Published in final edited form as:
Eur J Pharmacol. 2011 November 16; 670(1): 204–207. doi:10.1016/j.ejphar.2011.08.007.

On the quantitation of an agonist with dual but opposing components of action: Application to vascular endothelial relaxation

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Abstract

In this communication we show that the same principle that underlies the use of the isobolograph for assessing agonist interactions also leads to a method for analyzing the opposing effects of a single agonist. This is the principle of *dose equivalence* whose application is illustrated here and applied to the endothelium-dependent relaxing component of two putative vasoconstrictor peptides. These studies, employing angiotensin II and endothelin-1, were conducted with isolated preparations of rat aorta that were measured for agonist-induced isometric tension development in both endothelial-denuded and -intact vessels. The dose–effect relation of the relaxing component of each agonist, which should not be calculated from simple effect subtraction, was derived by the method described here.

Keywords

Isobole; Dose equivalence; Additivity; Endothelium; Angiotensin II; Endothelin-1

1. Introduction

Studies of interactions between two agonist drugs with overtly similar actions are common. Most often such studies employ isobolographic methodology, a graphical procedure introduced and used by Loewe (1927, 1928, 1953). The theoretical basis of the isobole, not represented in these early works, has been examined and extended in our more recent works (cited subsequently). One consequence of this extension of theory has led to new computational methods that include the topic of this communication, viz., quantitating the dual but opposing actions of a single agonist. This situation occurs in blood vessels when agonist-induced vasoconstriction on the smooth muscle cells is attenuated by relaxing mediators from the endothelium. This situation and the data we experimentally derived will serve as an example of the general computational methodology that grew out of our more

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There are no conflicts of interest.

detailed examination of isoboles for agonist drug combinations. That examination reveals a more basic concept, *dose equivalence*. This concept is the basis of the isobole, a graph of dose pairs that are expected to yield a specified effect magnitude. Details on this concept are given below in Section 2.1 *Theory*.

Much is known about the endothelium. For the purposes of this communication we note first that it consists of a layer of cells between the vessel lumen and the vascular smooth muscle cells. The endothelium is metabolically active and produces certain vasoactive mediators, most notably nitric oxide and prostacyclin (cf., Kim et al., 1992; Vanhoutte and Scott-Burden, 1994; Izumi et al., 1996; Shipley and Muller-Delp, 2005). Both mediators are known to be potent arterial vasodilators and therefore modulate the tone of blood vessels, endogenous release of these short-lived compounds is not easily accomplished, nor is it needed here since our aim is to quantitate their collective endothelial vasodilating effect that accompanies agonist action. There is also a third contributor referred to as endothelium-derived hyperpolarizing factor — a substance and/or electrical signal that is generated or synthesized in and released from the endothelium causing hyperpolarization of the underlying smooth muscle cells. This effect also reduces vascular tone. Collectively, the endothelium is acting like a second drug that produces effects on tension that are opposite to those produced by vasoconstrictors such as endothelin-1, angiotensin II, and norepinephrine. Our experimental design, derived from the concept of dose equivalence (Tallarida and Raffa, 2010), employs the same theory that underlies the isobole, and this leads to the determination of the dose–effect relation of the endothelial component as we will show in this communication. The experimental paradigm and the mathematics on which it is based are described below.

2. Material and methods

2.1. Theory

Our methodology follows from the same concept that underlies the isobole and, thus, we begin with a brief summary of that graph. In its common usage the isobole is a plot in Cartesian coordinates of dose b (for drug B) against dose a of drug A. It is a decreasing function because the presence of drug A reduces the quantity of drug B alone that is needed for the effect. In its common usage this decreasing function is taken to be a straight line with negative slope whose axial intercepts denote the individually effective doses. When the specified effect=1/2 of the maximum these intercepts are the respective D50 doses (or ED50 doses in quantal assays). However, the isobole is not necessarily linear (Grabovsky and Tallarida, 2004). It is only linear if the potency ratio of the constituent drugs is constant (as shown in Fig. 1) and, for some effect levels, this curved isobole may not have a second intercept. Whether linear or nonlinear, the isobole serves the purpose of distinguishing superadditive and sub-additive interactions by its comparison with experimentally derived dose combinations (a, b), the plotted points that give the effect. Points above the isobole indicate sub-additivity, whereas points below indicate super-additivity (synergism). In addition to the nonlinearity phenomenon, our expanded examination of theory has led to an additional application that is somewhat related to the current topic, viz., an analysis of a U-shaped dose effect curve such as that exhibited by buprenorphine antinociception in rodent

