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Endothelin-1-Induced Alterations in Phenylephrine-Induced Contractile Responses Are Largely Additive in Physiologically Diverse Rabbit Vasculature[1]

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ABSTRACT

Endothelin-1 (ET-1) is an important modulator of vasomotor tone that is thought to participate in the etiology of cardiovascular disease by virtue of its ability to amplify the contractile responses of vascular smooth muscle cells to the effects of other vasoactive agents. Despite this fact, few studies have quantitated the expected contribution of ET-1 to the enhanced contractile responses elicited in the presence of another spasmogen. As a first step in this direction, ET-1 and phenylephrine (PE) were used to evaluate the effects of co-activation of the $ET_{A/B}$ or *alpha*-1 adrenergic receptors, respectively, on contractile responses in isolated rings of rabbit aorta, mesenteric and femoral artery, or strips of corporal tissue. Cumulative steady-state concentration-response curves (CRCs) were constructed to PE alone before the construction of a CRC to ET-1 alone, or a mixture of PE and ET-1 using a previously described drug concentration paradigm. Computer fits of the logistic equation to CRC data revealed that in all vascular tissues examined, the partial substitution of PE with ET-1 was associated with a significant vessel-dependent ≈3- to 30-fold leftward shift in the CRC ($P < .01$, Student's *t* test for paired samples), as judged by a significant increase in the pEC_{50} (negative logarithm of the concentration of drug that elicits one-half of the calculated maximal effect), in the absence of any detectable effect on the calculated maximal contractile response (E_{max}) or the slope factor (ρ). A theoretical CRC constructed using the Pöch and Holzmann method for equiactive substitution demonstrated that the responses to mixtures of PE and ET-1 were often the result of simple additivity of agonist effects in these preparations, and thus, were "expected" based on detailed knowledge of the individual effects of these two agonists. Regardless of the precision of the Poch and Holzmann CRC in predicting the effects of this drug mixture in these vascular tissues, comparison of the "expected" contractile response with the "observed" response represents an important first step toward establishing a more uniform nomenclature for describing the physiological/pathophysiological effects of mixtures of drugs on diverse vasculature.

The contractile state of smooth muscle cells in the vascular wall is determined by a diverse array of neurotransmitters, neuromodulators and hormones. Clearly, release of norepinephrine from sympathetic adrenergic varicosities plays a primary role in the regulation of vasomotor tone. However, the effects of sympathetic neurotransmission on vascular smooth muscle tone are further modified by the presence of many vasoactive substances released from the underlying endothelium. Perhaps chief among this diverse class of endothelial-derived vasoactive substances is the constrictor peptide ET-1 (Yanagisawa *et al.*, 1988; Luscher and Noll, 1995; Maguire and Davenport, 1995; Ohlstein *et al.*, 1995). In fact, ET-1 is generally regarded as the most potent circulating vasoconstrictor (Relavic and Burnstock, 1993; Ohlstein *et al.*, 1995; Cesari *et al.*, 1996; Tamirisa *et al.*, 1997), and moreover, several reports have documented that ET-1 is capable of amplifying the contractile response to diverse vasoactive compounds (Nakayama *et al.*, 1991; Henrion and Laher, 1993; Consigny, 1990; Sudjarwo *et al.*, 1995).

Taken together, such observations suggest that ET-1 is likely to play a significant role in cardiovascular physiology and disease, by virtue of its ability to elicit marked alterations in vascular smooth muscle tone even in the face of relatively subtle alterations in its plasma concentration. Consistent with this supposition, elevated plasma levels of ET-1 have been reported to attend cardiovascular disease (Pernow and Wang, 1997; Tamirisa *et al.*, 1997; Cesari *et al.*, 1996; Shichiri *et al.*, 1990). However, despite the potential physiological/pathophysiological significance of these established facts, few studies have attempted to rigorously quantitate the "expected" contribution of ET-1 to the contractile responses elicited in the presence of another spasmogen(s). Such quantitative studies are clearly a prerequisite to eluci-

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ABBREVIATIONS: CRC, concentration response curves; FMR, fixed molar ratio; PE, phenylephrine; ET-1, endothelin-1.

dating physiological or pathophysiological mechanisms of ET-1-induced alterations in vascular smooth muscle tone.

As a first step in this direction, the goal of this investigation was to characterize steady-state contractile responses elicited by PE- (phenylephrine) and ET-1-induced activation of the *alpha*-1 adrenergic and $ET_{A/B}$ receptors, respectively, in physiologically diverse vasculature. To this end, we utilized a previously described drug concentration paradigm (Christ *et al.*, 1990; Christ and Jean-Jacques 1991; Kim *et al.*, 1995) to examine the effects of the co-administration of PE and ET-1 on steady-state contractile responses in rings of rabbit aorta, mesenteric artery and femoral artery, as well as corporal tissue strips. Comparisons were made between the expected CRC for simple additivity of agonist effects constructed using the Poch and Holzmann (1980) method of equiactive substitution, and the actual CRCs observed for mixtures of PE and ET-1. In short, these studies documented that the contractile responses elicited in physiologically diverse rabbit vasculature by mixtures of PE and ET-1 were often largely those "expected" based on detailed knowledge of their individual actions.

