *et al.*, 1983; Davidson *et al.*, 1984; Wilson, 1984). Although controversy attends the control of free catecholamine secretion by α-adrenoceptor mechanisms (Kalsner & Quillan, 1984; Kirpekar, 1984), α-adrenoceptor influences on conjugated catecholamines are unknown. The present experiments extended the study of circulating sulphoconjugated and glucuronoconjugated catecholamines to an amphibian species, observed the effect of the α-adrenoceptor antagonist prazosin on conjugated catecholamines in the circulation, then distinguished the regulation of free catecholamines from that of conjugates.

Methods

Animals and surgical preparation

Adult toads (*Bufo marinus*, 260–470 g) of either sex were maintained at 20–22°C on a 16L:8D photoperiod, fed commercial dog food, and allowed water *ad libitum*. Experiments were performed from September to November. The animals were anaesthetized with tricaine methane sulphonate, implanted with chronic indwelling polyethylene catheters in a sciatic artery (PE50) and a femoral vein (PE10), then allowed at least 2 days to recover.

Experimental procedures

All experiments were performed on unrestrained, conscious toads housed in opaque cages. Pulsatile arterial pressure was recorded continuously from the indwelling sciatic catheter. MAP was calculated as the sum of the diastolic pressure and one third the pulse pressure. Cardiac frequency was determined from the pressure trace.

Saline vehicle (0.65% sodium chloride) and peptide solutions were injected i.v. in volumes of 100 $\mu l\ kg^{-1}$, immediately followed by a 200 $\mu l\ kg^{-1}$ saline flush. To test the effect of Ang II on circulating catecholamine concentrations, 1 ml aliquots of arterial blood were sampled at 5 min after i.v. injection of saline vehicle or the hormone. To test the effect of α-adrenoceptor blockade on catecholamines, arterial blood was sampled at 60 min after i.a. injection of prazosin. The procedure for blood collection and assay of free catecholamines was exactly that of Wilson *et al.*, (1984). Sulphoconjugated catecholamines were determined by the arylsulphatase method (Johnson *et al.*, 1980). Glucuronoconjugated catecholamines were measured separately, using the β-glucuronidase procedure (Yoneda *et al.*, 1983). Assay specifically was confirmed by adding dopamine-3-sulphate or dopamine-4-sulphate to plasmas as internal standards (Wilson, 1984). Catecholamine concentrations for each plasma sample were calculated as the mean of duplicate determinations.

Statistical analyses

Data are reported as the arithmetic mean ± s.e. Factorial designs were used for the simultaneous evaluation of the data from each experiment by analysis of variance (Kirk, 1982). Five percent was the fiduciary limit.

Chemicals

Synthetic (Val^5)-angiotensin II (i.e. amphibian Ang II, Hasegawa *et al.*, 1983), sarcosine-substituted Ang II analogues, LHRH, and (D-$pGlu^1$, D-Phe^2, D-$Trp^{3,6}$)-LHRH were purchased from Peninsula Laboratories; sLHRH (i.e. Trp^7, Leu^8-LHRH, Sherwood *et al.*, 1983) was provided by Dr R. P. Millar; prazosin hydrochloride was donated by Pfizer Canada; (±)-propranolol, arylsulphatase and β-glucuronidase were purchased from Sigma. Reagents for the radioenzymatic assay of catecholamines were obtained from Upjohn Diagnostics. Dopamine-3-sulphate and dopamine-4-sulphate were gifts from Dr J. S. Kennedy, National Institute of Mental Health. All other chemicals were of the highest quality available.

Results

Injection of Ang II, LHRH or sLHRH produced dose-dependent increases in pulse pressure and MAP, while injection of the saline vehicle was without pressor effect (Figure 1). The maximal increases in pulse pressure evoked by the three peptides did not differ from each other. The maximal increases in MAP caused by LHRH and sLHRH were identical but Ang

