Br. J. Pharmacol. (1988), **95**, 569–577

Differences in agonist dissociation constant estimates for 5-HT at 5-HT_2-receptors: a problem of acute desensitization?

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1 The agonist dissociation constant for 5-hydroxytryptamine (5-HT) was estimated in the guinea-pig isolated trachea by the method of receptor inactivation. The value obtained ($pK_A = 6.45$) was significantly lower than estimates previously obtained in the rabbit aorta and rat jugular vein, although all three tissues are supposed to contain the same 5-HT_2 class of receptor.

2 The antagonist dissociation constant for α,α-dimethyltryptamine was also estimated in the guinea-pig trachea. The pK_B value (5.43) was not significantly different from previous estimates in the rabbit aorta and rat jugular vein, consistent with receptor homogeneity between the three tissues.

3 The effect-time profiles corresponding to individual 5-HT applications were more transient in the guinea-pig trachea than in the rabbit aorta. This difference could be accounted for using a simple model of acute receptor desensitization (Leff, 1986), assuming that the conversion of active agonist-receptor complexes into inactive ones was faster in the guinea-pig trachea than in the rabbit aorta.

4 Computer simulation of the desensitization model showed that the discrepancy of pK_A estimates for 5-HT between the rabbit aorta and guinea-pig trachea could also be explained using the same rate constant difference that accounted for the difference in effect-time profiles. This analysis indicated that the estimate made in the trachea was erroneously low, whereas that made in the aorta was concluded to be correct.

5 The apparent association between transience of response and pK_A estimates is discussed with particular attention to the reliability of agonist affinity estimates in receptor classification.

Introduction

In a number of recent articles (Leff & Martin, 1986; Leff *et al.*, 1986; 1987; Martin *et al.*, 1987) we have considered the use of tryptamine analogues in 5-hydroxytryptamine (5-HT) receptor classification. Essentially, these articles addressed the extent to which quantitative pharmacological information, namely affinity and efficacy estimates, can be obtained in isolated tissue assays, and the implications that this information has for 5-HT receptor classification in the context of information obtained using non-tryptamine 5-HT receptor ligands. An important factor in these considerations is the affinity of the natural ligand, 5-HT itself. This is particularly relevant when attempting to unify classification nomenclature schemes derived from isolated tissue studies with those emanating from binding studies, bearing in mind in the latter case, for example, that the division between 5-HT and $5HT_2$ binding sites was made partly on the basis of the affinity of 5-HT (Peroutka & Snyder, 1979). More generally, it can be argued, in the case of 5-HT receptors at least, that a physiological definition of the term 'receptor' is to be preferred; if so, the affinity of the natural ligand is an essential piece of information in receptor classification.

In a recent review of the literature (Leff & Martin, 1988) we found that estimates of the affinity of 5-HT_2-receptors in different isolated preparations varied over some 0.8 logarithmic units, with an average value (given as a pK_A) of 6.6. While this

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range appeared to be narrower than the range of affinity estimates for conventional 5-HT_2-receptor antagonists such as ketanserin, it is too broad for the affinity of 5-HT to be regarded as a parameter of sufficient stability to be of value in classification.

In this paper we consider one possible source of variation in agonist affinity estimation, namely acute desensitization or 'fade'. A theoretical model has previously been put forward (Leff, 1986) which showed how agonist concentration-effect curves can be rightward shifted (on the semilogarithmic agonist concentration axis) when acute desensitization operates, leading to underestimation of agonist affinity. Here we examine whether this phenomenon could contribute to variation in estimates of the pK_A for 5-HT in different tissues.

Methods

Tissue preparations

Rabbit aorta The thoracic aorta was removed from male New Zealand White rabbits (2.0–2.5 kg) which had been killed by injection of pentobarbitone sodium (Sagatal: 60 mg kg^{-1}) into a marginal ear vein. The vessel was cleared of adhering connective tissue after mounting on a polypropylene cannula (external diameter = 2.5 mm). For each experiment, six ring segments, approximately 3 mm wide, were prepared as described by Stollak & Furchgott (1983), preserving the plane of the circular smooth muscle.

