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Extended concentration-response curves used to reflect full agonist efficacies and receptor occupancy-response coupling ranges

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1 An approach is described for generating extended agonist concentration-response curves where the responses are unconstrained by the normal tissue maximum response. Functional antagonism is employed to hold the tissue state in the range where any change in stimulus can be translated into a measurable response.

2 The maximum response of these extended concentration-response curves provides an index of intrinsic activity reflecting the agonist efficacy and the receptor occupancy-response coupling range.

3 The use of this approach is illustrated with extended concentration-response curves for noradrenaline (NA), vasopressin, acetylcholine (ACh), and 5-methylfurmethide in the small mesenteric and tail arteries of the rat. Both NA and vasopressin can maximally activate the arteries, but the new protocol shows that NA can produce more cellular activation than vasopressin in the tail artery. Both ACh and 5-methylfurmethide are full agonists but ACh has a higher intrinsic activity than 5-methylfurmethide. The ACh muscarinic receptors in the mesenteric artery have a larger occupancy-response range than the ACh muscarinic-receptors in the tail artery, and the α-adrenoceptors in the tail artery appear to have a larger occupancy-response coupling range than those in the mesenteric artery.

4 This approach extends our ability to compare the efficacies of full agonists, and to compare the occupancy-response coupling ranges of receptors that can normally maximally activate the assay tissue. This is achieved without the use of an irreversible antagonist and should be applicable to many receptors and pharmacological assay systems where responses are stable and functional antagonists are available.

Keywords: Agonism; efficacy; intrinsic activity; receptor reserve; stimulus-response coupling; functional antagonism

Introduction

Concentration-response curves provide two useful measures of agonist activity. The location of the curve (e.g. the pEC_{50}) provides a measure of the agonist potency and the sensitivity of the assay system to an agonist (two different expressions of the same property). The maximum response provides an index of the agonist efficacy using Ariëns' scale of intrinsic activity (Ariëns, 1954). Because the maximum effect of the agonist can be constrained by the assay tissue, the intrinsic activity scale does not allow discrimination between full agonists, which are all assigned an intrinsic activity of 1. When an irreversible antagonist is available it is possible to expose any efficacy differences between full agonists by reducing receptor numbers until the agonists become effectively partial. However, such antagonists are not available for many receptor types.

An alternative approach exploits functional antagonism between agonists with opposite effects in the assay (Buckner & Saini, 1974; Broadley & Nicholson, 1979). These methods were primarily intended to provide affinity estimates for the agonists rather than efficacies, indeed the method of Broadley & Nicholson (1979) requires that the agonist affinity be calculated before the efficacy can be estimated. There are many levels at which functional interactions between agonists occur, and the patterns of concentration-response curves obtained are dependent on the nature of the interaction. Thus, there are theoretical considerations that limit the reliability of the affinity estimates obtained using functional antagonism (Mackay, 1981; Leff *et al.*, 1985) which makes the method of Broadley & Nicholson (1979) potentially unreliable. However, because efficacy estimates are useful on a relative rather than absolute scale, these considerations do not invalidate the use of functional antagonism for comparisons of efficacy between full agonists by the method of Buckner & Saini (1974). They compared the effects of isoprenaline and soterenol on carbachol concentration-response curves in guinea-pig trachea and found that isoprenaline caused a greater rightward shift than soterenol, indicating that isoprenaline has the higher efficacy. Because the comparison is between agonists acting at the same receptor in the same tissue with the same functional interaction, uncertainties about the form of the interaction could not result in the more efficacious agonist erroneously appearing less efficacious. At worst the difference in efficacy would not be discernible. Few studies have used functional antagonism to compare full agonist efficacies, despite the importance of agonist relative efficacies and the fact that functional antagonists are probably available for far more systems than irreversible antagonists. This may be the result of concerns about the theoretical limitations of functional antagonism in characterization of agonist properties, or the indirect nature of the efficacy index obtained from such studies.

The approach described in this paper uses functional antagonism in a different manner from that normally employed. A functional antagonist is applied as needed to keep the assay responses to the agonist of interest below the maximum level that can be expressed in the assay. This generates extended agonist concentration-response curves that are not bounded by the normal tissue maximum response. The protocol, called 'up/down', thus exploits functional antagonism between excitatory agonists ('up' agonists) and inhibitory agonists ('down' agonists) to hold the level of the assay response within the range where a change in the level of stimulus can be translated into a measurable response. The range of the extended concentration-response curves generated by this protocol is readily interpreted as an extension of Ariëns' scale of intrinsic activity (Ariëns, 1954). The functional antagonism interactions in the up/down protocol are the same as in other functional antagonism protocols, so the theoretical limitations of the values obtained are the same. However the up/down protocol produces a more

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straightforward display of the agonist efficacy, and smaller efficacy differences may be discerned than with the standard functional antagonism experiments.

Because intrinsic activity is dependent on both agonist and tissue properties, the up/down protocol can be used for at least three types of comparisons: (i) discrimination between high efficacy agonists acting at the same receptor; (ii) comparison of the stimulus-response coupling range of different receptors in the same tissue; (iii) comparison of the occupancy-response coupling efficiency of a single receptor type in different tissues. The experiments described in this paper illustrate the use of the up/down protocol in making each of these types of comparisons by (i) comparing ACh and 5-methylfurmethide as high efficacy ACh muscarinic (M)-receptor agonists, (ii) comparing vasopressin receptor-mediated responses with α-adrenoceptor-mediated responses within tissues, and (iii) comparing responses mediated by ACh M-receptors, vasopressin receptors and α-adrenoceptors between the rat small mesenteric and tail arteries.