species (Tallarida and Raffa, 2010). This is a situation in which a single agonist has a second nociceptive component of action that is activated at the higher dose levels, thereby resulting in the decline in antinociception. The work and theory used in analyzing that analgesic motivated our more detailed examination of other ongoing studies in our laboratory that deal with the vascular endothelium and its effect on vascular tone. This is a situation in which a single agonist results in opposite effects, and is an appropriate topic for illustrating the new methodology presented here. Further theoretical details are given in previous communications (Tallarida, 1992, 2000, 2001, 2002, 2006, 2007; Tallarida and Raffa, 1996, 2010). As stated above an isobole is a graph of dose pairs for a specific effect level (most commonly 1/2 E_{max}) and its linear form arises, as we now show, from the assumption that the potency ratio R is constant over the entire effect range; thus, R=A50/B50, the ratio of doses of drug A and drug B that individually give 1/2 E_{max}. It follows that a dose a of drug A is equally effective to an equivalent quantity a/R of drug B. This means that the needed B50 dose can be achieved by adding dose b and the equivalent: $b+a/R$=B50. Alternatively, $a+bR$=A50. From either of the above we get Eq. 1:

$$\frac{a}{\mathrm{A50}}+\frac{b}{\mathrm{B50}}=1. \quad (1)$$

This expression, derived from the concept of dose equivalence, is the familiar form that gives the straight line isobole but, clearly, this linearity applies only for the constant R condition since a varying potency ratio would lead to a different drug B-equivalent of dose a. The isobole is called “additive” because of the addition of the dose of one drug and its equivalent of the other is used, which means that no interaction has taken place. (It is notable that this is *not* direct addition on the effect scale.) Fig. 1 further illustrates the concept of dose equivalence that underlies the isobole by showing both the parent dose-effect curves and the resulting isobole.

The outcome of a dose combination can also be viewed on the effect scale, i.e., without an isobole, if we remove the constraint of a constant effect level. One can readily see how the same concept of dose equivalence leads to the *effect* of both components of a single agonist. This is illustrated in Fig. 2. In that figure we see the full constricting effect of dose b alone (following removal of the endothelium) as point P and the contribution of the endothelium for this dose which reduces the effect. That contribution is shown as the drop ΔE in the constricting effect. This view from the effect scale shows a drop because the second component exerts an effect in the opposite direction. We also show as a broken curve in the figure the reflection of drug B’s constricting dose effect curve in the negative effect range. From this broken curve one can readily determine how a drop ΔE and the corresponding dose change Δb are identified on the broken curve. Using dose equivalence, we therefore see that Δb occurs at effect level denoted by (−E*). Thus, point Q with coordinates (b, −E*) is a point on the dose–effect curve of the endothelial component. When the drops at several doses are determined experimentally, we get the corresponding set of E* values, thereby leading to the dose–effect curve of the endothelial component. This analysis, using the same theory that underlies the isobole, shows how doses (and effects) of an agonist compound and an opposing second component are related to the combined effect of both together.