Materials and Methods

Vessel collection. The thoracic aorta (6–8 mm strips), the superior mesenteric artery (3 mm), the femoral (5 mm) artery and the corpus cavernosum (3x3x10 mm strips) were excised from a total of 13 male New Zealand white rabbits (3–3.5 kg) obtained from Charles River Laboratories, St. Constant, Quebec, Canada. Animals were sacrificed by CO_2 asphyxiation and tissues were generally harvested the day prior to the experiment. Importantly, as previously reported for rabbit aorta (Christ *et al.*, 1990), preliminary studies documented that all of the rabbit vascular tissues used in these studies could also be stored overnight at room temperature in Kreb's buffer, without any detectable loss of viability or change in sensitivity to agonist administration (Christ G. and Gondré M., unpublished observations). All loosely adhering fat and connective tissue were removed from the otherwise adventitia-intact ring preparations of each vessel.

Corporal tissue collection. The rabbit's penis was removed by an incision at its base near the pubic symphysis and transferred into fresh buffer where it was cleaned of all loosely adhering connective tissue and skeletal muscle on the exterior of the tunica albuginea. The organ was bisected and carefully slit with a scalpel on either side to expose the surfaces of the corporal tissue. The two corporal strips were then removed and cut into sections of equal length.

Tissue pretreatment. All tissues were mounted onto the appropriate hooks and allowed to equilibrate for 1.5 hr at 37°C in a 20 ml organ bath chamber containing Krebs-Henseleit buffer of the following composition (in mM): (NaCl, 124; KCl, 5; $MgSO_4$, 1.3; $CaCl_2$, 2.5; NaH_2PO_4, 0.6; $NaHCO_3$, 25; and glucose, 11, maintained at a constant pH of 7.4 ± 0.1. Tissues were continuously bubbled with a 95% O_2-5% CO_2 mixture, and buffer was replaced at 20-min intervals. After tissues had stabilized at 2 g of baseline tension, they were primed by the addition of 3 μM PE to the organ bath. Tissues were then washed and the response to PE was reelicited. This pretreatment protocol reduces the variability of tissue responses to repeated agonist administration (*i.e.*, there are no time-dependent changes in the PE-induced steady-state response over the time course of these experiments). The absence of a relaxation response to carbachol (1 μM) administered during a steady-state contractile response to PE (3 μM) was utilized to confirm the absence of a functional endothelium. Tissue activity was measured isometrically with a FT-03 force transducer and recorded on a Grass model 7E or 7F Polygraph. ET-1 and L-PE were purchased from Sigma Chemical Co., St. Louis, MO.

Construction of CRCs. CRCs were constructed for each specimen by the cumulative addition of drug at half-log increments in the presence of indomethacin. Indomethacin had no effect on the measured tissue response to PE or ET-1, but reduced occasional oscillations of tissue during construction of the steady-state CRCs.

Because preliminary studies affirmed the presence of long-lasting and slowly reversible contractions characteristic of ET-1-induced responses in all preparations studied, CRCs to PE alone, ET-1 alone and mixtures of PE and ET-1 could not all be constructed on the same tissue. Thus, no more than two CRCs were ever performed on the same specimen. More specifically, the experimental paradigm was as follows: a PE CRC was performed on every tissue immediately prior to either a FMR (see below) CRC or an ET-1 CRC. This experimental design allowed each tissue to serve as its own control for the purposes of statistical analysis. This seems a reasonable research strategy in light of the fact that preliminary studies conducted on all four vascular tissues revealed that the logistic parameter estimates for the ET-1 CRC were the same regardless of whether or not PE CRC was performed on the same tissue. All CRCs were constructed at half-log increments, with a minimum of 10 to 12 points.

Construction of the FMR CRC. A previously described method was used for the construction of the FMR CRCs (Christ *et al.*, 1990, Christ and Jean-Jacques, 1991). Briefly, cumulative CRCs were constructed at half-log increments such that for any given total molar drug concentration in the CRC, a fixed ratio was selected for the PE:ET-1 mixture. For these experiments the ratio of the two drugs in the mixture was always 80:20 (PE:ET-1); please note that preliminary studies showed that similar results were also obtained with other FMRs (*i.e.,* 90:10 or 70:30). Furthermore, the rationale for using only FMRs in which the concentration of PE was higher than that of ET-1 was related to the following: the EC_{50} for PE was always 1 to 2 orders of magnitude greater than the EC_{50} for ET-1 on each preparation. Thus, we could only test FMRs in which the concentration of PE was more than that for ET-1. That is, PE:ET-1 FMRs less than 1 would have resulted in such low occupancies of the *alpha*-1 adrenergic receptor, for so much of the CRC, that it would have been impractical to accurately evaluate the contribution of PE to the response. Finally, to simplify the graphical comparison of control and mixture CRCs, all displayed concentrations represent either the concentration of PE alone, ET-1 alone or the mixture of PE + ET-1.

Construction of equiactive CRCs. The equiactive CRC was constructed in accordance with the method of Pöch and Holzmann (1980) as previously described (Christ and Jean-Jacques, 1991, Christ *et al.*, 1990; Kim *et al.*, 1996). At each point on the observed FMR, the response produced by a known concentration of ET-1 was calculated from the logistic equation that describes the ET-1 CRC. The concentration of PE necessary to produce this same response (equiactive) was then similarly calculated, and the concentration of ET-1 at every point of the FMR was replaced by an equiactive concentration of PE. The response produced by the sum of the two PE concentrations was calculated from the logistic equation and plotted *vs.* the total concentration for the corresponding FMR. For example, let us examine the 80% PE: 20% ET-1 (PE:ET-1) FMR CRC on a rabbit mesenteric ring. In this case, a 10 nM concentration on the 80:20 (PE:ET-1) FMR contains 2 nM ET-1 and 8 nM PE. 2 nM ET-1 produces the same effect that PE produces at a concentration of 82 nM. Thus, the response to 90 nM (*i.e.,* 8 + 82 nM) PE was plotted as the Pöch and Holzmann prediction of the response on the 10 nM FMR. This value represents the anticipated response if the contraction were a result of simple additivity.