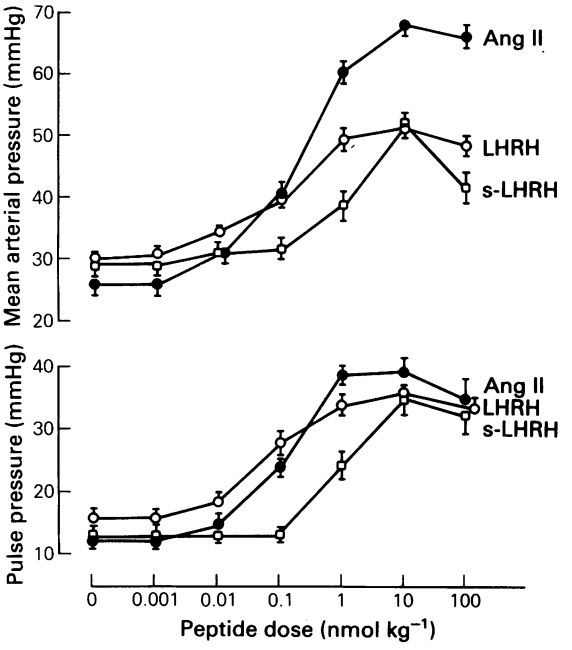

Figure 1 Pressor responses of conscious toads to synthetic angiotensin II (Ang II), luteinizing hormone-releasing hormone (LHRH) and salmon LHRH (sLHRH). The hormones were injected i.v. in increasing doses seriatim. Plotted are the peak MAP and the corresponding pulse pressure; each symbol represents the mean for the responses of 8 toads; s.e.means shown by vertical lines. Separate groups of toads were used to test each peptide. The ED_{50} for Ang II and LHRH were similar but sLHRH was significantly less potent. Ang II evoked a larger maximal MAP response than did either of the LHRH peptides.

II caused a 100% greater maximal MAP. The ED_{50} for both Ang II and LHRH were 0.1 nmol kg^{-1} while the ED_{50} for sLHRH was 1 nmol kg^{-1}. Blood pressure typically began rising within 3 min and peaked within 7 min following Ang II or LHRH administration whereas the effect of sLHRH developed at a slightly slower rate: the latencies to peak MAP after injection of the ED_{50} of Ang II, LHRH and sLHRH were 333 ± 30 s, 255 ± 51 s and 393 ± 48 s respectively. Preinjection cardiac frequency averaged 17 ± 1 min^{-1} and was unchanged by saline injection. The ED_{50} pressor dose of Ang II lowered cardiac frequency to 13 ± 1 min^{-1}. However, higher doses of Ang II increased cardiac frequency; e.g. 100 nmol kg^{-1} raised the frequency to 23 ± 2 min^{-1}. The LHRH peptides did not cause tachycardia; e.g. the 100 nmol kg^{-1} dose of sLHRH decreased cardiac frequency to 12 ± 1 min^{-1}.

In a second series of experiments the pressor actions of Ang II and LHRH were found to involve different receptor mechanisms. Responses to LHRH and sLHRH were blocked completely and reversibly by pretreatment with (D-pGlu[1], D-Phe[2], D-Trp[3,6])-LHRH. In contrast the LHRH-antagonist did not alter either pulse pressure or MAP responses to Ang II (Figure 2). Preliminary trials ($n = 7$ toads) with (Sar[1], Leu[8])-Ang II, (Sar[1], Ile[8])-Ang II and (Sar[1], Ala[8])-Ang II failed to identify a competitive antagonist for the Ang II receptor in the toad. In trials with the native hormone, Ang II injection did not affect subsequent pressor responses to sLHRH ($n = 5$ toads).

Through a third series of experiments Ang II was observed to increase arterial plasma NA concentration (Table 1). Significant proportions of total circulating catecholamines were observed to be sulphoconjugated and variable amounts were glucuronoconjugated, but only free NA was stimulated by Ang II. Elevated NA levels occurred coincidently with the