Methods

General

Male Wistar Kyoto or Sprague Dawley rats aged between 15 and 20 weeks were anaesthetized with carbon dioxide (80% CO_2, 20% O_2) and killed by exsanguination. A portion of intestine and mesentery was removed and place in cool Krebs solution (composition in mM: Na^+ 144, K^+ 5.9, Mg^{2+} 1.2, Ca^{2+} 2.5, HPO_4^- 1.2, Cl^-129, SO_4^-1.2, HCO_3^- 25, glucose 11) bubbled with 5% CO_2:95% O_2. Under a dissecting microscope, a mesenteric artery (three branch orders proximal to the arteries that enter the intestine) was carefully dissected free of the fat and connective tissue around it, and a 2 mm long segment was mounted on 40 μm wires in a Mulvany-Halpern style myograph (JP Trading, Aarhus, Denmark) and warmed to 37°C. In other experiments the tail of the rat was removed and the main caudal artery was dissected free under a microscope and segments mounted in a myograph as for the mesenteric arteries. The artery was incrementally stretched radially with about four steps, and the force measured and the arterial circumference calculated at each step. A diameter-force curve was thereby generated where the diameter is that of a circle with the same circumference as the vessel at each level of stretch. The diameter of the artery was then set to be 90% of the diameter predicted for distending pressure of 100 mmHg using standard calculations (Mulvany & Halpern, 1977). The rat mesenteric and tail arteries set up under these conditions do not develop any spontaneous active contractile force. Each artery was fully activated twice by either potassium depolarizing solution (KPSS, K^+ 120 mM substituted for Na^+) or NA (10 μM). The response to the second activation was always larger than the first and was used as an estimate of tissue maximum response, and used in normalising subsequent responses. This normalising response underestimates the true tissue maximum response pertaining to the subsequent concentration-response curves because the tissue maximum response of these arteries mounted in this fashion commonly increase with successive activations. This is evident in the fact that both NA and vasopressin (AVP) normal concentration-response curves have normalised maxima larger than one. In all experiments where NA was applied, desipramine (0.1 μM) was added to the Krebs solution to inhibit neuronal uptake. The mesenteric arteries had diameters of 317 μm ± 10 (mean ± s.d.) and the tail arteries 703 μm ± 68.

Concentration-response curves

Only one concentration-response curve (either normal or up/down) was constructed in each tissue. All drug additions were made in a cumulative fashion with 3.2 fold concentration increments. Normal concentration-response curves were constructed in the standard fashion. Extended concentration-response curves for vasoconstrictor and vasodilator agonists were constructed simultaneously to hold the active force close to 50% of tissue maximum response: the curve for the vasoconstrictor was started first, and when force exceeded 50%, the curve for the vasodilator was started. When the force declined below 50%, the next concentration of vasoconstrictor was applied and so on, until the full range of agonist concentrations was applied. Responses to each increment of agonist concentration were cumulated and a concentration-cumulated response curve generated. This protocol for drug application frequently resulted in a simple alternation between vasoconstrictor application and vasodilator application after the first few concentrations of each agonist. At very high concentrations of agonist no increase in response was found with increased agonist concentration, indicating that the maximum effect of the agonist had been obtained. When this occurred for the vasoconstrictor agonist before the vasodilator agonist, a second (or third) vasoconstrictor agonist was added. Similarly when the vasodilator agonist reached a maximum before the vasoconstrictor, a second vasodilator was added. Responses were calculated as the simple summation of the change in force elicited by each concentration increment. These cumulated responses were expressed as a fraction of the second KPSS or NA 10 μM response.

Protocols

Mesenteric arteries Normal concentration-response curves for NA, AVP and ACh were constructed for comparison with the up/down curves. For these normal ACh curves, the arteries were precontracted to 50–80% of tissue maximum response by AVP.

Up/down concentration-response curves for NA were constructed with ACh used as the down agonist. In these experiments the NA responses were exhausted before the ACh concentration-response curve was complete, so AVP and then endothelin-1 were added to allow completion of the ACh curve. This protocol yielded two sets of data, extended concentration-response curves for NA and for ACh.

Extended concentration-response curves for AVP were constructed with ACh used as the down agonist. The ACh concentration-response curve was not completed after the AVP responses were exhausted, so only the AVP extended concentration-response curve resulted from these experiments.

Tail artery Extended concentration-response curves for NA were constructed with either ACh or sodium nitroprusside as the first down agonist. When the responses to the first down agonist were exhausted, the other was added to extend the NA curve, and when both were exhausted, calcitonin gene-related peptide (CGRP) was added if needed. Three concentration-response curves were obtained from each of these experiments: NA; the first down agonist; and the second down agonist.

Extended AVP concentration-response curves were obtained as in the mesenteric arteries.

Extended NA concentration-response curves in the absence and presence (for at least 30 min) of various concentrations of prazosin (3, 30 and 300 nM) or yohimbine (100 and 1000 nM) were constructed with 5-methylfurmethide as the first down agonist. When the responses to 5-methylfurmethide were exhausted, sodium nitroprusside, and on one occasion, CGRP were added to allow completion of the extended NA curve. Each of these experiments yielded three concentration-response curves, one for NA, one for 5-methylfurmethide, and one for sodium nitroprusside as the second down agonist.

