Br. J. Pharmacol. (1993), **110**, 783–789

Characterization of endothelin receptors mediating the effects of the endothelin/sarafotoxin peptides on autonomic neurotransmission in the rat vas deferens and guinea-pig ileum

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1 To characterize the receptors mediating the effects of the endothelin/sarafotoxin family of peptides on the responses to electrical stimulation of the rat vas deferens (RVD) and guinea-pig ileum (GPI) we have used endothelin-1 (ET-1), ET-3, sarafotoxin 6b (SX6b) and SX6c as agonists and the endothelin-receptor antagonists BQ-123 (ET_A receptor selective) and PD 142893 (non-selective).

2 In the RVD, ET-1 and SX6b increased the twitches induced by field stimulation starting at a threshold concentration of 10^{-10} M while the threshold concentration for ET-3 was 3×10^{-9} M. SX6c (up to 3×10^{-8} M) did not potentiate the twitches. SX6b produced significantly ($P<0.05$) greater potentiations than ET-1 at concentrations of 3×10^{-9} M and higher, and 10^{-7} M ET-3 also produced a significantly greater effect than ET-1 at the same concentration. Thus, at threshold the rank order of peptides was ET-1 = SX6b > ET-3 >>> SX6c, and at concentrations of 3×10^{-8} M and higher, SX6b > ET-3 > ET-1 >>> SX6c.

3 In the presence of BQ-123 or PD 142893 (10^{-5} M) the threshold concentrations for ET-1 to augment the twitches were increased 30 fold. In the same conditions neither SX6b nor ET-3 potentiated the responses. The relative activities of the endothelin/sarafotoxin peptides and the effectiveness of the endothelin receptor antagonists are consistent with postjunctional ET_A receptors mediating these effects.

4 In the transmurally stimulated GPI the endothelin/sarafotoxin peptides produced two effects; an increase in the basal tension of the tissues and an inhibition of the twitch responses. To increase the basal tension the peptides had the order of potency ET-1 > SX6b >> ET-3 = SX6c. These direct effects of ET-1 or SX6b were strongly antagonized (100 fold) by either BQ-123 (10^{-5} M) or PD 142893 (10^{-5} M). Thus, ET_A receptors mediate contractions of the GPI induced by these peptides.

5 The endothelin/sarafotoxin peptides were approximately equipotent at depressing twitches of the GPI in response to transmural stimulation (EC_{50}s, 4×10^{-11} to 1.5×10^{-10} M). The depressions induced by ET-1 were unaffected by either BQ-123 (10^{-5} M) or PD 142893 (10^{-5} M). BQ-123 produced a small (three fold) antagonism of the inhibitory effects of ET-3 or SX6c. These results indicate that a receptor of the ET_B type mediates the inhibitory effects of the endothelin/sarafotoxin peptides on neurotransmission in the GPI.

6 Thus, both ET_A receptors and ET_B receptors mediate the effects of the endothelin/sarafotoxin peptides on neurotransmission.

Keywords: endothelin-1; endothelin receptors; endothelium-dependent relaxations

Introduction

The endothelin/sarafotoxin peptides potently affect responses to autonomic nerve stimulation. For instance, endothelin-1 (ET-1) inhibits the release of noradrenaline (Wiklund *et al.*, 1988; Tabuchi *et al.*, 1989) or acetylcholine (Wiklund *et al.*, 1989; Hiley *et al.*, 1989) from sympathetic or parasympathetic nerve endings. ET-1 also potentiates contractions through a postjuctional effect, as in the vas deferens of the rat (Hiley *et al.*, 1989; Maggi *et al.*, 1989; Wiklund *et al.*, 1990) or mouse (Rae & Calixto, 1990). The endothelin receptors mediating these effects have not been characterized although two receptors for the endothelin/sarafotoxin peptides have been identified. The ET_A receptor, is more selective for ET-1 or SX6b whereas the other, the ET_B receptor, is isopeptide non-selective (Ambar *et al.*, 1989; Arai *et al.*, 1990; Sakurai *et al.*, 1990; Saeki *et al.*, 1991; Williams *et al.*, 1991; Clozel *et al.*, 1992). BQ-123 (cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-) is a selective ET_A receptor antagonist (Ihara *et al.*, 1992) and PD 142893 (Ac-(3,3-D-diphenylalanine)-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp) has been shown to be a non-selective ET_A/ET_B receptor antagonist (Cody *et al.*, 1992), although we have found it to have little activity against ET_B receptors except for those present on endothelial cells (Warner *et al.*, 1993). Using these antagonists and ET-1, ET-3, sarafotoxin 6b (SX6b) and SX6c as agonists we have characterized the receptors that mediate the effects of the endothelin/sarafotoxin peptides on autonomic neurotransmission in the guinea-pig ileum and rat vas deferens.