Guinea-pig trachea Male albino guinea-pigs (Dunkin-Hartley; 300–350 g) were killed by a blow to the head. The entire trachea was excised from each animal and the middle portion cut into six rings, each three cartilage bands wide, which were then cut open to form a strip.

Aortic and tracheal tissue segments were mounted on 20 ml organ baths containing Krebs solution of the following composition (mM): NaCl 118.41, $NaHCO_3$ 25.00, KCl 4.75, KH_2PO_4 1.19, $MgSO_4$ 1.19, glucose 11.10 and $CaCl_2$ 2.50. This was maintained at 37°C and continuously gassed with 95% O_2:5% CO_2. Aortic rings were suspended between two wire hooks (platinum or stainless steel), and tracheal strips between two lengths of cotton thread, one end attached to a Grass FT03C force displacement transducer and the other to a stationary support in the organ bath.

Experimental protocols

Rabbit aorta At the beginning of each experiment, a force of 3 g was applied to the tissue preparations. During a subsequent stabilization period of 30 min, the force was re-established once and tissues were exposed to pargyline (500 μM) in order to inhibit monoamine oxidase irreversibly. Concomitant 30 min exposure to benextramine tetrahydrochloride monohydrate (BHC: 10 μM) also inactivated α_1-adrenoceptors, thereby preventing direct or indirect α_1-adrenoceptor stimulation by 5-HT (Innes, 1962; Apperley *et al.*, 1976; Fozard & Mwaluko, 1976; Marin *et al.*, 1981). At the end of the stabilization period, the inhibitors were removed by several exchanges of the organ bath Krebs solution. Tissues were then challenged with a near-maximally effective concentration of 5-HT (10 μM) to establish viability. After washout and restabilization, individual applications of 5-HT were made in order to obtain effect-time profiles at different concentrations of the agonist. The concentration-effect curve data used in this study were taken from a previous paper (Leff *et al.*, 1986).

Guinea-pig trachea At the beginning of an experiment, a force of 1 g was applied to each tissue and pargyline and BHC were applied as for aortic preparations. Tissues were then challenged with histamine (10 μM) in order to establish viability and also to provide a scale for measuring 5-HT-induced contractions (in antagonism experiments). The organ bath contents were subsequently exchanged for fresh medium until the resting tone of each tissue was re-established. In experiments using phenoxybenzamine, this agent (30 nM or 60 nM) or vehicle (absolute ethanol, 0.1% v/v) was applied to tissues for 30 min. Following washout, cumulative concentration-effect curves were obtained for 5-HT using 0.5 $\log_{10}$ unit increments.

In experiments using α,α-dimethyltryptamine, tissues were exposed to the drug for 60 min before construction of agonist concentration-effect curves. In a single experiment each of the six segments obtained from one trachea was treated with a different concentration of antagonist; replicate numbers, therefore, refer to the number of preparations.

Individual preparations were also used in other experiments to obtain effect-time profiles at different concentrations of 5-HT.

Analysis of data

Antagonist experiments Each E/[A] curve data set was fitted to a logistic function of the form;

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

in which α, $[A_{50}]$ and m are the asymptote, location and slope parameters respectively. Location parameters were actually estimated as logarithms

($-\log_{10}[A_{50}]$). Four individual experiments were conducted with α,α-dimethyltryptamine used as antagonist. On each occasion, a one-way analysis of variance tested for treatment effects on the computed estimates of α and m. If the treatments did not significantly ($P < 0.05$) modify these parameter estimates, then computed $\log_{10}[A_{50}]$ values were fitted to a linear form of the Schild equation:

$$\log_{10}[A_{50}] = \log_{10}[A^c_{50}] + \log_{10}(1 + [B]^n/K_B) \quad (2)$$

where $[A^c_{50}]$ is a control A_{50} value, [B] is the concentration of antagonist, K_B is its equilibrium dissociation constant and n is its apparent order of reaction with the receptor. If the average value of n from the four analyses was not significantly different from unity it was constrained to this value in order to obtain an estimate of pK_B ($-\log_{10} K_B$) in each experiment. These values were then averaged to provide a mean estimate with standard error. $[A_{50}]$ values obtained in these experiments are displayed in Clark plot form (Stone & Angus, 1978). This plot has advantages over the Schild plot in that it displays control $[A_{50}]$s and avoids the calculation of concentration-ratios which tend to over-weight the control values.