2.2. Experimental procedure

Adult, male Sprague–Dawley rats (Ace Animals, Boyertown, PA) were used in all studies to get in vitro measurements of developed isometric tension elicited by drug administration to isolated aortas. After acclimation to the animal facility, rats were euthanized via CO_2 asphyxiation, and their thoracic organs excised and placed in cold (4 °C) Krebs' buffer consisting of 120 mM NaCl, 25 mM $NaHCO_3$, 10 mM D-glucose, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, and 1.2 mM $MgSO_4$ (bubbled with 5% CO_2/95% O_2 to yield a pH of 7.4). A section of the aorta, from just below the aortic arch to the diaphragm, is then dissected for use. Minimal cleaning of connective tissue is performed, with the aim of avoiding damage to the adventitia and smooth muscle cells. In endothelium-denuded preparations, the lumen of the vessel is gently rubbed with a wooden dowel. The thoracic aorta segment is then cut into rings of ~3 mm length, and hung between two stainless steel hooks in a water-jacketed 15 ml organ chamber kept at 37 °C. One hook is anchored to a fixed position, and the other is attached to a force-displacement transducer (Grass Technologies, West Warwick, RI) mounted on a micrometer to allow for manual adjustment of tension. The transducers are connected in series to a signal amplifier, an analog-to-digital converter, and a PC. The data are visualized with Chart software (AD Instruments, Colorado Springs, CO). After an equilibration period of 30 min, the basal tension is adjusted to 2 g, and 30 min later the tissues are exposed to a modified 120 mM KCl Krebs' solution. This procedure is repeated after washing in triplicate, and an additional 30 min period of recovery. (The second KCl response was used to normalize tension in response to agonists). Following the two KCl responses, the rings are exposed to 1 μM phenylephrine, followed by 10 μM carbachol to elicit an endothelium-dependent vasodilatory response. Exclusion criteria for rings are as follows: a relaxation to carbachol of less than 70% of KCl contraction for endothelium-intact rings, and any visible relaxation to carbachol for endothelium-denuded rings. Following additional washes and recovery time, a single agonist of interest is tested. Because of the slow onset and prolonged duration of action, in addition to concerns of tachyphylaxis, only one concentration of endothelin-1 was tested per ring, yielding a non-cumulative dose response curve. Doses of angiotensin II, however, were added cumulatively.

3. Results

The dose–effect relation for angiotensin II and endothelin-1 are shown in Fig. 3 (*upper*) for both the normal endothelium-intact vessel and the endothelium-denuded vessel. It is seen that angiotensin II exhibits a strong endothelium-dependent vasodilatory tone. The E_{max} value in endothelium-denuded vessels is approximately three-fold higher than those with endothelium intact (52.06 vs 15.9% KCl). The data for endothelin-1, also displayed graphically, shows a prominent elevation of tension in the denuded case for most doses but differs from the angiotensin curves in that both the endothelium-denuded and -intact vessels approach the same maximum. For each compound, the drop in tension allows a determination of the endothelial component. The calculation is based on the dose equivalence concept, described in Section 2.1 *Theory*, which is the same as that used in isobolographic approaches. In this case the endothelial component acts like a second compound whose effect (relaxation) is opposite to that of the vaso-constrictor compounds.

In this calculation we know, for each dose, the positive effect magnitude for the smooth muscle component of action (endothelium-denuded condition), and the combination effect with both cell types contributing (attenuated tension with endothelium-intact). From these values we can determine the endothelium-dependent vasodilatory (relaxing) component of action. The methodology is further illustrated in Fig. 2 and the result of that calculation shows the endothelial component for each vaso-constrictor (Fig. 4) plotted in the negative effect range. It is seen that endothelin-1 induces a component of relaxation that begins at lower doses than those of angiotensin II.