Analysis

Concentration-response curves were analysed by fitting a four parameter logistic equation to the data to obtain location and slope parameters. The equation is

$$y = a + \frac{b}{1 + e^{-d(c + \log[A])}}$$

where A is the agonist concentration, a is the basal value, b is the vertical range, c is the pEC_{50}, d is the mid-point slope, and e is the base of the natural logarithm. Where the concentration-response curves started at zero, the basal value parameter, a, was deleted to give a three parameter logistic. When needed, any pEC_x was calculated from the logistic equation. Agonist potencies were compared as pEC_x values using an unpaired t test (2 tailed). Experimentally observed maximum responses were also compared by the unpaired t test (2 tailed). Data are shown in the figures as the mean ± s.e.mean.

Drugs

Acetylcholine bromide, desmethylimipramine hydrochloride, noradrenaline bitartrate, prazosin hydrochloride and yohimbine hydrochloride were purchased from Sigma Chemical Company (St Louis, MO, U.S.A.) and sodium nitroprusside from David Bull Laboratories (Mulgrave, Victoria, Australia). Arginine vasopressin, calcitonin gene-related peptide (human) were obtained from Auspep, (Parkville, Victoria, Australia) and endothelin-1 from Peninsula Laboratories (Belmont, CA, U.S.A). 5-Methylfurmethide iodide was a gift from Wellcome Research Laboratories (Beckenham, U.K.). All drugs were dissolved and diluted in water. Prazosin, yohimbine, 5-methylfurmethide and DMI were kept as 1 or 10 mM stock solutions at 5°C, and all other drugs were dissolved on the day of use.

Results

An example of the results from an up/down protocol obtained on the rat tail artery are shown in Figure 1. The active force was held in the range where changes in NA or ACh concentration always had the potential to elicit responses by incrementing the concentration of vasoconstrictor when the force was less than 50% of the tissue maximum response, and incrementing the vasodilator when the force was more than 50%. Figure 1b shows the data expressed as active force displayed as a family of NA concentration-response curves in the presence of various concentrations of ACh, and as a family of ACh concentration-response curves with various concentrations of NA. Figure 1c shows the data for the agonists cumulated to produce a single extended concentration-response curve for each. These graphs show the relationship between the up/down data and the data that would be obtained from a normal procotol using functional antagonism.

NA and AVP in mesenteric arteries

NA and AVP produce steep concentration-response curves in the rat mesenteric artery with the active force changing from about 10% to 90% over an agonist concentration-range of only ten fold. The time-courses of the responses were dissimilar, with the response to NA reaching a steady level in only 1–2 min and the responses to low concentrations of AVP taking more than twice as long. The maxima of normal concentration-response curves to NA and AVP in the mesenteric arteries were similar (20.0 ± 0.06 and 17.6 ± 1.9 mN respectively, $P = 0.30$, unpaired t test), and were approximately 10% larger than the responses to KPSS (Figure 2a), probably reflecting a time-related increase of the maximum response. Larger responses to other vasoconstrictor agonists have not been observed, so these maxima probably represent the maximum force that the mesenteric arteries can produce. The pEC_{50} values from these concentration-response curves were 6.07 ± 0.09 for NA and 9.01 ± 0.10 for AVP.

Responses to the vasoconstrictor agonists were measured over an extended concentration-range with ACh used as a functional antagonist with the up/down protocol. The cumulated response maxima for AVP and NA in the mesenteric artery were 2.01 ± 0.20 and 2.34 ± 0.35 times the force elicited by KPSS respectively (Figure 2b). The location of the up/down curves were measured as the $pEC_{0.55}$ (corresponding to the half-maximal response to NA and AVP applied in the normal manner) and these were found to be similar to the pEC_{50} values obtained from the normal concentration-response curves (6.25 ± 0.10, $P = 0.20$ and 9.23 ± 0.12, $P = 0.23$).

NA and AVP in tail arteries

The tail artery was more sensitive to NA than the mesenteric artery, with a threshold concentration of about 1 nM. Up/down concentration-response curves were constructed with either ACh or sodium nitroprusside as the vasodilator. Each of these vasodilators was exhausted well before the full range of NA concentrations was applied and thus both ACh and sodium nitroprusside were applied sequentially to each tissue to extend the NA concentration-response curve over a large range. In most of these experiments CGRP was also added after both of the other vasodilators to further extend the up/down protocol. Responses to NA were not noticeably affected by the sequence of vasodilator additions, but the responses to the vasodilators were affected by the prior application of the other vasodilators (see below). The cumulated response to NA was almost 4 times the response to KPSS (Figure 2c). In contrast to NA, the up/down concentration-response curve for AVP was completed with only a single vasodilator (ACh) and the maximum cumulated response was only 1.69 ± 0.23 times the response to KPSS (Figure 2c).

NA up/down concentration-response curves were constructed in the tail artery in the absence and presence of prazosin and yohimbine. The down agonists were 5-methylfurmethide, an ACh M-receptor agonist, followed by sodium nitroprusside. Prazosin caused a parallel rightward shift of the NA extended concentration-response curve (Figure 3a). When the data were analysed by the method of Stone & Angus (1978), it was found that the spacing of the curves was appropriate for a simple competitive interaction with a pK_B for prazosin of 9.14 ± 0.13. Yohimbine also caused a rightward shift of the NA concentration-response curve, but the spacing of the curves was inconsistent with simple competitive interaction (Figure 3b).