Operational model fitting The averaged E/[A] curve data measured in g force were fitted directly to the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985; Leff *et al.*, 1986):

$$E = \frac{E_m \tau^n [A]^n}{(K_A + [A])^n + \tau^n [A]^n} \quad (3)$$

in which K_A is the agonist dissociation constant, τ is the efficacy of the agonist in a particular tissue, E_m is the maximum possible effect in the receptor system and n determines the steepness of the occupancy-effect relation.

Comparison of pK_A estimates made in the two tissues was performed by analysing the residual sum-of-squares associated with each model-fit by F-ratios.

Computer simulation of effect-time profiles A simple model for acute receptor desensitization was used to simulate individual effect-time profiles obtained in each tissue at different concentrations of 5-HT. Full algebraic details are given elsewhere (Leff, 1986). Briefly, one of the schemes proposed by Katz & Thesleff (1957) is simplified by supposing that the recovery of desensitized receptors is negligible during agonist application:

$$A + R \rightleftharpoons AR \rightarrow AR_D$$

AR_D represents desensitized receptors. The differential equations which describe the scheme were solved to find [AR] (the concentration of agonist-occupied receptors) as a function of time. Note that in applying equation (11) in Leff (1986), the values of λ_1 and λ_2 which appear therein are the *negatives* of the roots of the auxiliary equation (13). Also, in the original model (Leff, 1986), [AR] was assumed to be related to pharmacological effect by a rectangular hyperbolic function although here, a more general, logistic function has been employed:

$$E = \frac{E_m [AR]^n}{K_E^n + [AR]^n} \quad (4)$$

which is the transducer relation assumed in the operational model of agonism (Black & Leff, 1983; Black *et al.*, 1985; Leff *et al.*, 1986). Thus, effect-time profiles corresponding to different values of k_1, k_{-1}, k_2 etc. could be generated. In addition, concentration-effect curves could be generated under conditions when desensitization operates.

Drugs and solutions

The following drugs were used: 5-hydroxytryptamine creatinine sulphate (Sigma Chemical Company, St Louis, MO., U.S.A.); pargyline hydrochloride (Sigma); benextramine tetrahydrochloride monohydrate (Aldrich Chemical Company Ltd, Dorset); phenoxybenzamine hydrochloride (Smith, Kline and French, Welwyn Garden City, Herts).

α,α-Dimethyltryptamine hydrochloride was synthesized by Dr H. F. Hodson, Medicinal Chemistry Department, Wellcome Research Laboratories, Beckenham, Kent.

Phenoxybenzamine was dissolved in absolute ethanol which attained a concentration in the organ bath of 0.1% v/v. This did not influence tissue responsiveness. All other drugs were dissolved and diluted in distilled water.

Results

Figure 1 shows the effects of phenoxybenzamine (30 nM and 60 nM, 30 min) on 5-HT-elicited increases in force in the rabbit aorta and in the guinea-pig trachea. The lines drawn through the points are best fits using equation (3) and they were obtained by fitting the average concentration-effect data from each of the two tissues separately. This analysis estimated the affinity of 5-HT to be higher ($pK_A = 6.88$) in the rabbit aorta than in the guinea-pig trachea ($pK_A = 6.45$). These estimates, along with the other