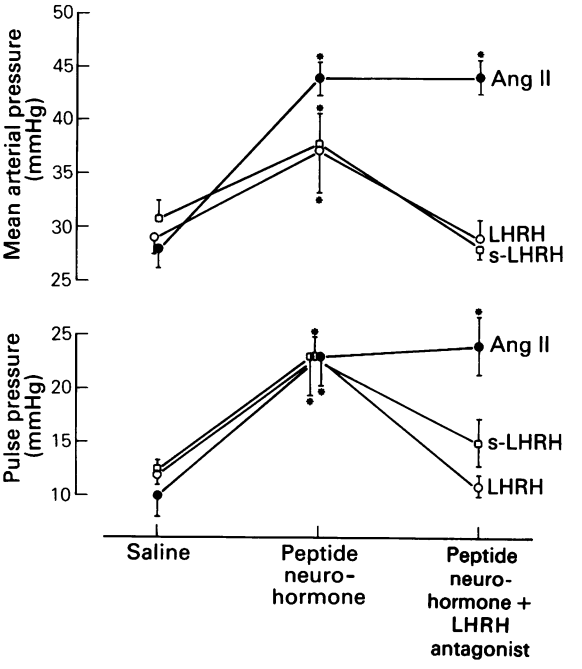

Figure 2 Pretreatment with (D-pGlu[1], D-Phe[2], D-Trp[3,6])-luteinizing hormone-releasing hormone (LHRH) inhibited toad pressor responses to LHRH (0.1 nmol kg^{-1}) and salmon LHRH (sLHRH, 1 nmol kg^{-1}) but not responses to angiotensin II (Ang II, 0.1 nmol kg^{-1}). Saline vehicle or the LHRH antagonist was injected i.v. 10 min before each hormonal injection. Plotted are the peak MAP and corresponding pulse pressure; each symbol represents the mean for 4 to 6 toads; s.e.means shown by vertical lines. $*P < 0.05$ compared to the response to saline vehicle injection.

Table 1 Concentrations (pg ml^{-1}) of free, sulphoconjugated and glucuronoconjugated catecholamines in toad arterial plasma

	n	*Dopamine* Free	*Dopamine* Sulphate	*Dopamine* Glucuronide
Saline	8	144 ± 74	439 ± 86	413 ± 141
Ang II (0.1 nmol kg^{-1})	8	174 ± 8	429 ± 25	332 ± 105
Saline	6	67 ± 13	725 ± 119	51 ± 35
Ang II (1 nmol kg^{-1})	6	120 ± 28	828 ± 148	190 ± 99
Prazosin	6	90 ± 22	731 ± 132	141 ± 84
Ang II (0.1 nmol kg^{-1})	6	170 ± 46	810 ± 190	154 ± 58
	n	*Noradrenaline* Free	*Noradrenaline* Sulphate	*Noradrenaline* Glucuronide
Saline	8	35 ± 9	23 ± 3	25 ± 11
Ang II (0.1 nmol kg^{-1})	8	565 ± 104*	142 ± 93	86 ± 48
Saline	6	13 ± 2	38 ± 8	8 ± 4
Ang II (1 nmol kg^{-1})	6	3048 ± 927*	800 ± 711	998 ± 898
Prazosin	6	54 ± 14	48 ± 16	4 ± 3
Ang II (0.1 nmol kg^{-1})	6	125 ± 82	49 ± 18	8 ± 4
	n	*Adrenaline* Free	*Adrenaline* Sulphate	*Adrenaline* Glucuronide
Saline	8	305 ± 89	351 ± 66	114 ± 43
Ang II (0.1 nmol kg^{-1})	8	201 ± 60	402 ± 63	61 ± 37
Saline	6	146 ± 49	810 ± 224	13 ± 8
Ang II (1 nmol kg^{-1})	6	109 ± 25	828 ± 255	21 ± 9
Prazosin	6	945 ± 142*	813 ± 271	238 ± 245
Ang II (0.1 nmol kg^{-1})	6	377 ± 28†	758 ± 215	112 ± 66

Values are the mean ± s.e. for 6 or 8 animals. To test the effect of Ang II on circulating catecholamines, 1 ml aliquots of blood were sampled at 5 min after i.v. injection of saline vehicle or the hormone; to test the effect of α-adrenoceptor blockade on catecholamines, blood was sampled at 60 min after i.a. injection of prazosin. Ang II increased free NA in controls and decreased free Ad in prazosin-treated toads; prazosin elevated free Ad but blocked the NA response to Ang II.

* $P < 0.05$ compared to control.

† $P < 0.05$ compared to prazosin.

peaking of blood pressure and both responses were dose-dependent. Ang II 0.1 nmol kg^{-1} increased pulse pressure from 12 ± 1 mm Hg to 22 ± 2 mm Hg, while Ang II 1 nmol kg^{-1} raised pulse pressure to 37 ± 4 mmHg; simultaneously MAP increased from 27 ± 2 mmHg to 44 mm Hg following Ang II 0.1 nmol kg^{-1} and to 59 ± 2 mmHg following Ang II 1 nmol kg^{-1}.

