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Evidence that the apparent complexity of receptor antagonism by angiotensin II analogues is due to a reversible and syntopic action

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1 The interactions between angiotensin II (AII), two non-peptide antagonists DuP 753 and IMI, and eight peptide analogues of AII were investigated on the rabbit isolated aorta assay. DuP 753 and IMI behaved as simple competitive antagonists (pK_B values 8.4 and 6.8, respectively). To different degrees, all the AII-peptide analogue interactions failed to meet the basic criteria for simple competition. In addition to rightward shift, the most significant feature was a concentration-dependent saturable depression of the upper asymptote of the AII concentration-effect curves.

2 'Washout' and combined dose-ratio analysis experiments, in which DuP 753 was used as a reference antagonist, indicated that the profile of peptide antagonism was solely due to a reversible and syntopic action at the AII receptor.

3 By use of an operational model of agonism (Black & Leff, 1983) as a starting point, it was possible to account for the data with a new model which describes reversible receptor occupancy and occupied receptor-determined, saturable reduction in the efficacy of AII. Model-fitting gave estimates of pK_B values for the peptide analogues and agonist affinity and efficacy parameters for AII.

4 The model was successfully tested by applying it to qualitatively similar results obtained in a cross-tissue analysis on guinea-pig aorta, ileum and stomach.

5 A 'molecular' interpretation of the efficacy changes, based on the concepts of receptor internalisation and expression, is offered.

Keywords: Angiotensin II; receptor; antagonist; agonist; aorta, ileum, stomach; DuP 753

Introduction

Angiotensin II (AII), peptide-analogue, antagonists were first described by Khairallah *et al.* (1970) and Marshall *et al.* (1970). Since then, although a large number of similar AII analogues have been described, all of them have shown more or less complex pharmacological behaviour. For example, it has been reported that some peptide analogues did not behave in a simple competitive manner with AII, but produced 'non-competitive' or 'insurmountable' antagonism (Regoli *et al.*, 1974; Freer *et al.*, 1980a,b; Koziarz & Moore, 1989; Bovy & Blaine, 1989). The antagonism of [Sar[1],Ile[8]]AII, however, has been described as both 'essentially competitive' (Turker *et al.*, 1972) and 'pseudo-irreversible' (Scanlon & Moore, 1988). Most peptide analogues have been found to produce partial agonist effects *in vivo* (Streeten & Anderson, 1984; Holck *et al.*, 1989; Bovy *et al.*, 1989) and some *in vitro* (Regoli *et al.*, 1974; Scanlon *et al.*, 1984; Criscione *et al.*, 1990).

In contrast, recently described, non-peptide, AII-receptor ligands, exemplified by DuP 753 (Chiu *et al.*, 1990; Timmermans *et al.*, 1991) and IMI (Koekpe *et al.*, 1990), have been described as simple competitive antagonists free from partial agonist activity.

In this paper we have used DuP 753 as a reference competitive antagonist to analyse the complex behaviour of eight peptide analogues of AII in isolated preparations of rabbit aorta and guinea-pig aorta, ileum and stomach.

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Methods

Isolated smooth muscle preparations

Rabbit and guinea-pig aorta ring preparations Aortic ring segments (approximately 3 mm long) from rabbit (male, New Zealand White, approximately 2.5 kg) and guinea-pig (male, Dunkin Hartley, 350 to 450 g) aortae were prepared following Stollak & Furchgott (1983). Briefly, descending thoracic aortae were excised, cleaned of extraneous tissues, and the endothelium was removed. Rings were suspended between 2 stainless steel wires in 20 ml organ baths containing Krebs-Henseleit (K-H) solution of the following composition (in mM): Na^+ 143, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 128, HPO_4^{2-} 1.0, SO_4^{2-} 1.2, HCO_3^- 25, D-glucose 10, constantly gassed with 95% O_2 and 5% CO_2, and maintained at 37°C. Tension was continuously recorded with isometric transducers, following the application of 1 g and 4 g pre-loads in the guinea-pig and rabbit preparations, respectively.

Guinea-pig ileum longitudinal muscle preparation Sections of the distal ileum were removed from guinea-pigs and prepared essentially as described by Rang (1964). Longitudinal muscle strips of approximately 2 cm were suspended between 2 stainless steel hooks in K-H solution at 30°C, and gassed as above. A 1 g pre-load was applied and isometric tension was continuously recorded.

Guinea-pig stomach smooth muscle preparation Whole stomachs were removed from guinea-pigs and the smooth muscle from the main body region was separated from the mucosa by injecting K-H solution to form a blister. Thin strips (approximately 0.5 × 1.5 cm) were cut from the blistered region and mounted on isotonic transducers, in

gassed K-H solution at 30°C, for continuous displacement recording.

Experimental protocols

Six preparations were used simultaneously and allocated to control and treatment groups by a randomised block design. Preparations were allowed to stabilize for 60 min during which the organ bath fluid was replaced with pre-warmed K-H solution at 15 min intervals.

Analysis

Logistic curve-fitting Agonist concentration-effect (E/[A]) data from individual preparations were fitted to a logistic function of the form,

$$E = \frac{\alpha\ [A]^p}{[A_{50}]^p + [A]^p} \quad (1)$$

to provide estimates of α (upper asymptote), $\log[A_{50}]$ (midpoint location) and p (midpoint slope index). These parameters, expressed as mean ± s.e., were used for subsequent analysis and display of data (see Black & Shankley, 1985 for details).

Competitive analysis Competitive analysis was performed according to the procedure described previously (Black *et al.*, 1985b). If the Schild plot slope parameter, b, was found to be different from unity, or if differences were found in the midpoint slope indexes or upper asymptotes of the AII E/log[A] curves in the absence and presence of an antagonist, a pA_2 value was estimated without prejudice to mechanism of action.

Combined dose-ratio (r) analysis This analysis was performed as described previously (Shankley *et al.*, 1988) with DuP 753 used as reference antagonist to test for syntopic, competitive, antagonism. In brief, the theoretical relations which describe the syntopic (additive, $r_{B+C} = r_B + r_C - 1$) and allotopic (multiplicative, $r_{BC} = r_B.r_C$) interaction between two antagonists were expressed as test statistics (S) in terms of the experimentally-estimable $\log[A_{50}]$ values,

$$S_A = \log[A_{50}]_{B+C} - \log([A_{50}]_B + [A_{50}]_C - [A_{50}]) \quad (2)$$

$$S_M = \log[A_{50}]_{BC} - \log[A_{50}]_B - \log[A_{50}]_C + \log[A_{50}] \quad (3)$$

Test statistic values of zero are expected for model compliance.