Some of these data were presented to the British Pharmacological Society (Allcock *et al.*, 1993).

Methods

Organ bath experiments

Male Wistar rats (250–400 g) or Dunkin-Hartley guinea-pigs (350–450 g) were killed with thiopentone sodium (Sagatal, 120 mg^{-1} kg^{-1} i.p. or i.v.). Rat vas deferens (RVD), *pars prostatica*, or sections of guinea-pig ileum (GPI) were mounted in isolated organ baths containing 10 ml of Krebs buffer under resting tensions of 0.75–1 g for the isometric measurement of contractions. The bathing Krebs solution contained bacitracin (3 mg l^{-1}), bovine serum albumin (50 mg l^{-1}), indomethacin (5×10^{-6} M), thiorphan (10^{-6} M), captopril (10^{-6} M) and bestatin (10^{-6} M) (Maggi *et al.*, 1989)

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to protect the peptides and was gassed with 95% O_2:5% CO_2 at a temperature of 37 °C. After an equilibration period of 60–90 min the tissues were electrically stimulated (ES) (Grass S48 Stimulator connected to a Med-Lab Stimu-Splitter II) via platinum electrodes positioned for transmural stimulation of the GPI (0.1 Hz, 0.5 ms, 80% of current for maximum response) or field stimulation of the RVD (0.1 Hz, 1 ms duration, 0.3 mA). When stable reproducible contractions of the tissues were obtained (approx. 10 min), BQ-123 (10^{-5} M), PD 142893 (10^{-5} M) or vehicle were added before the addition of cumulative concentrations of ET-1, ET-3, SX6b or SX6c (10^{-11} to 3×10^{-7} M). Changes in twitch tension were calculated as a percentage of the initial stable levels. Each tissue was used for only one curve.

Materials

The Krebs buffer used for the GPI had the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 1.17, $CaCl_2.6H_2O$ 2.5, $NaHCO_3$ 25, glucose 5.6. Krebs buffer without $MgSO_4.7H_2O$ was used for the RVD. BQ-123 and PD 142893 were synthesized by Parke-Davis, MI, U.S.A. ET-1, ET-3, SX6b and SX6c were purchased from Peptide Institute (Osaka, Japan). BQ-123 and the endothelin/sarafotoxin peptides were reconstituted in 0.9% w/v saline containing 1% w/v bovine serum albumin and 10 mM sodium bicarbonate. PD 142893 was reconstituted in dimethylsulphoxide (10^{-3} M) and added directly to the organ baths. All other compounds were obtained from Sigma Chemical Co. (Poole, Dorset) and dissolved in either distilled water or saline.

Statistics

Statistical differences between curves were determined by two-way analysis of variance and a $P<0.01$ was taken as significant. Statistical differences between points were determined by unpaired two-tailed Student's t test, and between points and basal by a one sample test. For both tests a $P<0.05$ was taken as significant.

Results

Rat vas deferens

ET-1 (10^{-11} to 3×10^{-7} M) potentiated twitches of the RVD induced by nerve stimulation in a concentration-dependent manner (threshold of 10^{-10} M, Figure 1). ET-3 also potentiated the responses (Figures 1 and 2) but with a higher threshold concentration (3×10^{-9} M). SX6b had a similar threshold to ET-1 but at concentrations of 3×10^{-9} M or greater was significantly ($P<0.05$) more active than ET-1 (Figure 1). The concentration-response curve for ET-3 also crossed that for ET-1 and at a concentration of 10^{-7} M ET-3 produced a greater increase in twitch size than did ET-1 at the same concentration ($P<0.05$). SX6c at concentrations of up to 3×10^{-8} M was without effect. Thus, at threshold the rank order of peptides was ET-1 = SX6b > ET-3 >>> SX6c, and at concentrations of 3×10^{-8} M and higher, SX6b > ET-3 > ET-1 >>> SX6c. The endothelin/sarafotoxin peptides were without significant effect on the basal tone of the RVD.