Vasodilators

ACh fully relaxed mesenteric arteries precontracted by NA, with a maximum response at 1 μM ACh, and a pEC_{50} of 7.6 ± 0.1 (Figure 4). When the up/down protocol was used, the cumulated maximum response to ACh was a relaxation of over four times the force elicited by KPSS, and relaxation responses were observed with ACh concentrations of up to 30 μM and the $pEC_{-0.5}$ was 7.8 ± 0.2 (Figure 4). ACh was less potent in the rat tail artery than in the mesenteric artery, and the maximum cumulated response was smaller, about 2.6 ± 0.1 times the force elicited by KPSS (Figure 5a). In the tail artery experiments where ACh was tested after sodium nitroprusside, the responses to ACh were greatly attenuated (Figure 5b). Similarly, the cumulated responses to sodium nitroprusside were much larger when it was applied before ACh rather than after, with cumulated responses of −3.1 ± 0.3 and −0.42 ± 0.07, respectively at 100 μM, the maximum concentration applied (Figures 5a and b). 5-Methylfurmethide caused a smaller maximal cumulated response in the tail artery than ACh (−1.85 ± 0.09, $P < 0.001$, Figure 5c). There was no effect of prazosin or yohimbine on the responses to 5-methylfurmethide. Pre-application of 5-methylfurmethide greatly reduced the effect of sodium nitroprusside (−0.77 ± 0.05 at 100 μM), but this response was significantly larger than the equivalent response in the presence of ACh ($P < 0.001$).

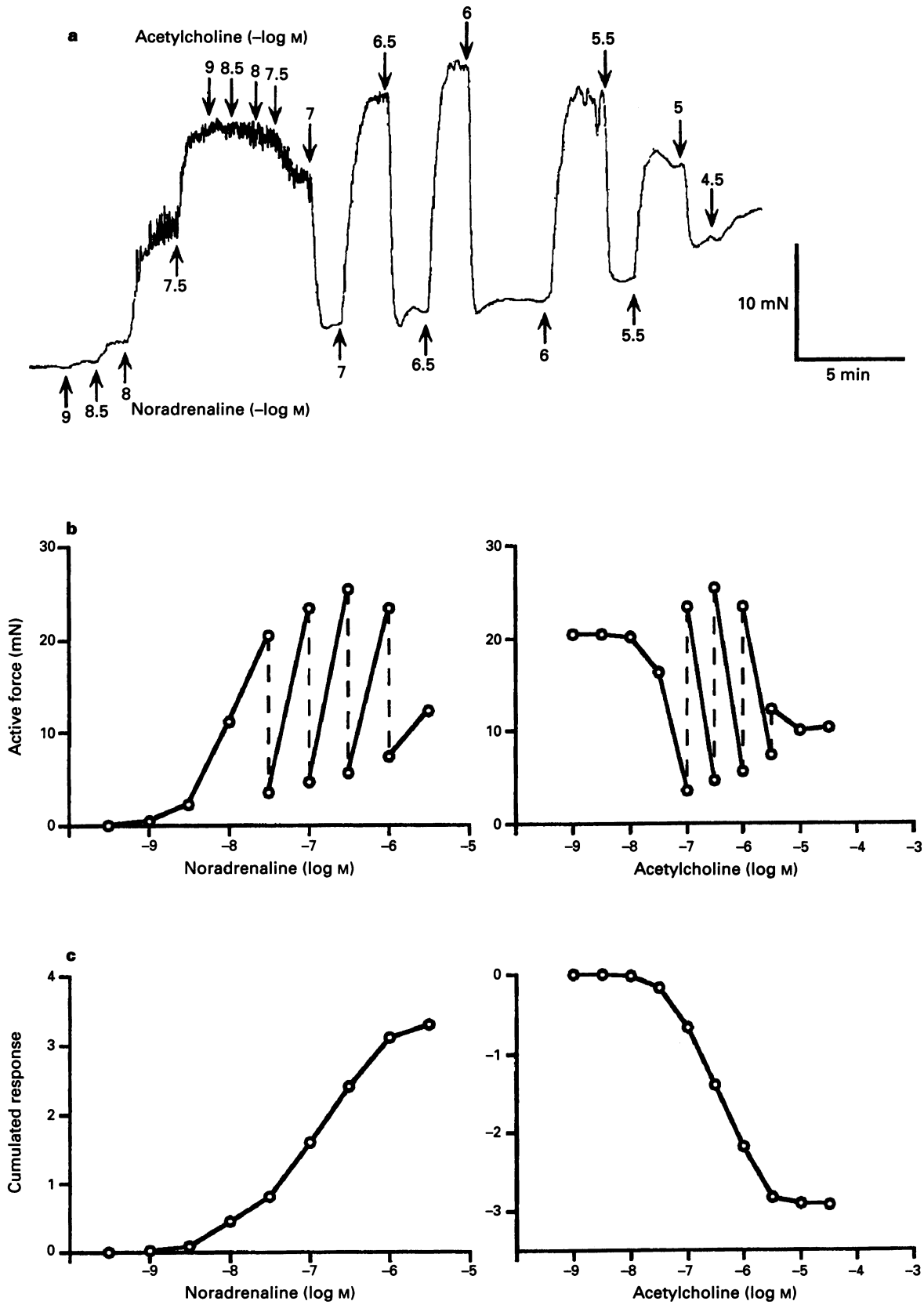

Figure 1 An example of an up/down experiment. (a) Chart record showing the active force produced by a rat tail artery in response to noradrenaline (NA) and relaxation produced by acetylcholine (ACh). (b) Data plotted to show the relationship between up/down data and data from 'normal' functional antagonism protocols. NA concentration-response curves in the presence of different concentrations of ACh (left) and ACh concentration-response curves in the presence of different concentrations of NA (right). (c) Data plotted using cumulated responses to produce extended concentration-response curves for NA (left) and ACh (right).