Experimental data were directly fitted to the mathematical models described below with the 'AR' programme (derivative-free, non-linear, regressional analysis) within the BMDP statistical software package (Dixon, 1990).

Drugs

Drug stock solutions were prepared in distilled water, stored frozen below −20°C and used within 4 weeks, except noradrenaline which was prepared freshly, stoichiometrically with ascorbic acid. The latter had no significant effect on tissue responsiveness in control experiments. The total volume added to the 20 ml organ bath did not exceed 400 µl. Drugs and their sources were as follows: AII (Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]), Cambridge Research Biochemicals, U.K.; [Sar[1],Ala[8]]AII, [Sar[1],Val[5],Ala[8]]AII, [Sar[1],Ile[8]]AII, [Sar[1],Leu[8]]AII and noradrenaline hydrochloride (NA), Sigma Chemical Co. Ltd., U.K.; [Sar[1], Tyr(Me)[4]]AII, a gift from Professor G.J. Moore, University of Calgary, Canada and Peninsula Laboratories Inc., U.K.; [Sar[1]]AII(1-7)NH_2 and IMI (4'((2-butyl-4-chloro-5-(hydroxymethyl)-1H-imidazol-1-yl)methyl)-(1,1'-biphenyl)-2-carboxylic acid), gifts from Dr P.R. Bovy, Searle Research and Development, U.S.A.; [Sar[1],Thr[8]]AII, a gift from Dr M.C. Khosla, The Cleveland Clinic Foundation, U.S.A.; [Sar[1],Phe(2,3,4,5,6-Br_5)[8]]AII, a gift from Dr E. Escher, University of Sherbrooke, Canada; DuP 753, also designated as losartan and MK 954, (1-(2'-(5-tetrazoyl)phenyl)benzyl-2-butyl-4-chloro-5-methanolimidazole, potassium salt), a gift from E.I. Du Pont De Nemours & Co., U.S.A.

Results

Angiotensin concentration-effect relations in rabbit aorta

AII concentration-effect (E/[A]) data obtained by cumulative dosing in half-log dose increments at peak responses, could be fitted to the general logistic function (equation (1)). As fitted, the AII E/log[A] curves were symmetrical sigmoids with mean (± s.e.) parameter values as follows: $\log[A_{50}] = -8.86 \pm 0.05$, $\alpha = 6.28 \pm 0.11$ g, $p = 1.55 \pm 0.07$ (d.f. = 59). The phasic relaxation seen with high concentrations did not appear to accumulate suggesting that rapid desensitization was not occurring. Thus, when AII was given as single additions, one per preparation, the average E/[A] curve was not significantly different from that obtained by cumulative dosing. AII behaved as a partial agonist in the contractile system having an α value of about 60% of the NA maximum response (Figure 1).

Simple competitive antagonism of AII-receptors in rabbit aorta

DuP 753 and IMI, non-peptides, classified as competitive AII-receptor antagonists with pA_2 values of 8.5 (Chiu *et al.*, 1990) and 7.1 (Koepke *et al.*, 1990), have been used to validate the assay and characterize the AII-receptor population. When AII E/log[A] curves were obtained after incubation for 30 min with these ligands they were, with respect to control curves, displaced in parallel with no depression of upper asymptotes as expected for simple competitive antagonism. These concentration-dependent displacements were fitted closely by the Gaddum-Schild equation and pK_B values of 8.44 ± 0.05 (d.f. = 34) and 6.81 ± 0.03 (d.f. = 28) were estimated for DuP 753 and IMI, respectively. Judged by goodness-of-fit and the value of the pK_B's the system was apparently in equilibrium at all antagonist concentrations (Figure 2).

Therefore, these non-peptide ligands are behaving as simple competitive antagonists of AII at a homogeneous population of receptors in rabbit aorta.

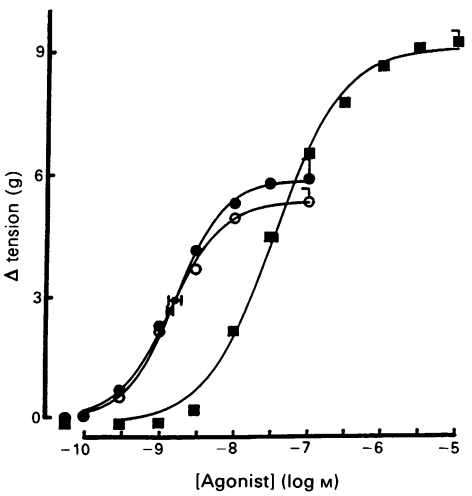

Figure 1 Angiotensin II (AII) E/[A] curves obtained by cumulative (●) and single (○) dosing. The noradrenaline (NA) E/[A] curve obtained by cumulative dosing (■) is shown for comparison (see text for details).

AII-receptor antagonism by peptide analogues of angiotensin II

Incubation times for each ligand were chosen by determining the time (in 30 min increments) required for the rightward shifting effect on the AII curve to saturate with the lowest concentrations (data not shown). All of the eight peptide analogues required longer periods of incubation than the non-peptides at the lowest concentrations tested. For most of them 60 min was sufficient but three needed 120 min (Table 1).