Data analysis. The magnitude of the contractile responses were empirically determined from the chart recorder and computer fit using the RS/1 software package (BBN Software, Cambridge, MA) on a Gateway 2000–486DX/33 computer to the general logistic equation:

$$E = E_{max}/1 + (EC_{50}/[A])^{\rho}$$

where E is the observed response, [A] is the agonist concentration, E_{max} is the fitted maximum response, EC_{50} is the concentration of agonist needed to obtain 50% of E_{max} and ρ is the slope index of the CRC.

Statistical analysis. Statistical comparisons were made using the Statview II software program on a MacIntosh Quadra 800. The EC_{50} values were expressed as the geometric mean ± S.E.M. (*i.e.,* pEC_{50}: negative logarithm of the EC_{50}), whereas all other parameters were expressed as their arithmetic mean ± S.E.M. A Student's *t* test for paired samples was used to compare E_{max}, EC_{50} and slope factor values for PE, ET-1, and PE + ET-1 (*i.e.,* FMR) CRCs performed on the same tissue strip; $P < .05$ was considered statistically significant in all cases.

Results

Analysis of PE and ET-1 CRCs. Cumulative CRCs were constructed for steady-state contractile responses elicited by PE alone and ET-1 alone on rings of rabbit aorta, femoral artery and mesenteric artery, as well as corporal tissue strips. Table 1 summarizes the calculated mean E_{max}, pEC_{50} and slope factor values derived from computer fits of the logistic equation to these CRC data. As shown, statistical analysis revealed significant differences in the location of the PE and ET-1 CRCs, as reflected by the significantly different pEC_{50} values ($P < .0001$; Student's *t* test for paired samples) for ET-1 when compared to PE in all four vascular tissues studied. Additionally, significant differences were detected between PE and ET-1 in the calculated E_{max} values for aortic and mesenteric rings, as well as corporal tissue strips ($P < .01$ in all cases; Student's *t* test for paired samples), but not for the femoral rings ($P > .05$). There was no detectable difference in the slope factor value that describes the PE and ET-1 CRCs in any of the preparations studied. The relationship among the ET-1, PE and 80:20 FMR CRCs are depicted in the representative examples illustrated in figure 1.

Comparison of the PE and 80:20 (PE:ET-1) FMR CRCs. CRCs were also constructed for PE before performing another CRC on the same isolated tissue preparation for a mixture of PE and ET-1 using a FMR protocol (see "Materials and Methods"). In this fashion, PE and 80:20 (PE:ET-1) FMR CRCs were constructed for all four vascular tissues, once again permitting each tissue to serve as its own control. Mean logistic parameter estimates for the PE alone and 80:20 FMR CRCs in each isolated vascular tissue are summarized in table 2, and representative examples are graphically depicted in figure 2. In short, the partial substitution of ET-1 for PE, revealed the following for the 80:20 FMR: 1) an ≈3- to 28-fold leftward shift in the pEC_{50} ($P < .05$, Student's *t* test for paired samples), in the absence of any detectable changes in either the E_{max} or slope factor values.

Construction of the expected CRC for simple additivity of agonist effects. The Poch and Holzmann method (1980) of equiactive substitution was utilized to further explore the nature of the observed leftward shift in the PE CRC in the presence of ET-1. As illustrated in figure 3, some discrepancy was observed in the point estimates that describe the mean response levels for the 80:20 FMR and the response expected based on simple additivity of agonist effects, respectively. However, despite this apparent discrepancy, the expected CRC for simple additivity of agonist effects fell largely within the 95% confidence interval for the mean 80:20 FMR in each of the vascular tissues examined. This fact indicates that simple additivity of agonist effects seems to provide a reasonable description of the effects of this drug mixture on the steady-state contractile responses observed in these vascular tissues (fig. 3).

Discussion

The potent and long-lasting nature of the ET-1-induced contractile response in vasculature has led many investigators to propose that ET-1 is likely to play a significant role in cardiovascular physiology and disease (Yang *et al.*, 1990; Consigny, 1990; Ohlstein *et al.*, 1995; Tamirisi *et al.*, 1997). In fact, in the complex hormonal milieu present *in vivo*, it is conceivable that ET-1 could elicit quite marked alterations in vascular smooth muscle tone even in the face of relatively subtle alterations in its plasma concentration. Consistent with such a hypothesis, several reports have shown that threshold or near threshold concentrations of ET-1 potentiate contractile responses to other vasoactive agents (Nakayama *et al.*, 1991; Henrion and Laher, 1993; Consigny, 1990; Yang *et al.*, 1990). However, insufficient information was provided in these seminal studies to determine whether or not the observed increase in the magnitude of the contraction was at all "expected." Such considerations are especially important when utilizing threshold or near threshold drug concentrations, because of the marked percent variability in tissue response observed near threshold. Moreover, despite the potential importance of these observations to vascular physiology and disease, to date, there has been no systematic and rigorous investigation into the "expected" contribution of ET-1 to the enhanced contractile response elicited in the presence of another vasoactive agent. As a first step in this direction, the goal of these studies was to evaluate contractile responses elicited during coactivation of the ET and *alpha*-1