Discussion

Maximum response as a constraint

The maximal effect measured for an agonist may, in general, be limited by three kinds of constraint. First, the maximum might reflect the response resulting from complete occupation of the receptors by the agonist. This case occurs when the agonist is partial, and the maximum is a useful index of the agonist efficacy. Second, the maximum may reflect the maximum level of the measured parameter that the assay system can express: the 'tissue maximum response'. An example of this type is where a vasodilator agonist is able to relax completely an arterial assay. No further response can be measured in response to increased agonist concentration, even though more receptors can be occupied and additional intracellular stimulus produced. Third, the maximum may be constrained by the attainment of a maximal possible, or maximally effective, level of an intermediate stimulus between the receptor and the measured response: the 'receptor transduction maximum'. This maximum can be less than the maximum produced in the same assay by activation of another receptor type. An example of this type of constraint is provided by α_2-adrenoceptor activation in canine saphenous veins, where B-HT 933 has a receptor reserve, and so is a full agonist, but does not cause as large a response as the α_1-adrenoceptor agonist, cirazoline (Ruffolo & Zeid, 1985).

The agonists used in this study can be shown to be full agonists in normal concentration-response curves. NA and AVP were able to activate maximally the mesenteric artery (Figure 2), ACh was able to relax AVP-induced precontraction completely (Figure 4) as was 5-methylfurmethide (data not shown). For each of these agonists, the assay maximum was therefore equal to the tissue maximum response. The receptor transduction maximum and agonist effiacy can be described only as greater than that needed to elicit the tissue maximum response. Data from normal concentration-response curves such as these do not allow useful comparisons between full agonists, between the receptor transduction maximum of different receptors, or between the receptor transduction maxima in different tissues.

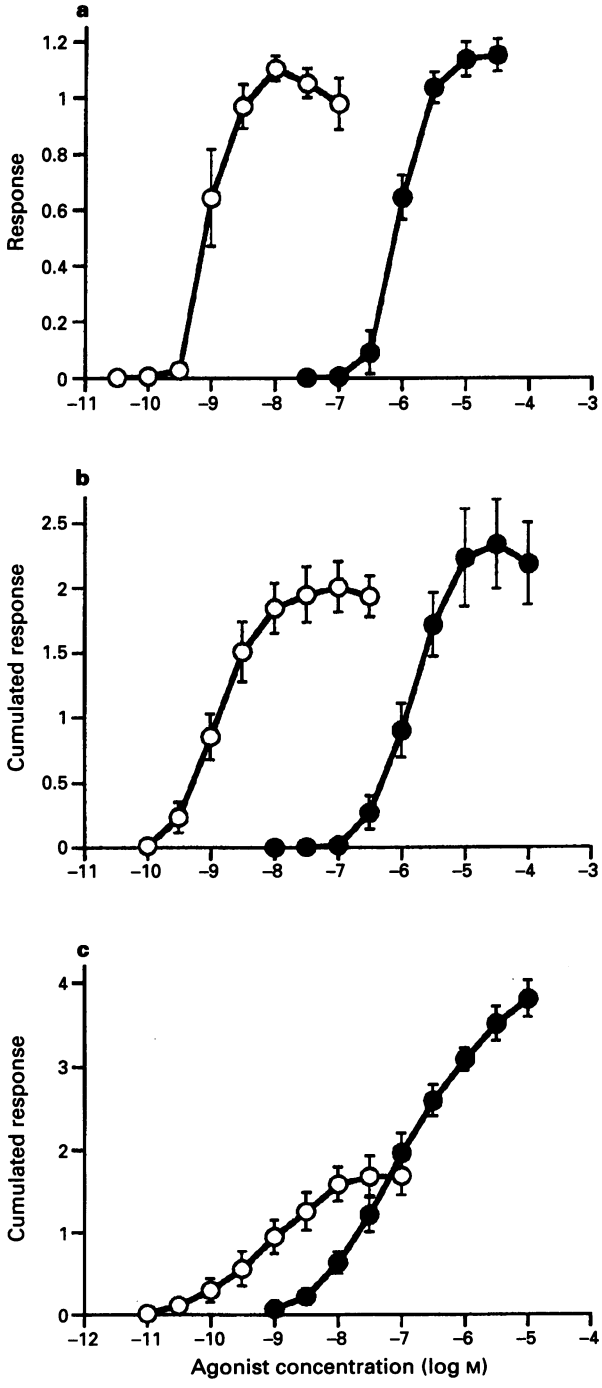

Figure 2 Concentration-response curves for vasopressin (AVP, ○) and noradrenaline (●). (a) Normal concentration-response curves in the rat mesenteric artery. (b) Up/down concentration-response curves in the rat mesenteric artery. (c) Up/down concentration-response curves in the rat tail artery. Responses are expressed as a fraction of the response to potassium depolarizing solution (KPSS). Values are mean ± s.e.mean ($n = 3$ to 7).