Injection i.a. of prazosin, during a fourth series of experiments, was followed within 60 min by elevation of arterial concentrations of free Ad (Table 1). Simultaneously prazosin increased pulse pressure from 12 ± 1 mmHg to 24 ± 1 mmHg, MAP from 29 ± 2 mmHg to 47 ± 4 mmHg, and cardiac frequency from 14 ± 1 min to 24 ± 3 min^{-1}. These effects were specific for prazosin because i.a. injection of saline vehicle was without sustained effect. After α-adrenoceptor blockade, injection i.v. of Ang II 0.1 nmol kg^{-1} had no effect on cardiac frequency but further raised pulse pressure to 30 ± 4 mmHg and MAP to 58 ± 5 mmHg. Ang II failed to elevate arterial plasma NA concentration in toads pretreated with prazosin; furthermore, the hormone lowered plasma Ad (Table 1).

The cardiovascular sequelae to α-adrenoceptor blockade were not artifacts of blood loss because similar effects of prazosin and Ang II were obtained in a fifth series of experiments, during which blood was not sampled (Figure 3). The changes in pulse pressure and MAP caused by prazosin and Ang II were again shown to be additive. These final experiments also indicated the role of β-adrenoceptor mechanisms: injection of propranolol into prazosin-treated toads returned pulse pressure and MAP towards control

levels. Propranolol also abolished the additivity between the pressor effects of prazosin and Ang II (Figure 3).

Discussion

The present results conflict with the finding of Grill *et al.* (1972) that Ang II causes little or no elevation of blood pressure when injected systemically into *B. marinus*. We observed that conscious adult toads responded to i.v. injection of Ang II, LHRH or sLHRH with increases in pulse pressure and MAP. The maximal pulse pressure responses to the three peptides did not differ from each other: these pulse pressures may be the largest which the toad heart is capable of producing under the experimental conditions. The maximal MAP response to Ang II was approximately double that elicited by the LHRH peptides, suggesting that Ang II and LHRH act through different mechanisms. The hypothesis of separate receptor mechanisms was confirmed by finding that an LHRH analogue completely blocked the cardiovascular effects of LHRH and sLHRH but not those of Ang II. There is some evidence for the evolution of LHRH receptor mechanisms: LHRH and sLHRH are equipotent in stimulating gonadotropin release from teleost fish pituitary (Mackenzie *et al.*, 1984), LHRH is 10 fold more potent than sLHRH in elevating amphibian blood pressure (present experiments), and LHRH is about 50 fold more potent than sLHRH in releasing luteinizing hormone from rat pituitary cells (Sherwood *et al.*, 1983).

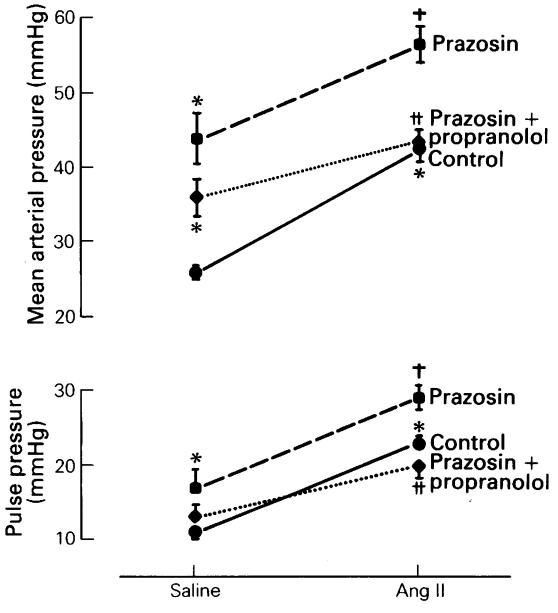

Figure 3 Effects of pretreatment with the adrenoceptor antagonists prazosin (25 μmol kg^{-1} i.a.) and (±)-propranolol (6.5 μmol kg^{-1} i.v.) seriatim on toad pressor responses to angiotensin II (Ang II, 0.1 nmol kg^{-1} i.v.). Plotted are the peak MAP and corresponding pulse pressure; each symbol represents the mean for 6 animals; s.e.means shown by vertical lines. * $P < 0.05$ compared to the response to saline vehicle injection. † P compared to the response to Ang II injection. †† $P < 0.05$ compared to the response after prazosin treatment.