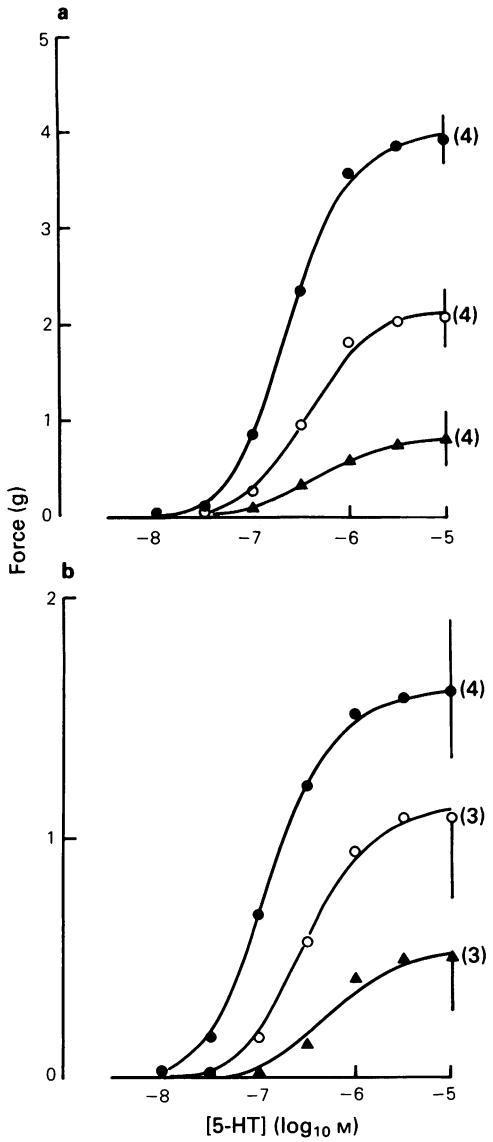

Figure 1 5-Hydroxytryptamine (5-HT) concentration-effect curves in (a) rabbit aorta and (b) guinea-pig trachea obtained in controls (●) and in tissues treated with phenoxybenzamine (○) (30 nM, 30 min) and (▲) (60 nM, 30 min). Average effect data are shown (replicates being indicated by each curve) together with the best-fit lines obtained by operational model-fitting to these averages. Data and fit in rabbit aorta were reproduced from Leff *et al.* (1986) with permission. Vertical lines indicate s.e.

parameter values, are summarized in Table 1. In order to assess the significance of this difference a subsequent analysis was performed in which equation (3) was constrained to fit data from both tissues with the same pK_A. Comparison of the residual sum-of-squares obtained in this 'constrained' fit with those obtained from the former 'unconstrained' fit indicated significant worsening (see Table 1) and, therefore, a significant difference between the two affinity estimates.

Table 1 Analysis of 5-hydroxytryptamine agonism by operational model-fitting

Parameter	*Rabbit aorta*	*Guinea-pig trachea*
pK_A	6.88	6.45
τ_1 (control)	1.20	3.31
τ_2 (30 nM Pbz)	0.81	1.38
τ_3 (60 nM Pbz)	0.55	0.63
E_m	6.40	1.81
n	2.95	1.76

This set of estimates corresponded to a fit with no constraints. In order to test the significance of the difference in pK_A estimates a second fit was performed in which only a single pK_A was made for both sets of data. Pbz = phenoxybenzamine.
The residual sum-of-squares for the unconstrained fit was 58.420 with 25 degrees of freedom. Therefore, the mean square error was 2.337.
The increase in residual sum-of-squares upon constraint was 13.887 with 1 degree of freedom. Therefore, the increase in mean square error was 13.887.
The F-ratio, $F_{1,25} = 13.887/2.337 = 5.942$, corresponding to $0.01 < P < 0.05$.

Figure 2a illustrates the effects of α,α-dimethyltryptamine on 5-HT concentration-effect curves in the tracheal preparation. Analysis of the curves using equations 1 and 2 indicated that all the criteria for simple competitive antagonism were fulfilled in each of the four separate analyses performed. The average pK_B estimate was 5.43 ± 0.17 (mean ± s.e., 3 d.f.). Figure 2b displays the data in Clark plot form. This affinity estimate was not significantly different from that obtained from the rabbit aorta (5.67; see Leff *et al.*, 1986) as assessed by Student's *t* test.

Effect-time profiles for 5-HT in the two tissues are illustrated in Figure 3a. The concentrations chosen correspond to the 5%, 50% and 95% effect level for 5-HT in each tissue. Evidently, 5-HT-induced effects were better sustained in the aorta than in the trachea. Attempts were made to simulate these data using the simple model of desensitization (Leff, 1986) outlined above. Although some of the features of the data could not be accommodated (see Discussion), the overall difference in effect kinetics was reasonably well explained by a difference in k_2, with no difference in k_1 or k_{-1}, between the two tissues (Figure 3b). The values of the parameters used in the simulation are given in the legend to Figure 3.