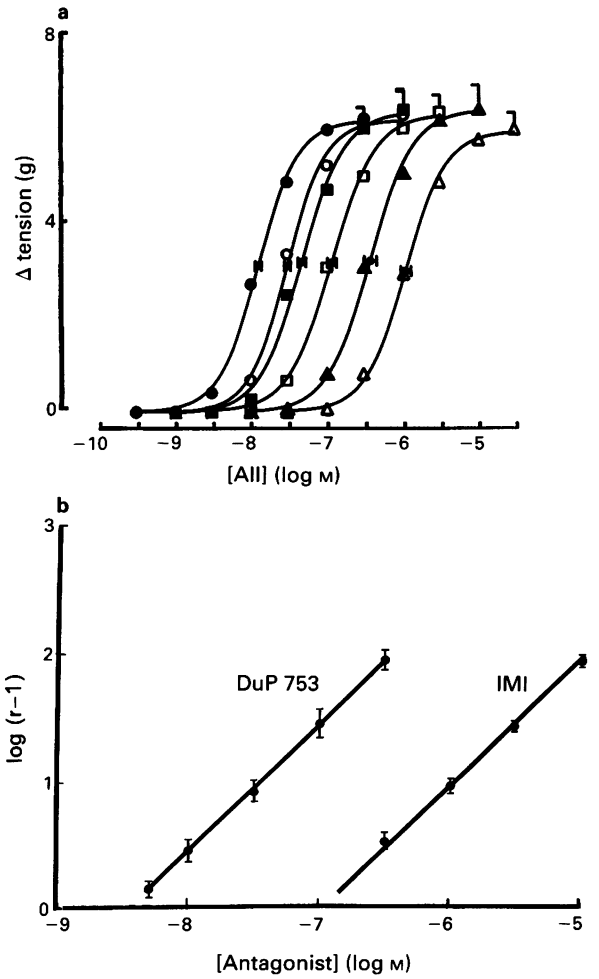

Figure 2 (a) Angiotensin II (AII) E/[A] curves obtained in the absence (●) and presence of 5 (○), 10 (■), 30 (□), 100 (▲), 300 (△) nM DuP 753. (b) Schild plots obtained from the interaction of AII with Dup 753 and IMI on rabbit aorta (see text for details).

Only two of the peptide analogues, [Sar^1,Tyr(Me)^4]AII and [Sar^1,Leu^8]AII, produced significant agonist responses (12 ± 4% and 3 ± 1%, respectively) which were totally blocked by DuP 753 at 0.3 μM. The partial agonist effect by [Sar^1,Leu^8]AII faded away during incubation (Figure 3d).

Simple competitive antagonism is manifested by parallel displacement of E/log[A] curves with no change in upper asymptote, so that only the log[A_{50}] parameter is changed. However, for this set of peptide analogues of AII although all of the data could be fitted by the logistic function, changes in all three of the parameters were found (Figure 3). In addition, in four cases the Schild plot slope parameters were significantly greater than unity (Table 1), another indication of the failure to adhere to the conditions of simple competitive antagonism. In spite of this and without prejudice to mechanism, we used the relationship between [B] and [A_{50}] in Schild plot space to estimate pA_2 values as a measure of the relative potencies of the ligands (Table 1).

For most of the compounds no significant change in the value of the slope index (p) of the AII E/log[A] curves could be detected by analysis of variance. However, with two compounds, [Sar^1,Ile^8]AII and [Sar^1,Leu^8]AII, a significant and apparently saturable, reduction in p occurred. These were also the compounds which produced the most depression of the AII E/log[A] curve maxima. Although, obviously, the relation between log [B] and log [A_{50}] did not saturate, it looked (Figure 3) as though the relationship between log [B] and the change in α, the depressant effect, did. The appearance of saturation was not due to inadequate incubation (i.e. equilibration) times because increased incubation did not result in either increased rightward shift or further depression of α (data not shown).

In order to define this observation more precisely, the interaction between one of the ligands which produced the most depression, [Sar^1,Leu^8]AII, and AII was re-examined using extra [Sar^1,Leu^8]AII concentrations (Figure 4). The data were fitted to the following logistic function.

$$\frac{\alpha - \alpha_B}{\alpha} = \frac{D_m \cdot [B]^{p'}}{[B_{50}]^{p'} + [B]^{p'}} \quad , \qquad (4)$$

where α and α_B are the upper asymptotes from the logistic curve-fitting (equation (1)) in the absence and presence of

Table 1 Analysis of angiotensin II (AII)-receptor ligands: rabbit aorta

Ligand	D_m[a]	pA_2[b]	$p[B_{50}]$[c]	*Model-fitting*[d] β	*Model-fitting*[d] pK_B
(incubation time)					
[Sar^1,Ala^8]AII (60 min)	37 ± 4	9.01 ± .06[e]	8.47 ± .02	0.43 ± .05	8.74 ± .06
[Sar^1,Val^5,Ala^8]AII (60 min)	34 ± 4	9.03 ± .06	8.34 ± .07	0.34 ± .05	8.69 ± .05
[Sar^1,Ile^8]AII (120 min)	82 ± 3	9.47 ± .10[e]	8.91 ± .14	0.68 ± .06	8.97 ± .05
[Sar^1,Leu^8]AII (120 min)	77 ± 2	9.04 ± .06[e]	8.86 ± .07	0.69 ± .05	8.93 ± .05
[Sar^1,Tyr(Me)^4]AII[f] (60 min)	21 ± 5	6.95 ± .09	6.94 ± .17	0.22 ± .05	6.75 ± .08
[Sar^1]AII(1–7)NH_2 (60 min)	42 ± 4	7.37 ± .08[e]	6.84 ± .12	0.49 ± .06	7.33 ± .06
[Sar^1,Thr^8]AII (60 min)	38 ± 4	8.80 ± .07	8.40 ± .08	0.35 ± .05	8.49 ± .05
[Sar^1,Phe(2,3,4,5,6-Br_5)^8]AII (120 min)	47 ± 2	8.41 ± .06	8.48 ± .02	0.41 ± .06	8.28 ± .06
DuP 753 (30 min)		8.44 ± .05			
IMI (30 min)		6.81 ± .03			

[a]The maximum percentage change from the mean control AII E/log[A] curve maximum (see text and equation (4) for details).
[b]Estimated by Schild analysis.
[c]-log concentration of ligand required to produce half maximum depression of α.
[d]Obtained by fitting of the mean experimental data to the model equations (10), (11), (15) and (16) (see text for details).
[e]Schild plot slopes greater than unity.
[f]Expressed significant partial agonism. Therefore, an estimate of τ was made ($\tau_B = 0.35 \pm .08$) (see text for details).