In the presence of BQ-123 (10^{-5} M) or PD 142893 (10^{-5} M) SX6b (Figure 3) and ET-3 (Figure 2) no longer potentiated the responses to nerve stimulation. In the presence of either antagonist the concentration-response curve for ET-1 was shifted to the same degree such that the new thresholds were both approx. 3×10^{-9} M (Figure 4). In addition, in the presence of PD 142893, but not BQ-123, the responses to concentrations of ET-1 of 3×10^{-8} M and 10^{-7} M were significantly greater than control ($P<0.05$, Figure 4).

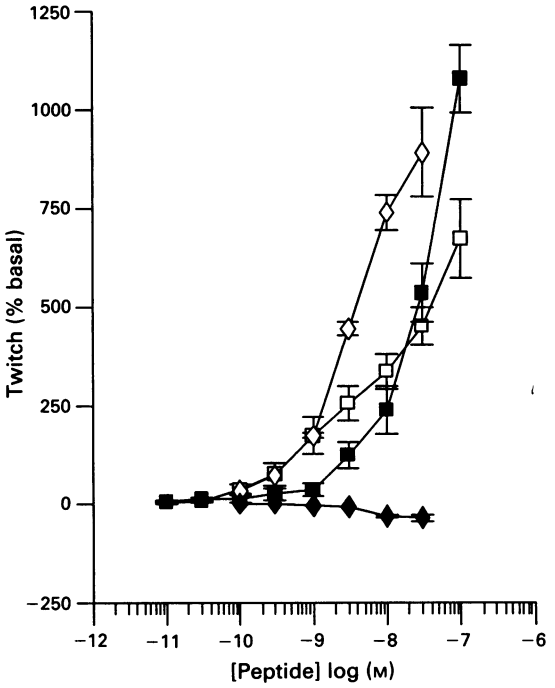

Figure 1 Comparison of the potentiating effects of the endothelin/sarafotoxin peptides on the twitch responses of the rat vas deferens to electrical field stimulation. Endothelin-1 (ET-1; □); ET-3, (■); sarafotoxin 6b (SX6b; ◇); SX6c (◆). Results are calculated as the mean (± s.e. mean, vertical bars) ($n \geqslant 6$).

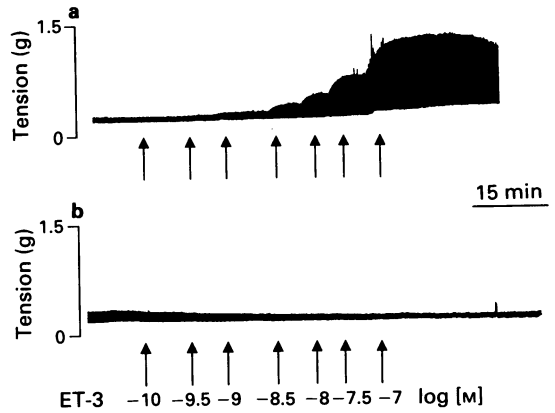

Figure 2 Effect of BQ-123 on the potentiation of twitch response in the rat vas deferens (RVD) induced by endothelin-3 (ET-3). (a) Cumulative concentrations of ET-3 (10^{-10} to 10^{-7} M) produced concentration-dependent increases in the twitch responses of the RVD in response to field stimulation. (b) In the presence of BQ-123 (10^{-5} M), ET-3 (up to 10^{-7} M) failed to potentiate the twitch responses of the RVD. Traces are from original experiments and are typical of at least 5 other preparations.