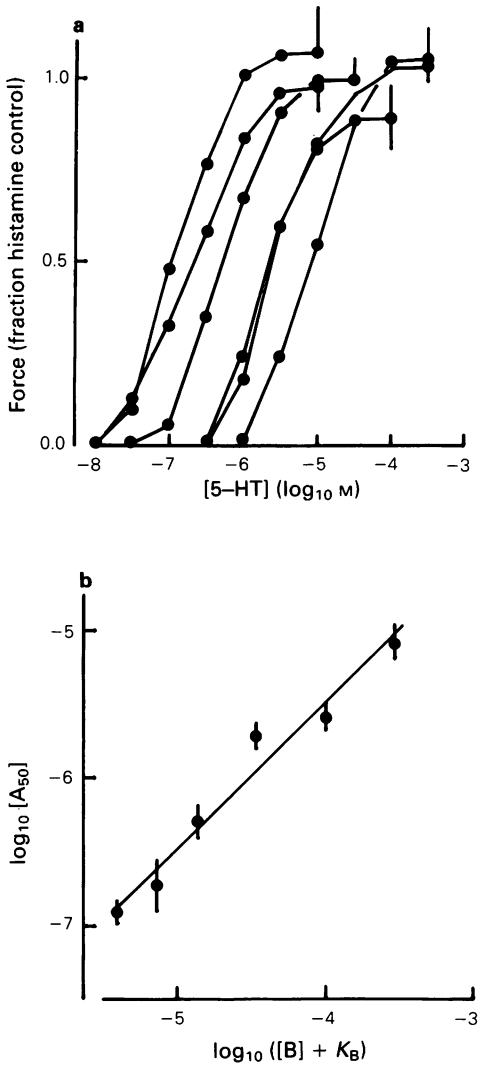

Figure 2 Antagonism of 5-hydroxytryptamine (5-HT) by α,α-dimethyltryptamine in guinea-pig trachea. Each of four animals provided six segments of trachea. In a single experiment, therefore, 5-HT concentration-effect curves were obtained at zero, 3 μM, 10 μM, 30 μM, 100 μM and 300 μM antagonist (a). Analyses of variance were performed on curve slopes and asymptotes, and accordance with Schild criteria were tested. In all four cases no significant deviations from parallelism were detected. Fitting curve $[A_{50}]$ values to equation (2) (see Methods) gave estimates of n of 0.91 ± 0.09, 0.72 ± 0.30, 1.00 ± 0.18 and 1.07 ± 0.32. Therefore, on each occasion the Schild plot slope criterion was fulfilled. pK_B estimates, obtained with n constrained to unity, were 5.16, 5.39, 5.35 and 5.74 giving a mean value of 5.43 ± 0.17. (b) Shows a Clark plot of all the $[A_{50}]$ values and the unit slope line corresponding to the average pK_B estimate. Vertical lines indicate s.e.

It was then examined whether this difference in k_2 could account for the discrepancy in pK_A estimates between the two tissues. In Figure 4 the data from Figure 1 are reproduced. The continuous lines drawn through the data were generated assuming the same parameter estimates (k_1, k_{-1}, k_2, etc.) as those used to simulate the effect-time profiles in Figure 3. Also shown are broken lines corresponding to the situation where no desensitization operates, that is, when $k_2 = 0$. Under this latter condition, no conversion of AR to AR_D occurs and so [AR] can achieve its equilibrium value. In the former case, where AR is converted to AR_D, AR cannot accumulate to its equilibrium value and, therefore, the concentration-effect curves are expected to depart from their equilibrium position (see Leff, 1986). In the case of the trachea the departure amounts to $0.5 \log_{10}$ units, whereas in the aorta the discrepancy is less than $0.1 \log_{10}$ units.

Discussion

This paper addresses a possible source of error in the estimation of agonist dissociation constants due to the occurrence of acute receptor desensitization.

It is evident from Figure 1 and analysis that the pK_A estimate for 5-HT was significantly lower in the guinea-pig trachea than in the rabbit aorta. In principle this difference could reflect receptor differences. However, the two tissues in question are supposed to contain the same 5-HT_2-receptor type (Bradley *et al.*, 1986). While it must be stated that the quantitative classification of the tracheal receptor has been compromised by the non-surmountable action of conventional antagonists such as ketanserin in this tissue (see Lemoine & Kaumann, 1986, for example), the present study has shown that α,α-dimethyltryptamine at least is able to behave as a simple competitive antagonist in the trachea and furthermore that it does so with an affinity indistinguishable from that demonstrated in the aorta (Figure 2 and analysis; Leff *et al.*, 1986). Therefore, we assume that the receptors in the two tissues are of the same type.