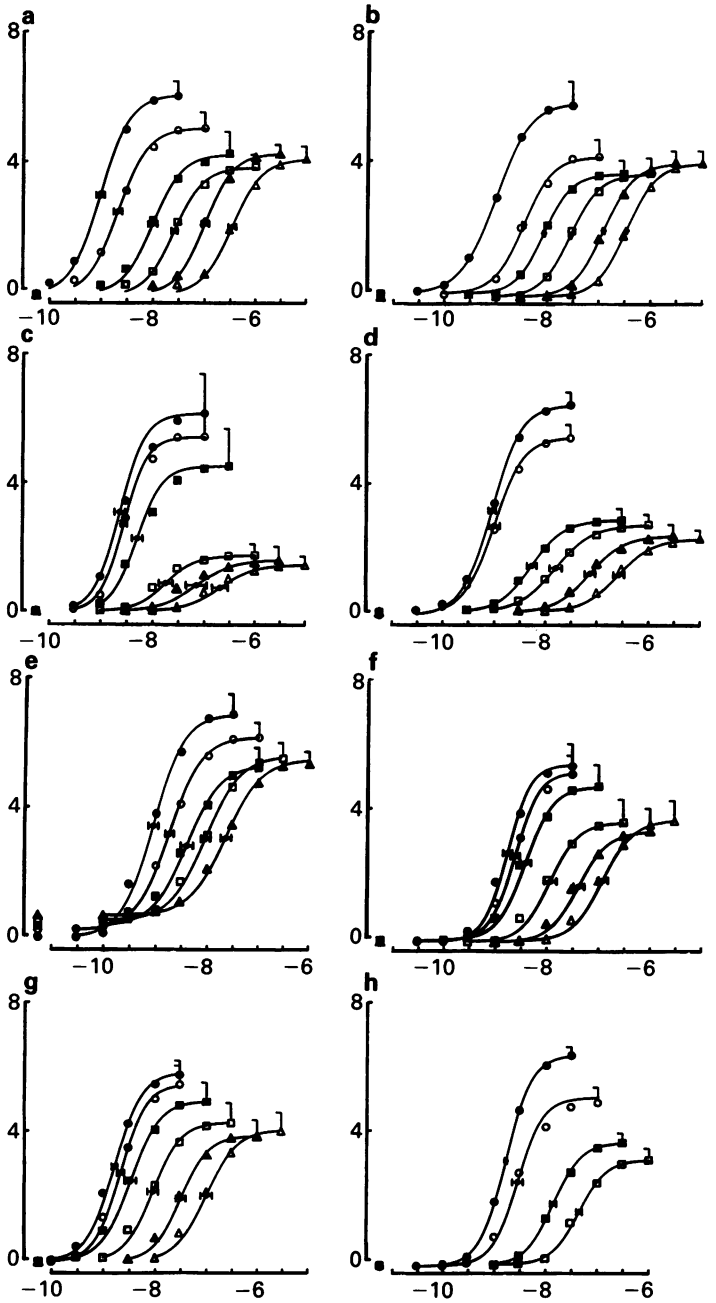

Figure 3 Angiotensin II (AII)-receptor antagonism by 8 peptide analogues of AII in rabbit aorta. AII E/[A] curves ($n = 6$ to 8) obtained in the absence (●) and presence (nM) of (a) [Sar^1,Ala^8]AII, 3 (○), 10 (■), 30 (□), 100 (▲), 300 (△); (b) [Sar^1,Val^5,Ala^8]AII, 3 (○), 10 (■), 30 (□), 100 (▲), 300 (△); (c) [Sar^1,Ile^8]AII, 0.3 (○), 1 (■), 3 (□), 10 (▲), 30 (△); (d) [Sar^1,Leu^8]AII, 0.3 (○), 1 (■), 3 (□), 10 (▲), 30 (△); (e) [Sar^1,Tyr(Me)^4]AII, 100 (○), 300 (■), 1000 (□), 3000 (▲); (f) [Sar^1]AII(1–7)NH$_2$, 30 (○), 100 (■), 300 (□), 1000 (▲), 3000 (△); (g) [Sar^1,Thr^8]AII, 1 (○), 3 (■), 10 (□), 30 (▲), 100 (△); (h) [Sar^1,Phe(2,3,4,5,6-Br$_5$)^8]AII, 3 (○), 30 (■), 100 (□). The curves drawn through the mean experimental data points were simulated using the parameter values obtained from the logistic curve-fitting procedure (see Methods). Error bars represent s.e.mean. For the incubation-times see Table 1. Abscissae: [AII]: log M. Ordinates: Δ tension (g).

[B]. D_m, $[B_{50}]$ and p′ are the fitting parameters for the maximum depression, midpoint location and midpoint slope index, respectively. From the logistic curve-fitting (Figure 4), estimates of D_m and $p[B_{50}]$ were obtained. Similarly, parameter estimates were obtained with the existing data (Figure 3) for the other peptide analogues (Table 1). The D_m values varied between 21 ± 5% and 82 ± 3%, but no correlation was found between this effect and the potency of the antagonists as defined by the pA_2 ($r = 0.62$, $t = 1.95$, d.f. = 6). However, a high correlation was found between the pA_2 and the potency of the depressant action as defined by the $p[B_{50}]$ ($r = 0.95$, $t = 7.45$, d.f. = 6) (see Table 1).

The effects of the peptide analogues on the AII E/log[A] curves were reversed following washout (washes at 15 min intervals for between 60 and 120 min, data not shown). Under all conditions, the α depression and the extent of rightward shift of the AII E/log[A] cuves were linked with respect to the concentration of antagonist.

Combined dose-ratio analysis of peptide analogues using DuP 753 as a reference antagonist

Cumulative AII E/log[A] curves were obtained in the absence and presence of [Sar^1,Ala^8]AII, 30 nM, (log dose-ratio, log