TABLE 1
Logistic parameter estimates for PE alone and ET-1 alone CRC data on the same vascular tissue preparation

		E_{max}	pEC_{50}	Slope Factor (ρ)
Aorta (4, 14)[a]	PE	9.81 ± 0.69	6.42 ± 0.07 (380 nM)	1.3 ± 0.07
	ET_1	7.49 ± 0.51[b]	8.12 ± 0.05[b] (7.59 nM)	1.28 ± 0.14
Mesenteric (4, 17)	PE	6.44 ± 0.8	6.27 ± 0.09 (537 nM)	1.98 ± 0.13
	ET-1	4.36 ± 0.63[b]	7.73 ± 0.21[b] (18.6 nM)	2.28 ± 0.18
Femoral (4, 17)	PE	5.67 ± 0.88	6.5 ± 0.07 (316 nM)	1.2 ± 0.05
	ET_1	4.93 ± 0.88	8.3 ± 0.07[b] (5 nM)	1.96 ± 0.18
Corpora (2, 11)	PE	2.14 ± 0.51	5.78 ± 0.1 (1.66 μM)	0.85 ± 0.05
	ET_1	1.38 ± 0.45[b]	8.16 ± 0.15[b] (6.92 nM)	0.83 ± 0.20

[a] Number to the left of comma refers to the total number of animals used, although the number to the right of the comma refers to the total number of vascular tissues used. All statistical comparisons were based on the total number of tissues.
[b] Denotes statistically significant difference from corresponding PE value, $P < .05$, Student's *t* test for paired samples.