An obvious difference in the expression of 5-HT agonism between the two tissues was the extent of fade operating. Figure 3 shows how 5-HT effects in the trachea were much more susceptible to fade than those in the aorta. Application of a theoretical model for acute receptor desensitization developed elsewhere (Leff, 1986) showed that this difference could be explained simply by a faster conversion of active AR into inactive AR_D complexes, that is, in model terms, a larger value of k_2 in the trachea than in the aorta. No differences in the association (k_1) or dissociation (k_{-1}) rate constants for the agonist-receptor interaction needed to be postulated and importantly

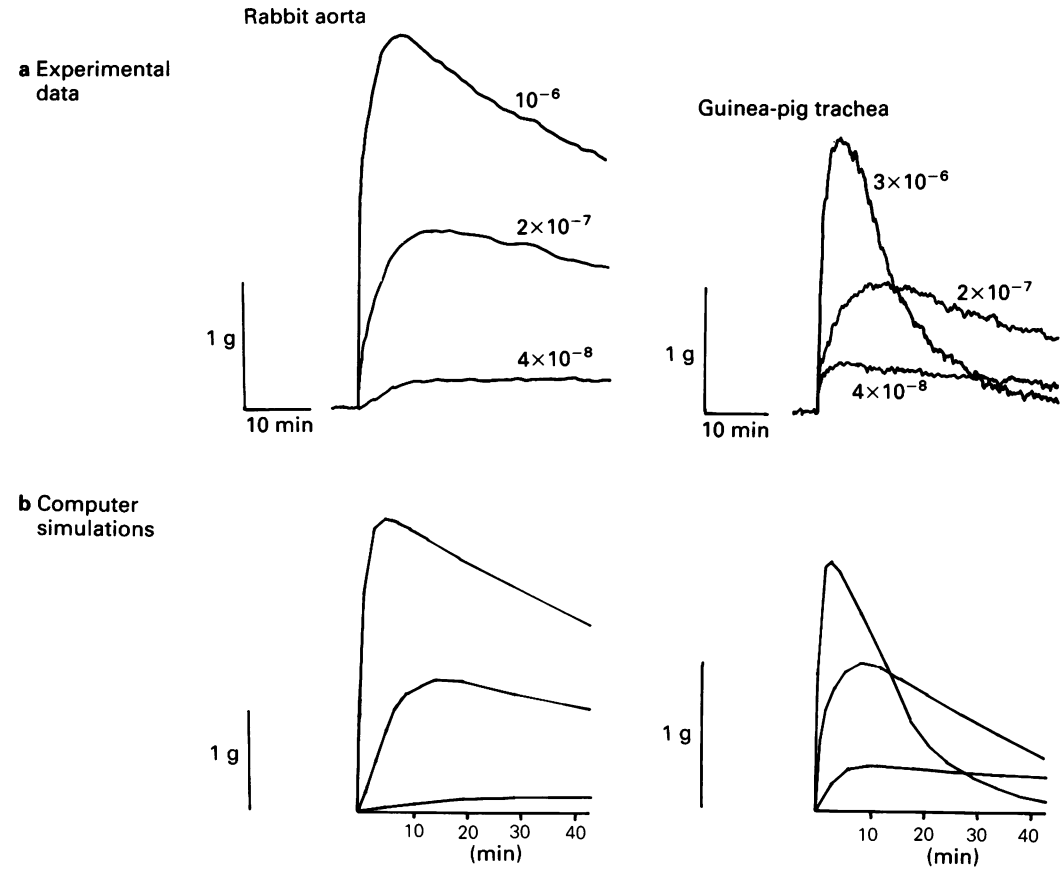

Figure 3 Effect-time profiles for 5-hydroxytryptamine (5-HT) in the rabbit aorta and guinea-pig trachea: (a) illustrates typical tracings obtained at the concentrations indicated in each tissue; (b) illustrates simulations of the simple model of desensitization described in Methods and elsewhere (Leff, 1986). The parameters used in the simulation were as follows. For both tissues $k_1 = 0.7 \times 10^6\,M^{-1}\,min^{-1}$ and $k_{-1} = 0.09\,min^{-1}$, the ratio k_{-1}/k_1 being equivalent to a pK_A value of 6.89. For the aorta $k_2 = 0.008\,min^{-1}$ and for the trachea $k_2 = 0.07\,min^{-1}$. Values of E_m, τ and n were respectively 6.4, 1.20 and 2.95 for the aorta and 1.81, 3.31 and 1.76 for the trachea. These values were taken from Table 1.

the values used in the simulation (see Figure 3) corresponded to the pK_A estimate for 5-HT in the aorta (6.88) and not to that in the trachea (6.42); the ratio k_{-1}/k_1 was 1.29×10^{-7} M which is equivalent to a pK_A value of 6.89.