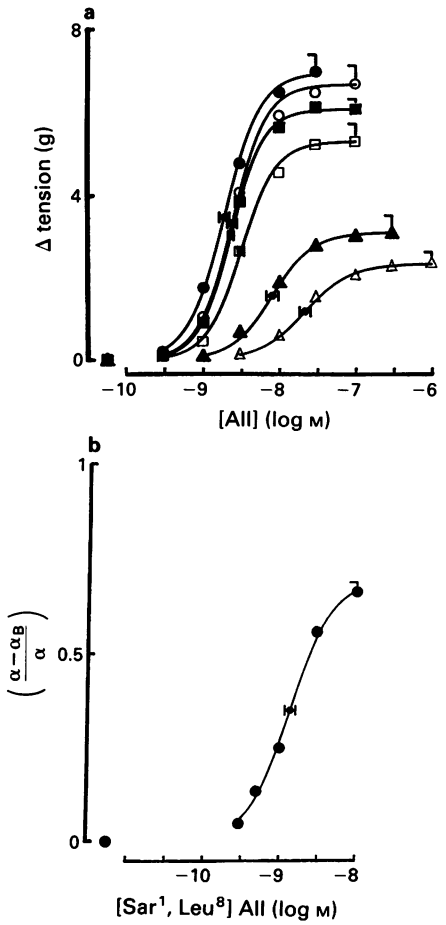

Figure 4 Analysis of the relationship between peptide ligand ([Sar¹,Leu⁸]AII) concentration and the depression of the angiotensin II (AII) E/[A] curve maxima. (α). (a) AII E/[A] curves ($n = 6$) obtained in the absence (●) and presence (nM) of [Sar¹,Leu⁸]AII, 0.3 (○), 0.5 (■), 1 (□), 3 (▲) and 10 (△). (b) The change in α has been expressed with reference to the control curve value α_B in the presence of the peptide ligand. The curve shown superimposed on the experimental data was obtained by logistic curve-fitting (see text for details).

$r_B = 1.43$) or DuP 753, 100 nM, (log $r_C = 1.46$) or both (log $r_{B+C} = 1.52$) (Figure 5). When these data were inserted in equations (2) and (3), the S_A value ($S_A = 0.02 \pm 0.10$, d.f. = 23) but not the S_M value ($S_M = -0.88 \pm 0.02$, d.f. = 23), was not significantly different from zero, indicating a syntopic interaction. Not only did DuP 753 act syntopically with [Sar¹,Ala⁸]AII in dose-ratio terms but also partially antagonized [Sar¹,Ala⁸]AII-induced depression of the AII maximum. Therefore the antagonism of AII by [Sar¹,Ala⁸]AII must be regarded, by this test, as not only syntopic but competitive as well. No significant differences were found by repeating the combined dose-ratio analysis using different orders of incubation. Similar results were obtained with [Sar¹,Ile⁸]AII (10 nM) and DuP 753 (100 nM). In addition, DuP 753 (100 nM) was found to antagonize totally the depression of the AII curve maxima by a lower concentration (1 nM) of [Sar¹,Ile⁸]AII.

Development and application of an explanatory model

To what extent could the features of the antagonism of AII by the peptide analogues be accounted for, qualitatively and quantitatively, within the framework of pharmacological concepts of ligand-receptor interactions? We have used the modelling process previously described by Black & Leff (1983). The AII E/[A] curves, in the absence and presence of all the

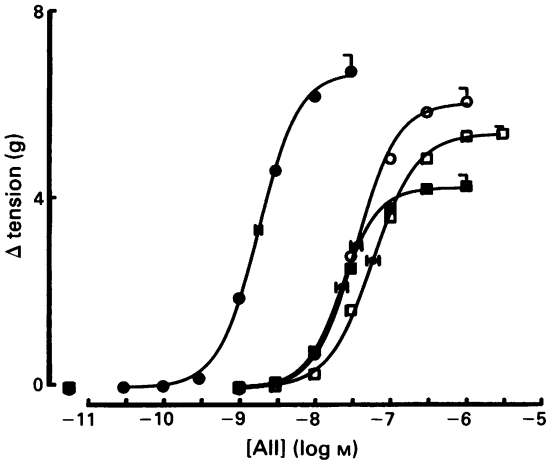

Figure 5 Combined dose-ratio analysis of angiotensin II (AII)-receptor antagonism in rabbit aorta. AII E/[A] curves obtained in the absence (●) and presence of DuP 753 (100 nM) (○), [Sar¹, Ala⁸]AII (30 nM) (■) and a combination of DuP 753 (100 nM) with [Sar¹,Ala⁸]AII (30 nM) (□).

antagonists could be adequately described by the logistic function (equation (1)). Therefore, the starting point was the form of the model of agonism (Black & Leff, 1983) which accounts for logistic E/[A] curves,

$$E = \frac{E_M[A]^{n\tau n}}{(K_A + [A])^n + [A]^{n\tau n}} \quad (5)$$

where E_M is the maximum effect achievable in the system; K_A is the equilibrium dissociation constant of an agonist A; τ is the transducer ratio which provides a measure of the efficiency of A in the system; n is the midpoint slope parameter of the transducer function. The problem now reduced to determining which of the four parameters needed to be changed to account for the observed profiles of AII antagonism.

Because all the effects of the ligands were concluded to be a consequence of reversible and syntopic receptor binding which had attained equilibrium, this component of the antagonism could be expressed in the model by multiplying K_A by the factor $(1 + [B]/K_B)$ in the usual way,

$$E = \frac{E_M[A]^{n\tau n}}{(K_A(1 + [B]/K_B) + [A])^n + [A]^{n\tau n}} \quad (6)$$

where [B] is the concentration of a peptide analogue and K_B, its equilibrium dissociation constant. This competition at the receptor level can only account for parallel rightward shift of E/log[A] curves with no change in the upper asymptote or slope index.

The experimental parameters α, p and $[A_{50}]$, obtained by logistic curve-fitting, are related to the model parameters as follows:

$$\alpha = \frac{E_M.\tau^n}{1 + \tau^n} \quad (7)$$

$$p = \frac{n(2 + \tau^n)\ ((2 + \tau^n)^{1/n} - 1)}{(2 + \tau^n)^{1/n}(1 + \tau^n)} \quad (8)$$

$$[A_{50}] = \frac{K_A}{(2 + \tau^n)^{1/n} - 1} \quad (9)$$

Changes in all three of these logistic parameters were detected (see Table 1). Therefore, because K_A and E_M only appear in two of the three equations above, an effect of the antagonist on either of these model parameters alone was not sufficient to account for the data. Only the model parameters

τ and n, because they appear in all three equations, could theoretically and singularly account for the data. In practice, only changes in τ could account for the data; changes in n can be ruled out because the midpoint slope index of the E/log[A] curves, p, is more sensitive than α to changes in n (equations (7) and (8)) yet large changes in α were detected for only small changes of p in the analysis.

The ligands appeared to be equipotent in terms of both reduction of α and the rightward shift, that is $p[B_{50}] \simeq pA_2$ (Table 1). Therefore, we assumed that these two events are both related to receptor occupancy. Further, because the reduction in α was saturable, whatever the mechanism, a simple relationship between the receptor occupancy and a change in τ could be expressed as,

$$\tau' = \tau \left(1 - \frac{\beta[B]}{K_B + [B]}\right) \quad (10)$$

where, τ' is τ in the presence of a peptide analogue, β is a proportionality constant included to define the maximum reduction in τ that each ligand can produce.