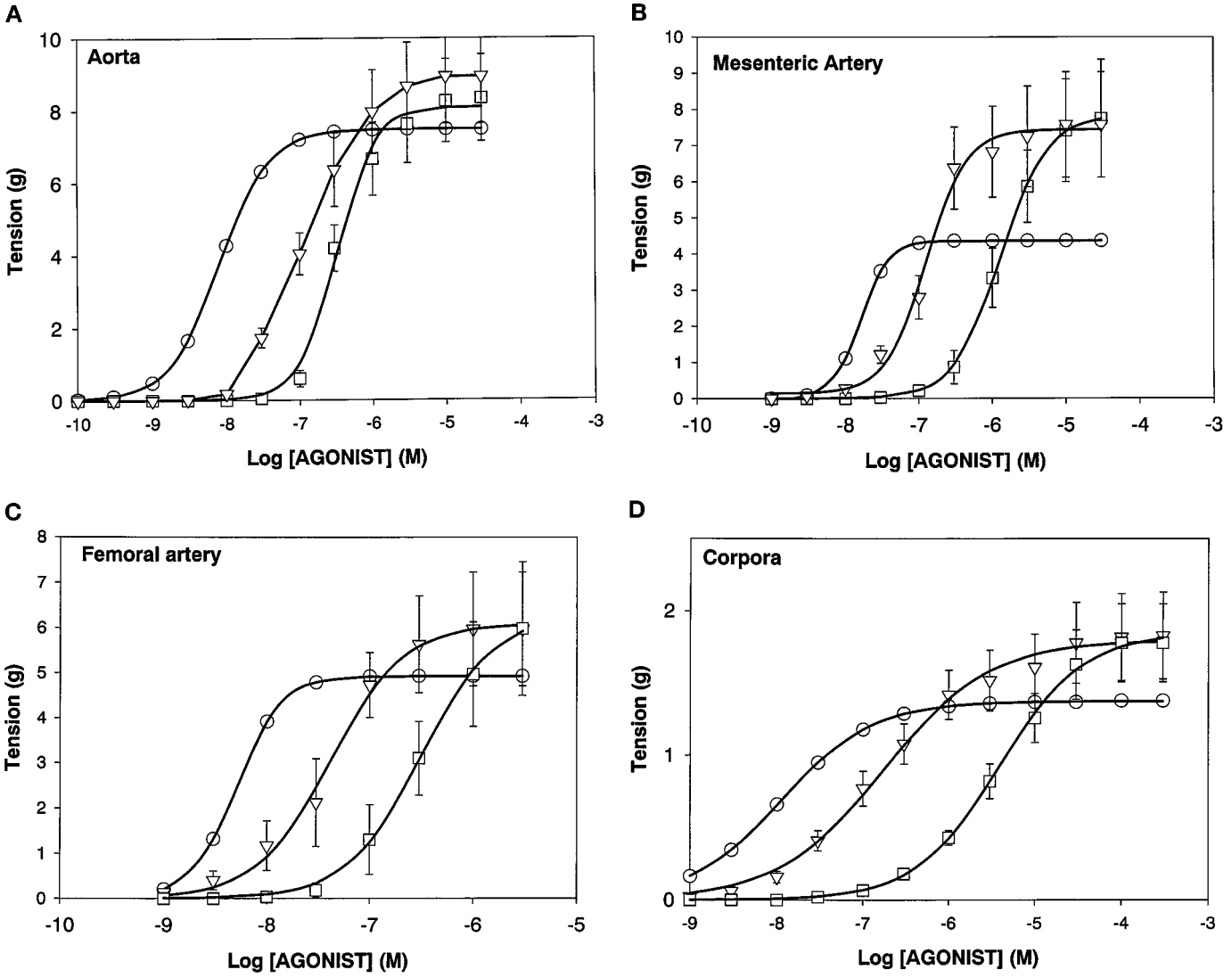

Fig. 1. Illustration of the relationship between the control ET-1 CRC (open circle; see "Materials and Methods"), a representative PE CRC (open square) and a representative 80:20 FMR (PE:ET-1) CRC (open inverted triangle) on the same tissue preparation a, Aorta; b, mesenteric artery; c, femoral artery and d, corporal tissue. In all cases, the ET-1 CRC was constructed from mean values obtained in the experiments summarized in table 1, and each point on the FMR and PE CRCs represents the mean ± S.E.M. for the contractile responses obtained at that concentration. a, Aorta. CRCs were obtained on the same three aortic rings from the same animal. Computer fits of the logistic equation to the PE CRC revealed values for E_{max}, $EC_{50,}$ and slope factor (ρ) of 8.11g, 0.3 μM and 1.63, respectively, although for the 80:20 FMR, these values were 8.94 g, 0.13 μM, 1.08, respectively. b, Mesenteric artery. CRCs were obtained on the same three mesenteric artery rings from the same animal. Computer fits of the logistic equation to the PE CRC revealed values for E_{max}, EC_{50} and slope factor (ρ) of 7.85 g, 1 μM, 1.36, respectively, although for the 80:20 FMR, these values were 7.47 g, 0.1 μM, 1.47, respectively. c, Femoral artery. CRCs were obtained on the same three femoral artery rings from the same animal. Computer fits of the logistic equation to the PE CRC revealed values for E_{max}, EC_{50} and slope factor (ρ) of 6.21 g, 0.3 μM and 1.27, respectively, although for the 80:20 FMR, these values were 6.21 g, 0.04 μM and 1.17, respectively. d, Corporal tissue. CRCs were obtained on the same six corporal strips from the same animal. Computer fits of the logistic equation to the PE CRC revealed values for E_{max}, $EC_{50,}$ and slope factor (ρ) of 1.85 g, 4.0 μM, 0.89, respectively, although for the 80:20 FMR, these values were 1.8 g, 0.73 μM and 0.47, respectively.

adrenergic receptors using a previously described drug concentration paradigm.

To do so we chose to use a previously described FMR protocol, rather than the single concentration method originally used by Poch and Holzmann. As discussed elsewhere (Christ *et al.*, 1990) the FMR method conveys a distinct advantage for evaluating the potential physiological relevance of the interaction of a drug mixture. That is, with the one concentration method, as originally described by Poch and Holzmann, one is evaluating the effects of increasing the concentration of one drug in the presence of the *same* concentration *(i.e.,* stimulus) of a second drug. The observed effects are thus very dependent on the fixed concentration of drug that is initially chosen; this can be especially critical when one is evaluating "threshold" effects. In contrast, the FMR ratio method evaluates the interaction between two drugs at a variety of different concentrations of each, throughout much of their respective CRCs. Thus, as long as the EC_{50} values for the two drugs of interest are within 1 to 2 orders of magnitude, the latter allows a more complete evaluation of the nature of the interaction between a drug mixture, from threshold to maximally active concentrations of the two drugs of interest. Of course it should be pointed out that the systematic method of describing/evaluating simple

TABLE 2
Logistic parameter estimates for PE alone and 80:20 FMR CRC data on the same vascular preparation

		E_{max}	pEC_{50}	Slope Factor (ρ)
Aorta (5, 15)[a]	PE	9.80 ± 0.48	6.42 ± 0.04 (380 nM)	1.44 ± 0.09
	FMR	11.5 ± 0.7	6.89 ± 0.07[b] (129 nM)	0.98 ± 0.04
Mesenteric (5, 11)	PE	7.01 ± 0.62	5.68 ± 0.09 (2.1 μM)	1.7 ± 0.14
	FMR	7.13 ± 0.56	6.74 ± 0.07[b] (182 nM)	1.73 ± 0.32
Femoral (5, 12)	PE	5.49 ± 0.71	6.41 ± 0.07 (389 nM)	1.64 ± 0.13
	FMR	5.70 ± 0.70	7.82 ± 0.11[b] (14 nM)	1.67 ± 0.28
Corpora (5, 18)	PE	2.50 ± 0.34	5.4 ± 0.05 (4.0 μM)	0.85 ± 0.03
	FMR	2.23 ± 0.30	6.45 ± 0.12[b] (355 nM)	0.63 ± 0.04

[a] Number to the left of comma refers to the total number of animals used, although the number to the right of the comma refers to the total number of vascular tissues used. All statistical comparisons were based on the total number of tissues.
[b] Denotes statistically significant difference from corresponding PE value, $P < .05$, Student's t test for paired samples.