However, the important issue was whether the difference in k_2 required to account for the kinetic differences would also explain the difference in estimates of 5-HT's affinity between the two tissues. Theoretically the operation of acute desensitization can lead to a rightward shift of agonist concentration-effect curves away from the positions that they would occupy if pharmacological effect could be measured at equilibrium [AR] (Leff, 1986). In experiments using irreversible receptor inactivation both the "control' and 'treated' curve are expected to be frame-shifted leading to an underestimation of pK_A. According to the model the extent of the shift and, therefore, underestimation is dependent upon the difference between the transient, peak values of [AR] achieved and the equilibrium values of [AR] which would have been reached in the absence of desensitization. In the present case the 5-HT concentration-effect curves obtained in each tissue with and without phenoxybenzamine treatment were fitted acceptably well employing the same rate constant and parameter values that were used to simulate the individual effect-time profiles (Figure 4). The fact that both the aortic and tracheal data were fitted assuming a 'real' pK_A of 6.89 meant that the

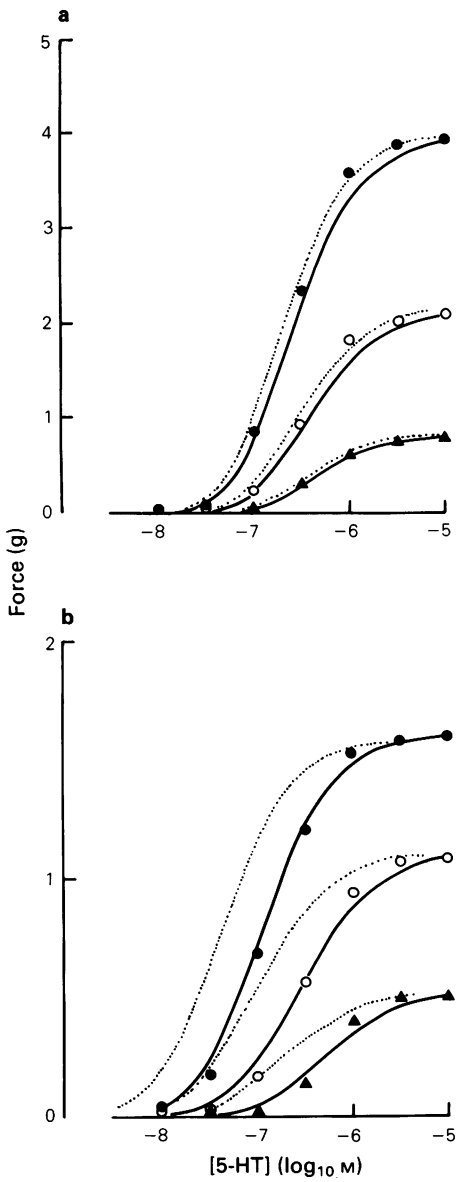

Figure 4 Simulation of agonist concentration-effect data using the model of desensitization. Experimental data from Figure 1 are reproduced; (a) rabbit aorta, (b) guinea-pig trachea. The continuous lines were generated by simulating the model employing the rate constants and parameter values used in Figure 3. A set of effect-time profiles was generated at the agonist concentrations used experimentally and peak effect levels were recorded. These levels were then used to construct a concentration-effect curve. The process was repeated for each curve in each tissue. The broken lines correspond to simulations where k_2 in the model was set to zero, that is, where no desensitization operates.