The model may then be represented as follows, where τ' is defined by equation (10),

$$E = \frac{E_M \tau'^n [A]^n}{(K_A(1 + [B]/K_B) + [A])^n + [A]^n \tau'^n} \quad (11)$$

In this new model, the upper asymptote of the E/log[A] curve, when $[A] \rightarrow \infty$ is given by,

$$\alpha = \frac{E_M \tau'^n}{1 + \tau'^n} \quad (12)$$

and the midpoint location parameter, [A] for 0.5α is given by,

$$[A_{50}] = \frac{K_A(1 + [B]/K_B)}{(2 + \tau'^n)^{1/n} - 1} \quad (13)$$

where τ' remains as defined in equation (10).

The model expectation for changes in τ could then be deduced. The effects of a reduction in the value of τ on the expression of competitive antagonism are dependent on the initial value of τ, that is the efficacy of the agonist; the system behaves as though irreversible receptor antagonism occurs with concurrent, competitive antagonism (Black *et al.*, 1985b). For high efficacy agonists and when the maximum τ reduction is not sufficient to reduce significantly the value of α, the midpoint location of the E/log[A] curve approximates to,

$$[A_{50}] = \frac{K_A(1 + [B]/K_B)}{\tau'} \quad (14)$$

Under these conditions, dose-ratios will be greater than expected. For low efficacy systems, as the value of τ is reduced so α will be reduced according to equations (10) and (12), but the effect on the $[A_{50}]$ is diminished as the value of the control $[A_{50}]$ approaches asymptotically $K_A(2^{1/n} - 1)$ (see Black *et al.*, 1985a). The behaviour of the model with a high and low efficacy agonist is illustrated in Figure 6.

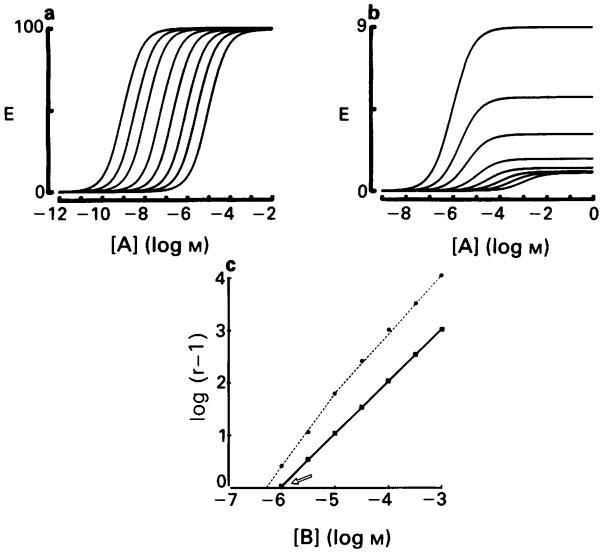

Figure 6 Simulation showing the effect of concurrent affinity and efficacy reduction in the operational model (equations (10) and (11)) for both (a) high and (b) low efficacy values. Parameters were set as follows: $E_M = 100$, $pK_A = 6$, $\beta = 0.9$, $n = 1$. (a) $\tau = 1000$ (b) $\tau = 0.1$. (c) Schild plots corresponding to this simulated data shown in panels (a, ●....●) and (b, ■—■).

Model fitting: rabbit aorta

One of the peptide analogues, [Sar^1,Tyr(Me)^4]AII, expressed significant agonist activity and, for completeness, this was accounted for in the model-fitting programme by adding the following system of equations to equations (10) and (11),

$$E_B = \frac{E_M \tau_B^n [B]^n}{(K_B(1 + [A]/K_A) + [B])^n + [B]^n \tau_B^n} \quad (15)$$

$$E_T = E + E_B \quad (16)$$

where E_T is the total effect produced by [Sar^1,Tyr(Me)^4]AII (still given as B) and AII (A). These equations, with equation (11), simultaneously describe the competitive interaction between one agonist (AII) and another with significantly lower efficacy ([Sar^1,Tyr(Me)^4]AII) such that the expression of efficacy by the latter compound does not significantly perturb the expression of its competitive antagonism (Jenkinson, 1978; Kenakin, 1987). The addition of these equations adds one extra estimable parameter, τ_B, the efficacy of [Sar^1,Tyr(Me)^4]AII.

The model assumes a reduction in τ. Therefore, the same principles apply for model-fitting of these data as they do when agonist K_A value estimates are made using irreversible antagonist pretreatment (Black *et al.*, 1985b). The model fit was set up to provide single values of the AII-dependent parameters, K_A and τ, single values of the tissue-dependent parameters, E_M and n, and individual values of the peptide analogue-dependent parameters, β and K_B. In this way, β was the only peptide analogue-dependent parameter allowed to vary between compounds to account for the reduction in τ and hence the depression of α, the changes in E/log[A] slope index and the steep Schild plots. In practice, log values of the equilibrium dissociation constants (K_B and K_A) were estimated as these are assumed to be normally-distributed.

Ideally, we would have liked to fit all the data simultaneously to the model. However, regardless of other problems, with such a large data set (circa 6000 E/[A] values) and large number of parameters to be estimated (26), we had insufficient computer capacity to perform the task. However, we were able to fit the data obtained with each peptide separately (Figure 7). In addition, two tests of the model were made by allocating the eight peptides into two groups based on the behaviour of the ligands. In particular, the data were divided to investigate the effects of different degrees of depression and the expression of efficacy by [Sar^1,Tyr(Me)^4]AII on the quality of the fit. The mean experimental data from each of these groups were fitted to the model (equations (10), (11), (15) and (16)). It was found that consistent estimates of the ligand and tissue dependent-parameters (K_A, τ, E_M and n) could be obtained regardless of the grouping, indicating the robustness of the model. The estimates of the peptide-analogue-dependent parameters (β and K_B) and their variance obtained from fitting the mean data in two groups (a, c, g, h and a, d, e, f referring to Figure 3 legend) are presented in Table 1.