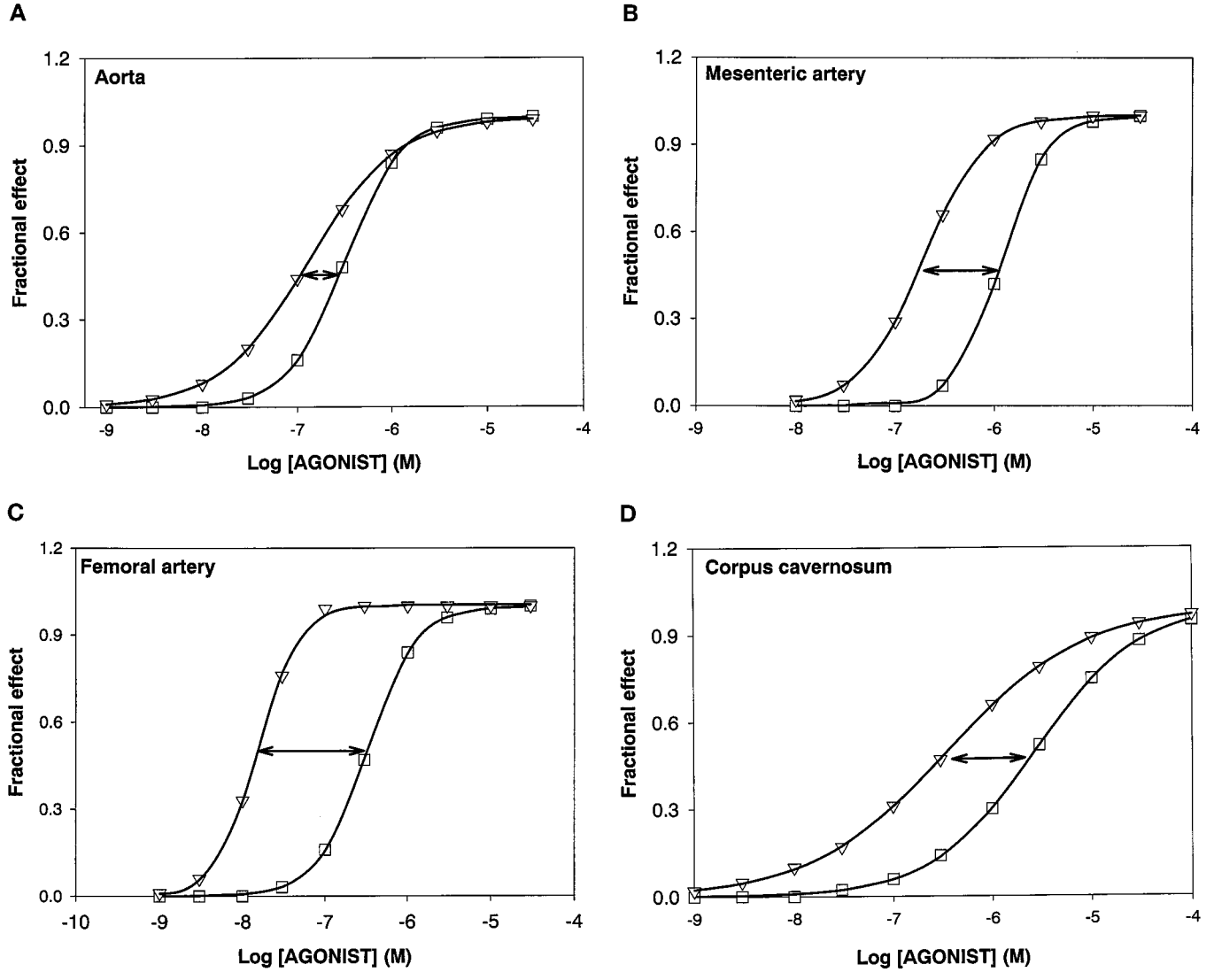

Fig. 2. ET-1-induced amplification of the PE-induced contractile response in diverse rabbit vasculature. Computer simulations of a CRC for PE alone (open square) and an 80:20 FMR CRC (PE:ET-1) (open inverted triangle) were generated by the logistic equation (see "Materials and Methods"), using the mean parameter estimates displayed in table 2. As illustrated, the magnitude of the leftward shift of the PE alone CRC in the presence of ET-1 varied by over an order of magnitude among these physiologically distinct vascular tissues.

additivity of agonist effects as originally proposed by Poch and Holzmann applies regardless of the exact protocol used to evaluate the drug mixture.

In this regard, a detailed pharmacological analysis of the CRCs that describe PE- and ET-1-induced steady-state contractile responses in each tissue was a prerequisite to evaluation of the "expected" effects of a mixture of these same drugs. Logistic analysis revealed an ≈4- to 5-fold range of values for both the calculated E_{max} and pEC_{50} parameter estimates for the PE and ET-1 CRCs, respectively, among these four distinct isolated vascular tissues (see fig. 1). In all tissues, the location (*i.e.,* EC_{50}) of the ET-1 CRC was signif-