4. Discussion

We have used the same theoretical approach that is used in isobolographic analysis in an experimental design and analysis that derived the dose–effect relation of the endothelium component of vascular tension reduction in response to two different vasoconstrictors. Endothelial cells influence vessel tone by releasing nitric oxide, and other substances, in response to shear stress or agonist stimulation. These vasodilatory mediators are released and act upon the smooth muscle cell to inhibit contraction. A single agonist can thus simultaneously possess two mechanisms of action relating to vessel tone: a vasoconstriction component via activation of the smooth muscle cells, and a vasodilatory component via activation of the endothelial cells. Importantly, since the endothelial influence over vessel tone is indirect (i.e., requires the presence of the smooth muscle cells to measure its functional effect on vessel tone), it cannot be measured directly. Our experimental design, built from the same theory that underlies the isobole, led to the procedure for getting the dose-effect relation of the endothelium-dependent vasodilation component. The methodology is also applicable for any agonist pair that produce effects in the opposite direction. Its application here has special relevance because the relaxing component of the endothelium due to vasodilators is indirect and difficult to measure. We found that the endothelial dose–effect curves derived from angiotensin II and endothelin-1 are different, a finding that implies that different vasoconstrictors affect endothelial release in different ways. It has been reported that nitric oxide is more potent than prostacyclin as a vasorelaxing substance released from the endothelium (Goldman et al., 1995; Izumi et al., 1996). Our derivation of the dose–effect curve of the endothelial component does not distinguish between nitric oxide and prostacyclin in producing relaxation, but the derived curves for the two vasoconstrictors that we tested show more potent relaxation when the stimulating compound is endothelin-1. This might suggest that endothelin-1 releases more nitric oxide than does angiotensin II. The methodology we developed and used here has additional applications, e.g., an examination endothelial relaxation induced by other endogenous vasoconstrictors such as norepinephrine and urotensin II. The methodology has even more extensive applications, e.g., a further examination of urotensin II and angiotensin II, a combination that we previously showed was synergistic (Lamarre and Tallarida, 2008). Another question for future study asks whether the released endothelial component effects vary with different vascular beds, and how is this affected by sustained hypertension? These questions are currently being investigated in our laboratory using the computational methodology and experimental designs described here. The most salient conclusion from the

work presented here is the demonstration that the dose-equivalence concept that is the theoretical basis of isobolographic theory also has this additional application.

Acknowledgments

This work was supported by NIH grants DA013429 (R.J.T.) and DA07237 (N.S.L.).

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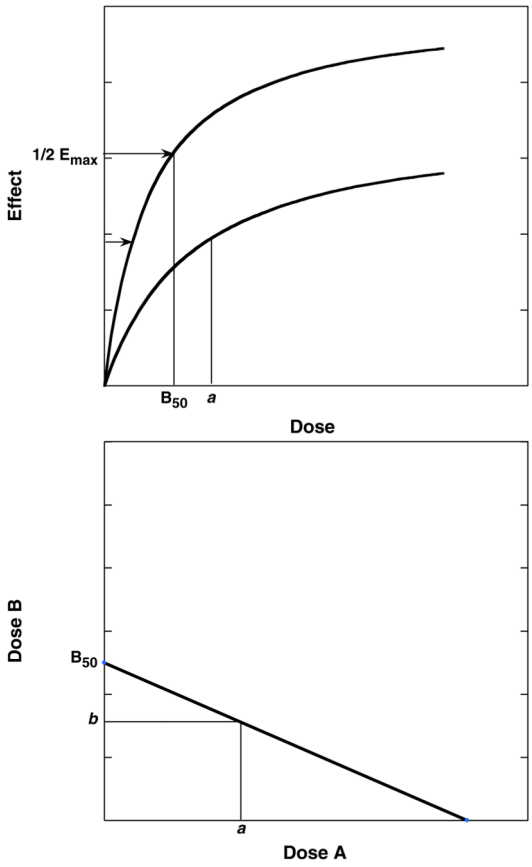

Fig. 1.

Dose equivalence and the isobole. *Upper* subpanel shows dose effect curve of two drugs, denoted A (lower curve) and B (upper curve), and illustrates graphically the drug B-equivalent of dose *a* of drug A (short arrow). The B50 of drug B is also indicated (long arrow). The contribution of drug A's equivalent means that a quantity of drug B less than B50 is needed to attain the specified effect level. This lesser quantity (difference between arrow lengths) is dose *b*. When shown on the isobologram (*lower*) we see the reduction from B50 to *b* and thereby illustrate why the isobole is a decreasing function of dose *a*.