Elevation of blood pressure by LHRH and sLHRH was accompanied by bradycardia. Low and moderate pressor doses of Ang II also suppressed cardiac frequency. However, high doses of Ang II (10 to 100 nmol kg^{-1}) simultaneously increased both blood pressure and cardiac frequency. Previously tachycardia induced by Ang II in animals with intact baroreflexes had been observed with ducklings (Wilson, 1983), dogs and sheep (Lumbers & Potter, 1983). Thus Ang II may inhibit the baroreflex control of cardiac frequency.

Significant concentrations of sulphoconjugated catecholamines were detected in toad plasma during the present experiments. For dopamine, in particular, sulphoconjugates comprised major portions of the circulating catecholamine and occurred at higher concentrations than did free dopamine. Plasma levels of glucuronoconjugated catecholamines varied markedly between individual toads. Previously conjugation had been observed in birds and mammals. Duck arterial plasma contained glucuronoconjugated dopamine, NA and Ad while chicken plasma contained both glucuronide and sulphate esters of each catecholamine (Wilson, 1984). Rat plasma contained low concentrations of sulphoconjugated dopamine, NA and Ad, higher concentration of glucuronoconjugated dopamine but no detectable glucuronoconjugated NA and Ad (Wang *et al.*, 1983). Sulphation was the sole mode of conjugation for dopamine, NA and Ad measured in human plasma (Wang *et al.*, 1983). Thus the pattern of catecholamine conjugation differed between species.

The physiological importance and regulation of catecholamine conjugation are unknown. Human experiments indicated that during exercise, plasma levels of sulphoconjugated NA and Ad decrease while free NA and Ad increase (Davidson *et al.*, 1984). An investigation in rats showed that conjugation with sulphate and glucuronide increases during stress when large amounts of free catecholamines enter the plasma (Alexánder *et al.*, 1984). However, the present experiments dissociated changes in free catecholamines from circulating levels of their conjugates: Ang II increased free NA concentration in toad arterial plasma by 5 min post-injection and prazosin increased

free Ad by 60 min post-injection, yet neither treatment augmented conjugated catecholamines.

The catecholamine response to Ang II in control toads comprised only a rise in free NA. This may indicate the adrenal glands are stimulated by Ang II because amphibian plasma NA is derived from adrenal paraneurones, Ad serving as the transmitter in peripheral nerves (Burnstock, 1969). Crossover of Ad into the NA assay is not detectable and cannot account for the NA concentrations measured. Although the elevation of free NA occurred coincidentally with the rise in blood pressure following Ang II injection, experiments with α- and β-adrenoceptor antagonists showed that mobilization of NA did not cause the increase in pressure. A novel observation of the present experiments was the suppression of free Ad by Ang II in prazosin-treated toads; this large effect was detected in each of the 6 toads tested but its explanation is unknown.

Injection i.a. of the α-adrenoceptor antagonist prazosin resulted in prolonged elevation of toad plasma Ad, cardiac frequency, pulse pressure and MAP. The same dose of prazosin injected i.v. had less marked effect on arterial pressure (Wilson *et al.*, 1984, and unpublished observations). Previously α-adrenoceptor blockade had been observed to increase the rate of sympathetic neurosecretion in mammals (Kalsner & Quillan, 1984; Kirpekar, 1984). Also the sympathomimetic actions of an α-adrenoceptor antagonist, phenoxybenzamine, was shown to increase the rate and force of contractions in rat isolated hearts (Chang, 1968). The present experiments showed that in toads the mobilization of Ad during prazosin treatment activates β-adrenoceptor mechanisms, because the cardiovascular sequelae were partially reversed by propranolol. Part of the cardiovascular stimulation following prazosin was not reversed by propranolol, however, consistent with an earlier report that sympathetic stimulation of the toad heart is mediated both by β-adrenoceptors and by receptors that are neither α- nor β- types (Morris *et al.*, 1981).

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