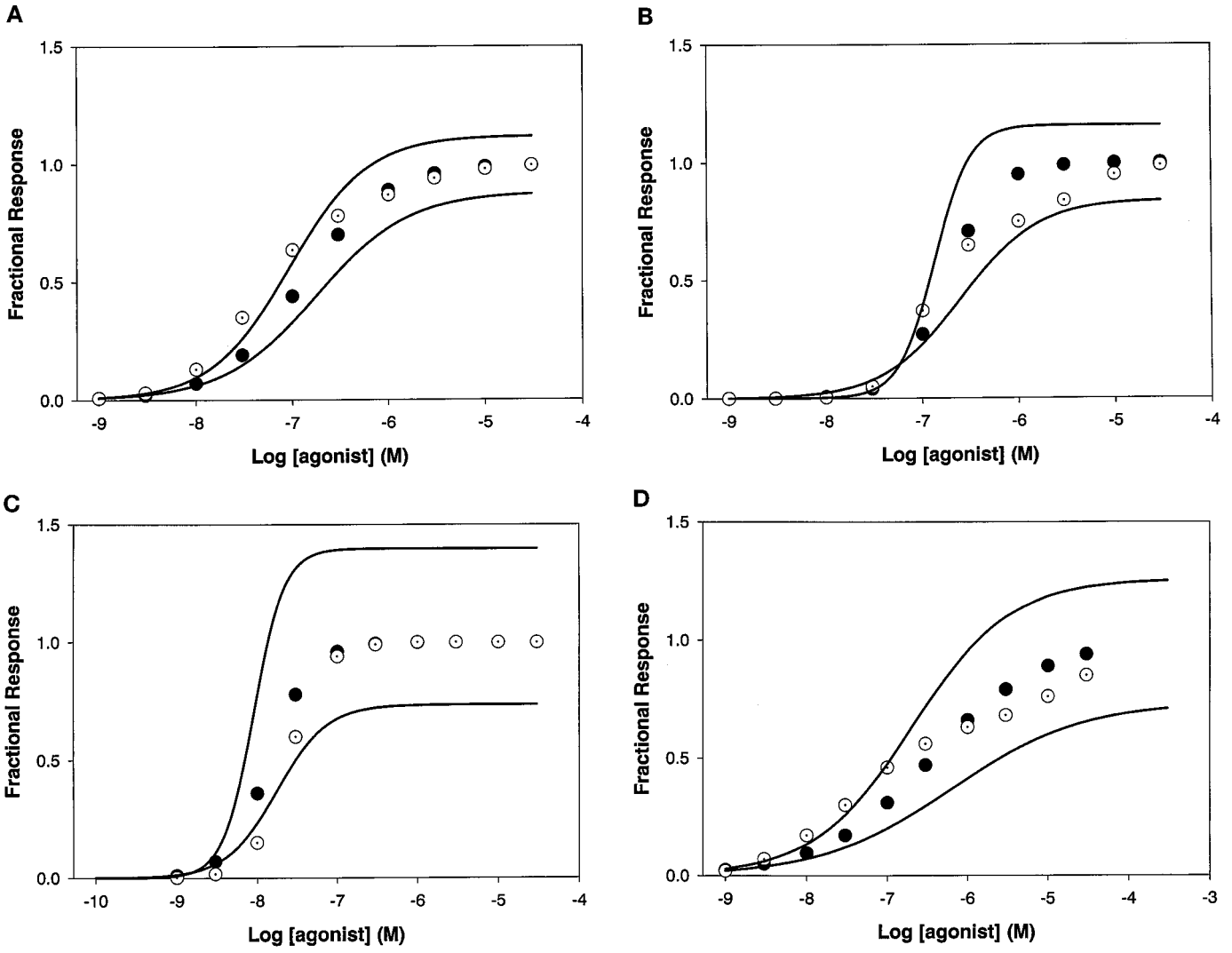

Fig. 3. Comparison of observed CRCs elicited by a (PE:ET-1) 80:20 FMR CRC (filled circle) with its corresponding 95% confidence intervals (solid lines) and the CRC predicted by the Pöch and Holzmann (1980) method for simple additivity of agonist effects (dotted circle). Each point on the FMR curve represents the fractional contractile response observed at each concentration, as calculated from the mean logistic parameter estimates listed in table 2 for each vascular tissue. The corresponding 95% C.I. was constructed by similarly calculating the fractional contractile response for each vascular tissue using mean logistic parameter estimates that were ± 2 S.E. from the mean values for each tissue as listed in table 2. The Pöch and Holzman CRC was constructed as described in "Materials and Methods," using the mean logistic parameter estimates for the ET-1 CRC as listed in table 1, and the mean logistic parameter estimates for the PE CRC as listed in table 2. For a, aorta; b, mesenteric artery; c, femoral artery; d, corporal tissue. Note that for the most part, the Pöch and Holzmann predictions fall within the boundaries defined by the 95% confidence interval for FMR CRCs in all the vascular tissues; indicating that the coadministration of PE and ET-1 results in contractile responses that are reasonably well predicted by simple additivity.

icantly to the left of the PE CRC, and with the exception of the femoral artery, the calculated E_{max} value for PE-induced contractions was significantly greater than that for ET-1-induced contractions (table 1).

Having established the characteristics of the PE- and ET-1-induced steady-state CRCs in each preparation, a previously described FMR protocol was used to study steady-state contractile responses elicited by a single mixture of these two compounds [*i.e.,* the 80:20 (PE:ET-1 FMR); see "Materials and Methods" for details]. In short, construction of CRCs to PE alone, as well as mixtures of PE and ET-1, on the same tissue from the same animal, revealed an apparently vessel-dependent 3- to 22-fold leftward shift of the EC_{50}; with no detectable effect on E_{max} or slope factor (fig. 2; table 2).