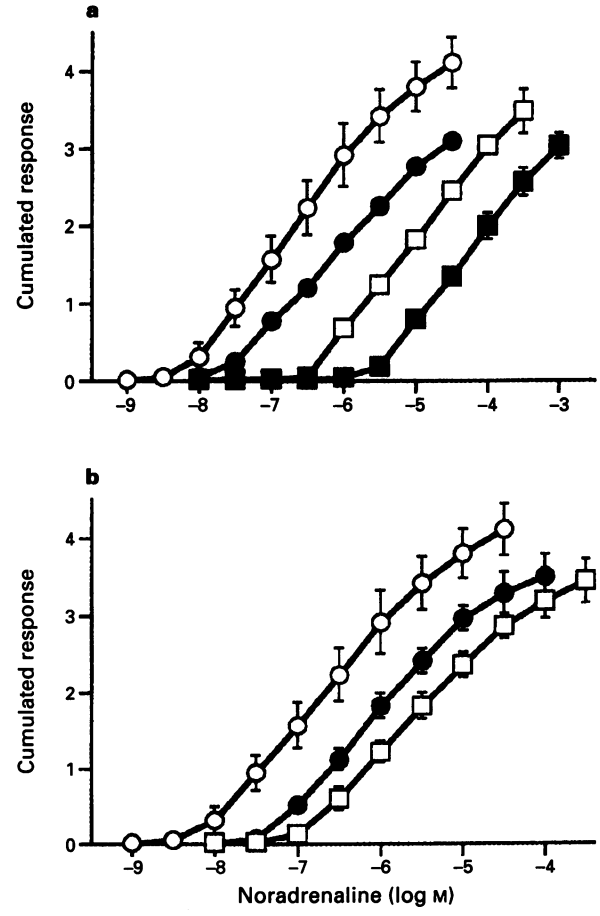

Figure 3 Up/down extended concentration-response curves in the rat tail artery for noradrenaline in the presence of (a) prazosin 0 (○), 3 (●), 30 (□) and 300 nM (■), or (b) yohimbine 0 (○), 0.1 (●) and 1 μM (□). Values are mean ± s.e.mean ($n = 3$, 2, 4 and 4 respectively for prazosin, and $n = 3$, 4 and 4 for yohimbine).

A different 'response'

In normal concentration-response curves the absolute level of the measured parameter (force or active force in the present experiments) or the change of the measured parameter from its initial value are used as the 'response'. However, to generate an up/down concentration-response curve the 'response' to each agonist concentration increment is the change of the measured parameter from its value immediately prior to the concentration increment. Cumulation of these responses allows the data to be expressed as a single concentration-response curve rather than a family of partial curves (Figure 1).

The resulting range of the up/down concentration-response curve will reflect the agonist efficacy or receptor transduction maximum, whichever is less. In the latter case two agonists of different efficacies at the same receptor would elicit the same maximum cumulated response, a situation directly analogous to full agonism in normal concentration-response curves. Nonetheless, for two drugs acting at the same receptor (in the absence of extraneous complications such as secondary drug actions), a larger range can result only from a higher efficacy. Thus the range of the up/down concentration-response curve is an open-ended extension of the intrinsic activity scale (Ariëns, 1954), increasing discrimination between high efficacy agonists in a straightforward manner. The up/down data show that ACh has a higher intrinsic activity than 5-methylfurmethide (Figure 5), and if they act at the same muscarinic receptors, then ACh has a higher efficacy than 5-methylfurmethide.

The up/down protocol also extends the range of comparisons between tissues and between receptors. The muscarinic receptors on the endothelium of the mesenteric artery can produce a larger response (scaled to the force elicited by KPSS) than the same system in the tail artery. In these arteries the maximum possible effect of muscarinic receptor stimulation in normal concentration-response curves is always 100% relaxation of the precontraction, so comparisons of response ranges from normal concentration-response curves between the tissues are meaningless. NA caused larger cumulated responses in the tail artery than in the mesenteric artery, and in the tail artery NA was able to elicit larger responses than AVP. 'It only makes sense to relate in one scale the intrinsic activities for compounds that interact with the same receptors' (Ariëns *et al.*, 1960), so it should be concluded that the α-adrenoceptors have a larger transduction maximum than the AVP receptors, rather than NA having a higher intrinsic activity than AVP. It is likely that the NA responses in the tail artery were large because of the presence of both α_1- and α_2-adrenoceptors (Medgett & Langer, 1984). The expriements with prazosin and yohimbine were intended to test this possibility, but were not really conclusive. Prazosin behaved competitively with a pA_2 appropriate for α_1-adrenoceptors (Flavahan & Vanhoutte, 1986). Yohimbine did not behave in a simple competitive fashion, but the antagonism by yohimbine at the lower concentration (0.1 μM) was consistent with a pA_2 of about 7.7, close to what would be expected for α_2-adrenoceptors. As it is often difficult to obtain clear evidence for the coexistence of two different receptor subtypes in experiments such as these using a non-selective agonist (Kenakin, 1992), these experiments do not rule out the presence of both receptors in the tail artery. Indeed, if 'responses mediated via postjunctional α_2-adrenoceptors in [vascular smooth muscle] are dependent upon a degree of vascular smooth muscle stimulation by some other receptor system' (Dunn *et al.*, 1991), then it is easy to see how

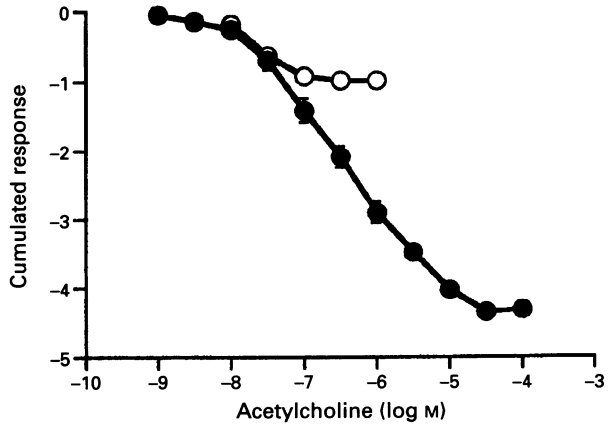

Figure 4 Normal concentration-response curve (○) and extended up/down concentration-response curve (●) for acetylcholine in the rat mesenteric artery. Values are mean ± s.e.mean ($n = 6$ and 4).