desensitization explanation for the discrepancy in affinity estimates was plausible. This is illustrated by comparing the positions of the experimental curves in each tissue with the theoretical curves generated using the same parameter values except with $k_2 = 0$, that is, with no desensitization operating (see Figure 4). In the aorta the two sets of curves lie very close to each other implying that the rate of AR to AR_D conversion is not sufficient to create a large discrepancy between equilibrium [AR] and the peak transient value at each concentration of agonist. In the trachea there is a discrepancy for each of the curves of $0.5 \log_{10}$ units which corresponds reasonably closely to the increment (0.43) by which the pK_A estimate for 5-HT differed in the trachea from that in the aorta. The implication here is that the relatively large value of k_2 causes a substantial departure between peak values of [AR] and their equilibrium counterparts.

The present explanation for the difference in 5-HT affinity estimates is clearly based on the application of a particular theoretical model. Although the model accounts for the dominant features of the data, namely the more rapid fade in the trachea than in the aorta, and the variant pK_A estimates, the simulated effect-time profiles do not follow the experimental transients as accurately as they might. For example, the experimental traces tend to approach a finite effect level in the limit (except in the case of 3 μM 5-HT in the trachea) when the agonist is left in contact with the tissue. The model, by assuming the AR to AR_D conversion to be irreversible, forces [AR] to decline to zero rather than a steady state value. Also, no allowance is made for rate limitation either at the diffusion or the post-receptor level; agonist occupancy is assumed to be the rate determining step which may be unjustified. Generalising the model to incorporate these features would improve its ability to fit the experimental data more exactly and this may be required in order to apply it in other cases. Evidently, however, the model is suitable in its present form in providing a basis for interpreting the data in question, and we consider the use of more complex versions unnecessary for the present.

Obviously, this analysis can only offer a possible explanation for the discrepancy in pK_A estimates. However, the association between transience of response and apparent underestimation of agonist affinity is maintained in at least two further 5-HT_2-receptor-containing tissues. In the rat jugular vein, although fade is evident, agonist effects are relatively well sustained and the data obtained for 5-HT in that tissue could be fitted assuming the same pK_A (6.88) for 5-HT as that estimated in the rabbit aorta (Leff *et al.*, 1986). Whereas in the rat caudal artery, where responses are more transient, a pK_A estimate

of 6.2 was made (Martin & Leff, unpublished). A similar association between response transience and agonist affinity estimates is apparent in studies of α_1-adrenoceptors in different tissues (Leff, 1986). As in the present case, a competitive antagonist demonstrated the same affinity for the receptors in the different tissues while agonist affinity appeared to vary.

Although the present explanation for tissue-dependent agonist affinity estimates cannot be proven, it makes sense to place less weight on a pK_A estimate obtained in a tissue where responses cannot be sustained than on an estimate made under 'stable' conditions. However, the choice between more and less meaningful estimates becomes more difficult when desensitization operates but when effect-time profiles do not display overt fade, a situation which can exist, at least in theory (Leff, 1986). Under these conditions, if a choice exists, the higher affinity estimate should be accepted. This presumes that precautions would have been taken to avoid other sources of error. For example, post-receptor actions by a receptor inactivating agent could lead to overestimation of agonist affinity due to functional antagonism (Leff *et al.*, 1985).

Ideally, other steps would be taken to obtain independent affinity estimates when errors such as those considered here are suspected. A method for estimating agonist affinity which, in principle, avoids problems associated with the expression of agonism itself was suggested by Furchgott & Bursztyn (1968). It involves the effective conversion of the agonist in question into a competitive antagonist using irreversible receptor inactivation, then estimating its dissociation constant as an antagonist. This method requires the availability of an agonist with sufficiently higher efficacy than the test agonist for it to be active after receptor inactivation. Unfortunately this presents a problem in quantification in the case of 5-HT_2-receptors at least, because there appear to be no agonist mimetics of 5-HT with substantially higher efficacy than the natural agonist.

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(Received November 23, 1987
Revised April 15, 1988
Accepted May 19, 1988)