The values of τ (1.42 ± 0.20), n (2.20 ± 0.30) and E_m (9.3 ± 1.0 g) were consistent with AII behaving as a partial agonist at the AII-receptor ($\alpha = 68\%$ E_m) in rabbit aorta and a pK_A value of 8.94 ± 0.10 was obtained. The effects of the small changes observed in τ are amplified in the system

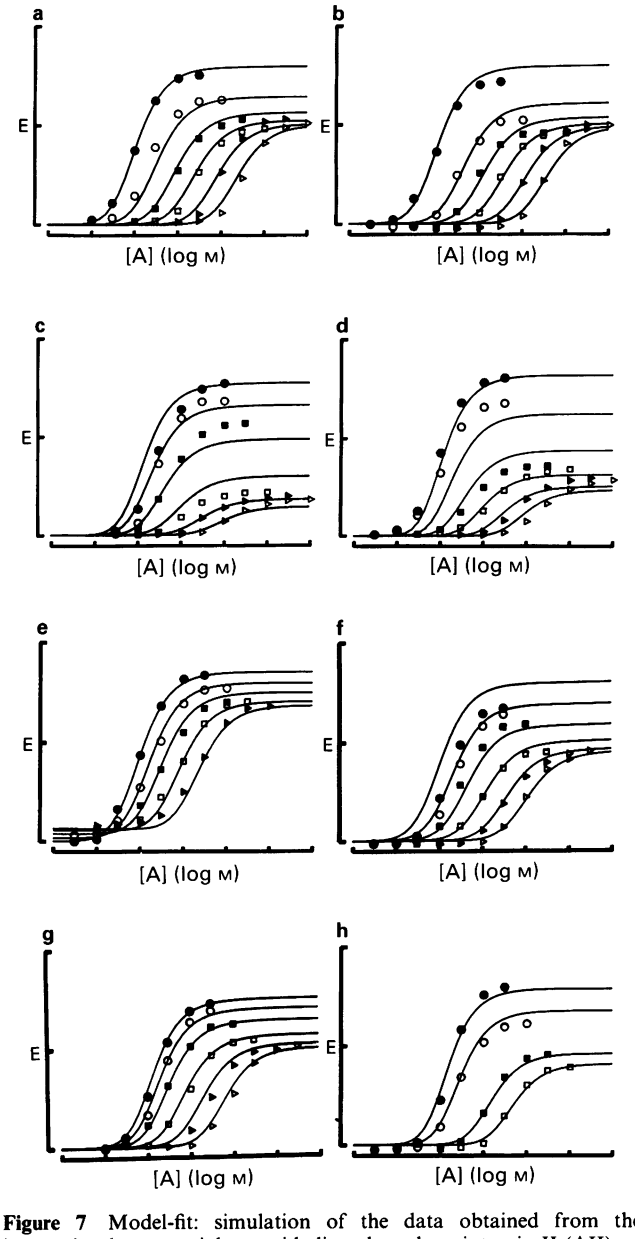

Figure 7 Model-fit: simulation of the data obtained from the interaction between eight peptide ligands and angiotensin II (AII) on rabbit aorta. The curves shown superimposed on the mean experimental data points were obtained using the parameter estimates, presented in Table 1 and the text, inserted into equations (10), (11), (15) and (16). (See Figure 3 legend for peptide analogue identification and concentrations).

because AII is a partial agonist and, in particular, because of the steep transducer function slope, n, in the model (see Black *et al.*, 1985a). The low value of τ accounts for the results that the model-fit derived pK_B values were close to the pA_2 values estimated by the Schild method (also see Figure 6).

Model fitting: cross-tissue analysis

Having established that the model could account for all the data obtained on the rabbit aorta assay, a useful test was established by attempting to fit the data obtained with one of the most frequently-quoted peptide analogues ([Sar^1,Ala^8]AII) on the guinea-pig aorta, ileum longitudinal and stomach smooth muscle assays. The location of the AII control E/log[A] curve varied significantly across these assays and although, qualitatively, a similar complex profile of antagonism was obtained, the degree of depression of α also varied across the assays. The non-peptide antagonist, DuP 753, behaved as simple competitive antagonist in all the assays with no significant difference between the pK_B values estimated, and acted syntopically with [Sar^1,Ala^8]AII as judged by combined dose-ratio analysis (data not shown).

For the model to have utility it would be expected that satisfactory fits could be obtained by only allowing the tissue-dependent parameters E_M, τ and n to vary between assays. Therefore in the model-fits the ligand-dependent parameters, pK_B, β and pK_A, were constrained to those values obtained on the rabbit aorta assay.

The model could apparently account for the data as judged by inspection of Figure 8. However, the estimates of the model parameters E_M and τ in each tissue were associated with extremely high variance (Figure 8 legend). This follows from the nature of the data set. The control curve log[A_{50}] values on the three guinea-pig assays are greater than the $\log K_A$ estimated in rabbit aorta. In the operational model of agonism this can only occur when n is significantly greater than unity and for relatively low τ values (Black *et al.*, 1985a). Reliable estimations of E_M and τ requires knowledge of how, experimentally, [A_{50}] changes with α to define the underlying transducer function. When the competitive element, $(1 + [B]/K_B)$, due to the peptide analogues is removed then in the three guinea-pig assays the [A_{50}] values are indistinguishable from the control [A_{50}] values. In terms of the model, this indicates that AII is operating through the pseudolinear region of the transducer function. Under this condition, therefore, there is minimal information available to define the transducer function asymptote, E_M and the absolute value of τ. However, the uncertainties in the parameter estimates do not invalidate the model test which is that satisfactory fitting can occur with fixed values of β, pK_A and pK_B.

Discussion

Previously, several explanations have been offered for the complex profile of antagonism observed with AII-derived peptide antagonists. Only non-equilibrium conditions and/or allotopic actions, have been proposed. Mechanistically, Koziarz & Moore (1989) suggested that [Sar^1,Ile^8]AII binds to a secondary site quite distinct from the primary AII-receptor binding site. Similarly, Timmermans *et al.* (1991) invoked an allosteric model to account for the depression. This latter model, first used to explain the pharmacological behaviour of 'non-competitive' 5-HT-S_2-receptor-ligand interactions (de Chaffoy de Courcelles *et al.*, 1986), was derived by analogy to enzymological models of allosteric interactions.