To evaluate the nature of the leftward shift in the FMR CRC for each vascular tissue type, the 80:20 FMR CRC was compared to the CRC for simple additivity of agonist effects, as constructed using the Poch and Holzmann (1980) method of equiactive substitution (fig. 3). Even though the average point estimate for the Pöch and Holzmann CRC did not necessarily directly coincide with the corresponding mean value for the 80:20 FMR, for the most part, the expected CRC for simple additivity of agonist effects (fig. 3) did fall within the 95% C.I. for the 80:20 FMR CRC on each preparation. As might be expected, the discrepancies between the 95% C.I. for the 80:20 FMR and the Poch and Holzmann CRC mostly occurred on the linear portion of the FMR CRC (see fig. 3a, c and d). The deviation of the Pöch and Holzmann CRC from the 95% C.I. for the 80:20 FMR was more frequently under-additive (fig. 3a and d) than over-additive (fig. 3c). Thus, overall it appears that the steady-state contractile response to mixtures of PE and ET-1 in diverse rabbit vasculature is

reasonably well codified by the Pöch and Holzmann (1980) method of equiactive substitution.

Certainly PE and ET-1 can each elicit contractile responses after activation of more than one alpha-1 adrenergic ($\alpha_{1a,\ b}$) (Pepperl and Regan, 1994) or ET ($ET_{A/B}$) (Seo and Luscher, 1995; Hay *et al.*, 1996; Moreland *et al.*, 1994; Ladouceur *et al.*, 1993) receptor subtype. Despite this fact, no attempt was made in these initial studies to delineate either the particular receptor subtype(s) present or their relative contributions to the actions of PE and ET-1 in these vascular tissues. This seems reasonable given the fact that the pharmacological analysis applied here [*i.e.,* the Pöch and Holzmann (1980) additivity analysis] is independent of such considerations, and moreover, that the focussed aim of these initial studies was solely to compare the "expected" effects of this drug mixture to the "observed" effects in these physiologically diverse vascular tissues.

The conclusions of this report are similar to that of a previous study in isolated human corporal tissue strips (Kim *et al.*, 1996). In that study, Kim *et al.*, (1996) reported that FMR CRCs to PE and ET-1 was also largely predicted by the Pöch and Holzmann (1980) method of equiactive substitution. As such, simple additivity of agonist effects seems to be characteristic of steady-state contractile responses to mixtures of PE and ET-1 in diverse rabbit vasculature, as well as the specialized vascular tissue of the human corpus cavernosum. However, even though the Pöch and Holzmann CRC was also sufficient to describe steady-state contractile responses elicited on rat aortic rings by a distinct drug mixture (*i.e.,* PE and 5-HT), it cannot be assumed that simple additivity reflects a general principle governing normal vascular pharmacology. As a case in point, previous studies conducted in rabbit aorta demonstrated that for mixtures of PE and 5-HT, the resulting FMR CRC was always more than additive on the linear portion of the CRC (Christ *et al.*, 1990). That is, in the same vascular tissue, the rabbit aorta, the location of the Pöch and Holzmann (1980) CRC indicates that the PE:ET-1 FMR CRC is largely additive or under-additive (see fig. 3), although the PE:5-HT FMR CRC is actually over-additive (Christ *et al.*, 1990).

Such observations clearly demonstrate that even when vasoactive agents act through the same putative effector pathways (*i.e.,* activation of the α_1-adrenergic (Timmermans and Thoolen, 1987), $ET_{A/B}$ (Goto *et al.*, 1989; Griendling *et al.*, 1989; Nambi *et al.*, 1995; Tamirisa *et al.*, 1997) and $5\text{-}HT_2$ (Feniuk and Humphrey, 1987) receptor subtypes are all thought to result in contractile responses that are mediated, in large part, by activation of the inositol trisphosphate, diacylglycerol and protein kinase C pathway, the resultant contractile responses are still not necessarily predictable. These results are not surprising when one considers the manifold events that occur between receptor activation and tension development in isolated vascular tissues. In fact, although signal transduction pathways for activation of distinct membrane receptors may have considerable overlap [*i.e.,* PE and 5-HT (Christ *et al.*, 1990)], they may not be identical. As such, it is conceivable that subtle dichotomies in the signal transduction pathways mediated by activation of distinct membrane receptors could well lead to "unexpected" contractile responses to one drug mixture (*i.e.,* PE and 5-HT in rabbit aorta), although simultaneously eliciting "expected," *i.e.*, additive responses to another drug mixture (*i.e.,* PE and ET-1 as reported in rabbit aorta) in the same vessel. For example, as described elsewhere (Kenakin, 1997; Kenakin and Morgan, 1989; Weiss *et al.*, 1996), differences in the observed contractile responses elicited by different drug mixtures might result when distinct membrane receptors activate either more than one G protein, or possibly, a slightly different complement of G proteins.

In fact, therein lies the main importance of the knowledge gained from the current studies. That is, application of the Poch and Holzmann (1980) method of equiactive substitution provides a critical conceptual framework for making comparisons between the "expected" and "observed" effects of mixtures of physiologically relevant agonists on the same or distinct vascular tissues. Clearly it remains to be determined what impact age or disease might have on the relationship between the "expected" and "observed" responses of these vascular tissues to PE and ET-1.

Undoubtedly, the *in vitro* experimental analysis reported here greatly oversimplifies the *in vivo* situation. Nonetheless, these initial observations do provide a general background for beginning to address such complex and physiologically relevant issues. Future studies in vasculature from aged or diseased animals is the next logical step in identifying the boundary conditions for the relevance of this type of analysis to the understanding of normal vascular physiology *in vivo*, as well as perhaps identifying some mechanistic aspects of vascular disease.

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