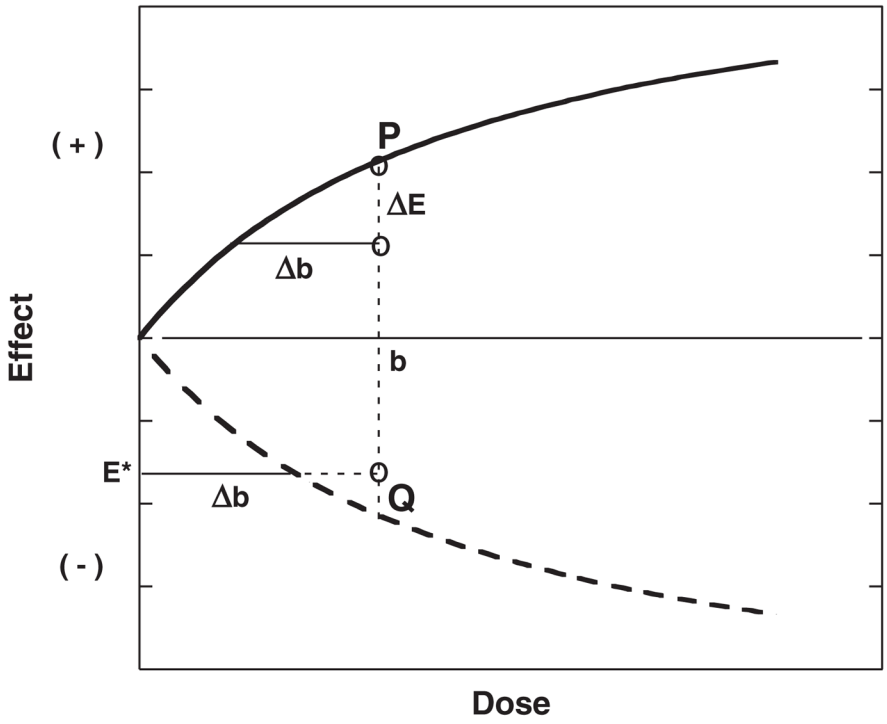

Fig. 2.

Deriving the endothelial component. A decrease ΔE in constricting effect corresponds to a reduction Δ*b* of dose *b*. That magnitude of dose reduction is transferred to the reflected dose–effect curve (shown broken in the negative range) which is used to locate point Q, a point that identifies the endothelial component at effect level (E*). Thus, for this drop (ΔE) we derive the point Q for the endothelial relaxing component of this agonist. In other words, the endothelial component of relaxation at point Q reduces the constricting component of dose b by the amount ΔE.