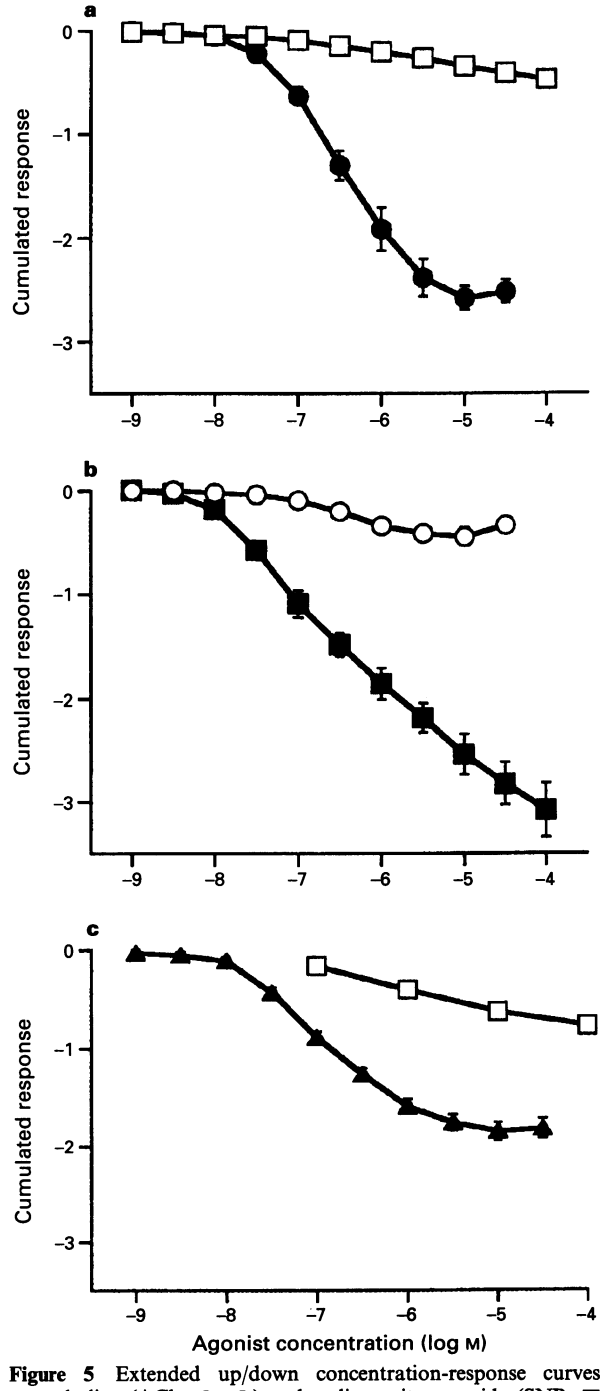

Figure 5 Extended up/down concentration-response curves for acetycholine (ACh, ○, ●) and sodium nitroprusside (SNP, □, ■) and 5-methylfurmethide (▲) in the rat tail artery. In each experiment both drugs were applied sequentially. (a) ACh followed by SNP ($n = 4$); (b) SNP followed by ACh ($n = 4$); (c) 5-methylfurmethide followed by SNP ($n = 12$). Values are means ± s.e.mean.

prazosin might appear competitive over the whole concentration-range even where both α_1- and α_2-adrenoceptors are involved in the response.

The possibility of NA acting through more than one receptor highlights a potential complication in using the up/down protocol. Agonists concentrations in an up/down concentration-response curve cover a larger range than in a normal concentration-response curve. This leads inevitably to the possibility of secondary actions of the agonists influencing the results. This problem can exist for any concentration-response curve, but is more likely during the up/down protocol. Where an agonist is acting at more than one type of receptor, the resulting up/down curve more properly reflects the overall tissue stimulus-response coupling than the agonist efficacy.

Spare receptors

The concept of spare receptors or receptor reserve results directly from the existence of a ceiling to the range of a concentration-response curve. This means that the up/down protocol will reduce or eliminate spare receptors. This can be seen by comparison of normal and up/down concentration-response curves for ACh. In the normal curve, ACh elicits a maximum response at 1 μM, but in the up/down curve it continues to elicit responses at concentrations up to 30 μM. If removal of the constraint of tissue maximum response leads to the occupancy-response curve becoming unconstrained overall, then there can be no receptor reserve in an up/down curve. However, while the up/down protocol removes the constraint of tissue maximum response, it does not necessarily remove all constraints. As assay where agonist responses are normally constrained by the tissue maximum may be constrained by the receptor transduction maximum in an up/down protocol. The up/down curve of ACh in the tail artery probably almost saturates the cyclic GMP system in the smooth muscle cells, on the evidence that sodium nitroprusside is nearly ineffectual after ACh. 5-Methylfurmethide does not reduce the effectiveness of sodium nitroprusside quite as much, as would be expected from its lower efficacy. These data probably indicate that there is a maximally effective, or maximal achievable, concentration of cyclic GMP, with neither ACh nor 5-methylfurmethide being sufficiently powerful to produce that concentration of cyclic GMP without further stimulation by sodium nitroprusside. It is possible that in another tissue the cyclic GMP system would be saturated by both ACh and 5-methylfurmethide, and thus their efficacy difference would not be exposed by the up/down protocol, and both agonists would have spare receptors.