In contrast to the above, the current analysis suggests that the behaviour of all the peptide analogues could be accounted for by a reversible and syntopic action at the AII-receptor which results in a decrease in both the apparent affinity and efficacy of AII. The model is useful in allowing us to estimate pK_B values from otherwise uninterpretable data and also to account for cross-tissue differences. However, this model begs the question of how it can be that an antagonist could combine these properties by a single site action.

Speculation about the molecular basis for these effects is possible from the nature of the efficacy parameters and the known biochemical properties of AII-receptor interactions. In the operational model of agonism (Black & Leff, 1983), the efficacy parameter, τ, is given by the ratio of the concentration of receptors ([R_0]) and the midpoint location parameter of the transducer function (K_E). Therefore, changes in either of these parameters could account for the data. However, because the interaction with DuP 753 pointed to the occupancy-determined nature of the reduction of efficacy, we prefer to ascribe the effects to changes in [R_0]. Furthermore,

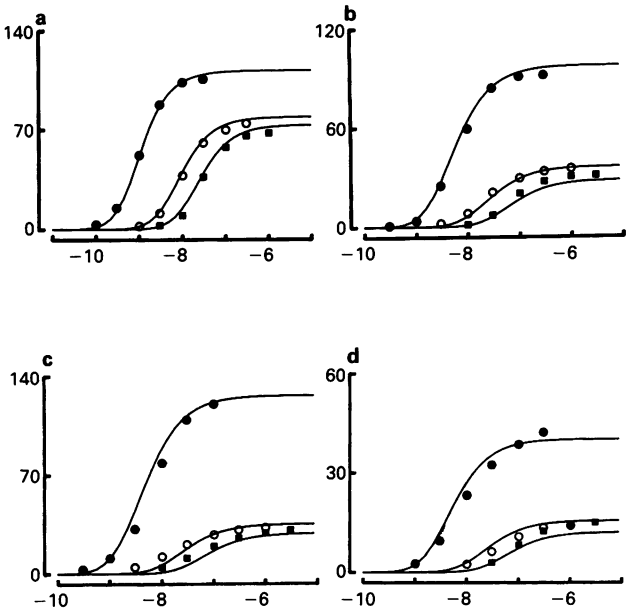

Figure 8 The interaction between [Sar^1,Ala^8]AII (10 and 30 nM) and angiotensin II (AII) on the rabbit aorta (a) and guinea-pig aorta (b), ileum (c) and stomach (d). The curves shown superimposed on the mean experimental data points, with (●) 0, (○) 10 and (■) 30 nM [Sar^1,Ala^8]AII, were obtained by model-fitting of the data to the system of equations (10) and (11). Abscissae: [AII]: log M. Ordinates: % of sighter (response to AII 30 nM (a), 300 nM (b, c, d) before the experiments). In the fits, the parameters pK_B (8.74), pK_A (8.94) and β (0.43) were fixed. The other parameters (± s.e.) were estimated as follows:

Assay	τ	n	E_M
Rabbit aorta	1.83 ± 0.24	2.22 ± 0.29	7.76 ± 1.02
Guinea-pig ileum	0.31 ± 1.65	2.88 ± 0.60	3480 ± 137000
Guinea-pig stomach	0.34 ± 1.58	2.41 ± 0.94	521 ± 4830
Guinea-pig aorta	0.31 ± 1.12	2.49−0.67	2270 ± 10800

biochemically, AII-receptors have been reported to be internalised after occupation (Crozat *et al.*, 1986; Bianchi *et al.*, 1986; Griendling *et al.*, 1987; Delafontaine *et al.*, 1987). Internalisation has the modelling effect of producing an irreversible reduction in the value of $[R_0]$. Physiologically, this is imagined to be offset by the expression of unoccupied receptors into the cell membrane. The regulation of, and the relationship between, the processes of receptor internalisation and expression is unknown to us. Nevertheless, without that knowledge, we may imagine that a ligand-generated imbalance between the rates of internalisation and expression would produce a reduction in $[R_0]$.

If our assumption is correct that the interaction between AII and DuP 753 was simply competitive, internalisation of AII-receptors, following receptor occupation by AII cannot, alone, account for the complex interaction with the peptide analogues. The complexity has to be related to an intrinsic property of the peptide analogues. Any explanation has to account for the saturability of the depression and its reversal by both DuP 753 and 'washout' but not by AII itself. An answer to all of these is suggested by assuming that the peptide analogues, although lacking contractile efficacy, can also internalise the AII-receptor (Ullian & Linas, 1990). The saturability and the reversing effects of both DuP 753 and 'washout' could be explained by the receptor expression assumed to be occurring concomitantly. The failure of AII to surmount the antagonism by the peptide analogues would be due to the replacement of peptide analogue receptor occupancy by AII-receptor occupancy. Unlike DuP 753, AII can effectively maintain the receptor deficit by internalisation.

The feature of the results is the ligand-dependent nature of the complexity. The extent of depression was independent of the level of receptor occupancy and therefore it is as though rates of internalisation can be influenced by an 'efficacy-like' property of the ligands themselves. This property appears quite independent of the efficacy for the contractile process. The greater the capacity of the internalisation process, the longer it would be expected to take for the system to come into equilibrium. The compounds that produced the greatest depression required the longest periods of incubation (see Table 1).

Of particular interest was the model-derived finding that AII behaves as a partial agonist in all four of the assays investigated. This interpretation is reinforced by the fact that the pK_A value estimated for AII was within the same nM range as the pK_B values estimated for the structurally-related antagonists. It may not be a coincidence that in the rabbit aorta assay the intrinsic activity (α) of AII was the same, approximately 60%, with respect to both the model-fit-derived system maximum effect (E_M) and the maximum response obtained with noradrenaline.

Although there is aesthetic satisfaction in producing a model which provides a simplifying and unifying explanation for the complex behaviour of the peptide ligands, it does not follow that the new explanatory model is more likely to be correct. However, because the model can be specified and, therefore, expressed formally, it does allow it to be objectively tested and rejected or refined, as appropriate. In this case, one test of the model was carried out by applying it to the results of the cross-tissue analysis of the interaction between AII and [Sar^1,Ala^8]AII on the guinea-pig aorta, ileum and stomach assays. The outcome was consistent with the presence of a homogeneous AII-receptor population within and between these tissues.

The derived model and the molecular mechanism may be general for peptide receptor systems. It also remains to be seen whether the complexity reported and the receptor internalisation inferred will be restricted to peptide ligands.

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