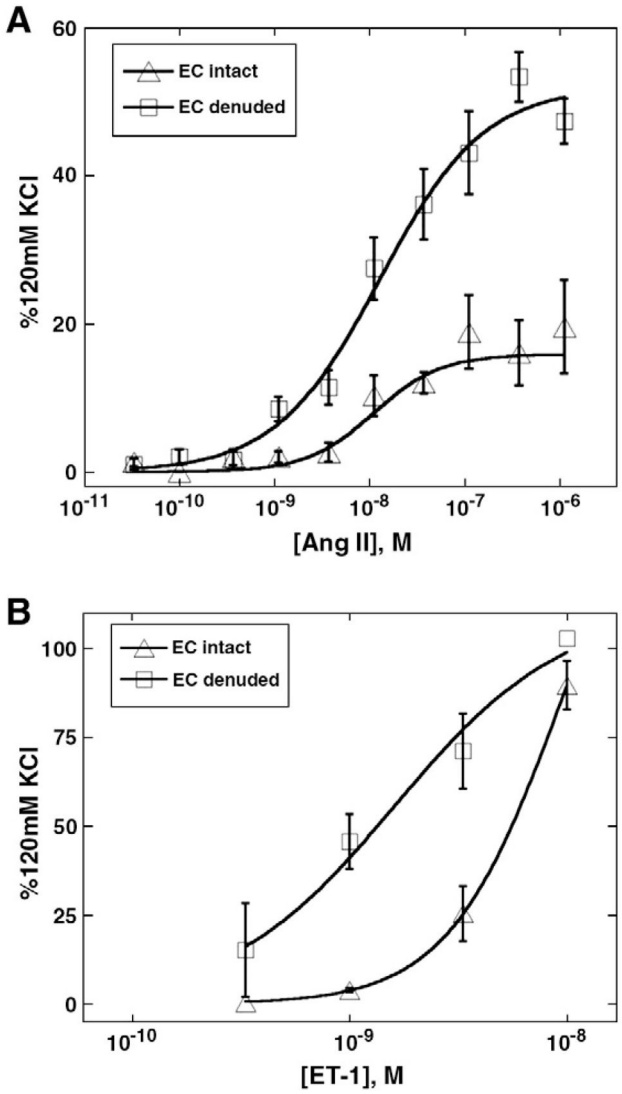

Fig. 3.

Dose–effect curves. The dose–effect curves for the endothelium (EC)-denuded and -intact preparations are shown for both angiotensin II (A) and endothelin-1 (B).

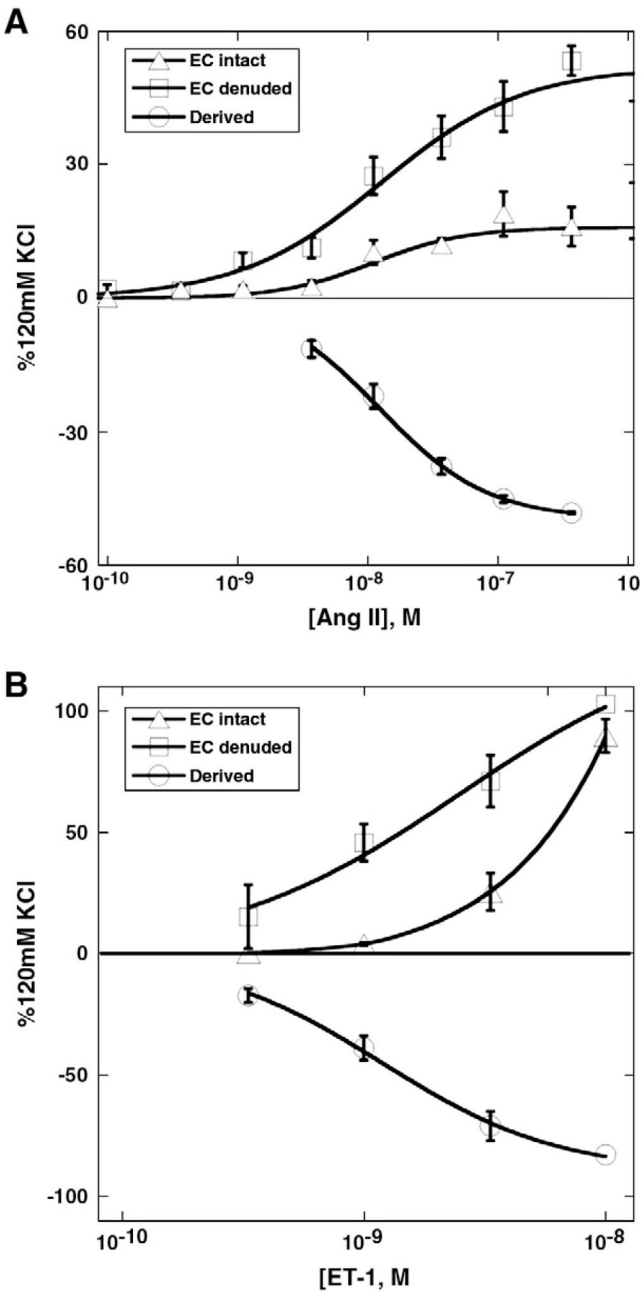

Fig. 4.

The endothelial component. The derived endothelial-dependent vasodilatory component of angiotensin II (A) and endothelin-1 (B) are shown as curves in the negative effect range. (The positive effect range shows the same curves that are displayed in Fig. 3.)