Functional antagonism

Agonst dissociation constants (K_As) have been assessed using functional antagonism with limited success (Buckner & Saini, 1974; Broadley & Nicholson, 1979; Broadley & McNeill, 1983). It has been demonstrated that valid estimates of pK_As can be obtained from functional antagonism experiments with only some forms of functional interaction (Mackay, 1981; Leff *et al.*, 1985). However, even with those forms of functional interaction agonist affinity cannot be estimated directly from the pEC_{50} of an up/down concentration-response curve for two reasons. First, where the range of the curve is constrained by the receptor transduction maximum, then there can be a receptor reserve, and pEC_{50} of the resulting concentration-response curve will not approximate to the pK_A. Second, even where the responses are not constrained by the receptor transduction maximum, the slope of the occupancy-response relationship probably will not be constant over the whole range of agonist concentrations. Thus the response level that corresponds to agonist occupancy of half of the receptors will not be half of the maximum. Changes in the shape of the occupancy-response relationship during the protocol can be expected as a result of non-linear interactions of second messenger systems, so the form of the functional interaction will, in part, determine the slope of the occupancy-response relationship. This means that the location of the extended concentration-response curve can be dependent on the choice of functional antagonist. The pEC_{50} of an up/down curve cannot be relied upon to correspond to the pK_A of the agonist.

Agonist efficacies have also been estimated from functional antagonism experiments in the past (Buckner & Saini, 1974; Broadley & Nicholson, 1979; Emmerson & Mackay, 1981; Broadley & McNeill, 1983). The method used by Broadley involved calculation of K_A from functional antagonism data, and calculating relative efficacies using these values and the method of Besse & Furchgott (1976). The problems with K_A estimations from functional antagonism data probably make this strategy unreliable. In principle, the relative efficacy of an agonist used as a functional antagonist can be estimated, at least qualitatively (Mackay, 1981) from the amount that it can shift the concentration-response curve of another agonist (Van der Brink, 1973). Thus, the efficacy of a test drug of interest would be estimated from the potency ratio of a standard agonist in the absence and presence of a maximally effective concentration of the test drug. In practice, useful data can only result where the test drug has a low enough efficacy that it does not obliterate the responses to the standard drug. Furthermore, the confidence band for the potency ratio can be rather wide because its variability is the product of the variability of efficacy of both the test drug and the standard drug. Estimations of relative intrinsic activities using the up/down protocol are not subject to either of these limitations. If the test drug has enough efficacy to obliterate the responses to the standard drug, then another agonist acting in the same direction as the standard agonist can be added. The variability of efficacy of the standard agonist in the up/down protocol should have a negligible effect on the estimate of intrinsic activity for the test drug because the standard agonist is titrated to effect, and the concentration needed for that effect is not important.

Comparisons between assays (scaling)

For comparisons between agonists or receptors within a single assay tissue, no normalisation of the data is necessary, and absolute units should be used. When comparing between tissues, however, some sort of normalisation of the data is necessary to control for between tissue differences that are irrelevant to the comparison being made. The difference in tissue maximum active force between the mesenteric artery and the tail artery is an irrelevant difference. Two possible normalisation methods are applicable to the between artery comparisons: calculation of 'equivalent distending pressure' from the force and arterial dimensions (Mulvany & Halpern, 1977; Lew & Angus, 1992), and simple expression of the force as a fraction of the tissue maximum response. The latter method was chosen because it would be applicable to all types of assay (not only to vascular tissues), and because the resulting intrinsic activities for the agonists are effectively part of the same scale as that for which the concept of intrinsic activity was originally defined (Ariëns, 1954). The use of this normalisation will not have affected data comparisons within tissue (other than possibly decreasing variance), as essentially the same concentration-response curves would be seen with the data expressed as cumulated active force.

Conclusions

The up/down protocol described in this paper circumvents the tissue maximum response as a constraint on the maximum effect of an agonist. This extends the scale of intrinsic activity to allow discrimination between high efficacy agonists and should be a useful approach in comparisons of agonist efficacies, in comparisons of tissue stimulus-response coupling efficiencies between tissues, and within tissues between disease states. Interpretation of any experiment involving functional interactions between agonists is more complex than interpretation of normal concentration-response curves, but the

information available froma normal concentration-response curve is also available from an up/down concentration-response curve. The first few concentrations of each agonist in the up/down protocol are applied exactly as they would be in a conventional concentration-response curve, so the threshold concentration of an agonist determined from an up/down curve is identical to that obtained in the conventional curve. Further, because the concentration of each agonist is increased until the force of contraction crosses the 50% of tissue maximum response, the pEC_{50} that would be obtained from the normal concentration-response curve can be obtained from the up/down concentration-response curve. Thus, the up/down concentration-response curves for NA, AVP and ACh contain all of the information that can be obtained from the normal concentration-response curves for these agonists. The range of the up/down concentration-response curve is additional information. This method does not require the use of an irreversible antagonist, so it is applicable to most receptor types. The protocol is demonstrated in this paper using small vascular tissues, but it has been used successfully in other small and large arteries measuring either isobaric diameter or isometric force, and in cardiac tissues measuring either atrial rate or driven force. As long as responses of the assay do not fade excessively during the protocol and a functional antagonist is available, this approach may increase the information gained from agonist concentration-response curves.

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