Guinea-pig ileum

ET-1, ET-3, SX6b and SX6c were equipotent in inhibiting twitches of the GPI in response to transmural stimulation (EC_{50}s 2×10^{-10}, 1.5×10^{-10}, 8×10^{-11}, 1.2×10^{-11} M, respectively, Figures 5, 6a and b). In addition, ET-1 and SX6b, but to a much lesser extent ET-3 and SX6c, increased the basal tension of the GPI (Figure 7). The increases induced by ET-1 (Figure 8) or SX6b (data not shown) were strongly antagonized by either BQ-123 or PD 142893. For instance, the EC_{50} for ET-1 increased from 10^{-9} M in control to 4×10^{-8} M in the presence of BQ-123 (10^{-5} M) or PD 142893 (10^{-5} M).

In the presence of PD 142893 (10^{-5} M) the inhibitory effects of all the endothelin/sarafotoxin peptides were unaffected (e.g. Figure 9a, $P>0.01$, $n=3-7$ for each). BQ-123 (10^{-1} M) caused small, but significant ($P<0.01$) shifts in the concentration-response curves for ET-3 (Figure 9b) and SX6c (Figure 9c).

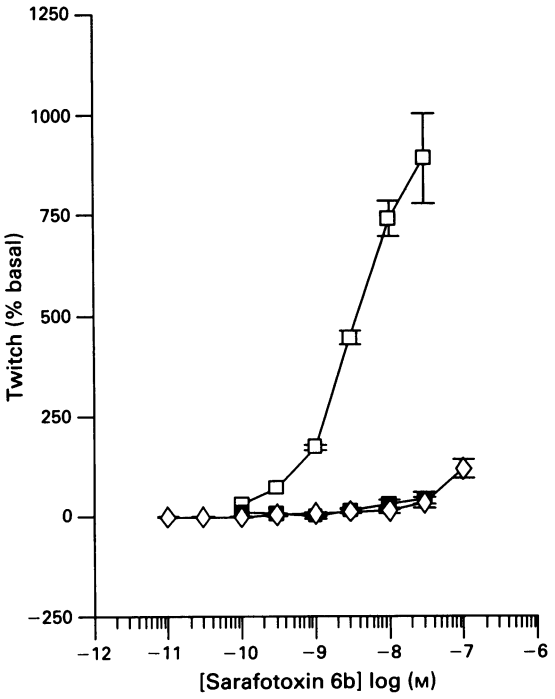

Figure 3 Effects of BQ-123 and PD 142893 on the potentiating effects of sarafotoxin 6b on the twitch responses of the rat vas deferens. Control, (□); +BQ-123 (10^{-5} M), (■); +PD 142893 (10^{-5} M), (◇). Results are calculated as the mean (± s.e. mean, vertical bars) ($n \geq 6$).

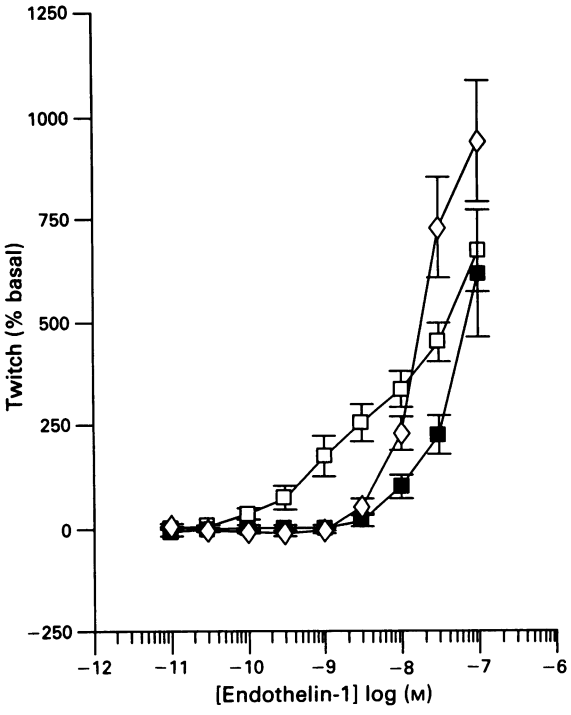

Figure 4 Effects of BQ-123 and PD 142893 on the potentiating effects of endothelin-1 on the twitch responses of the rat vas deferens Control, (□); +BQ-123 (10^{-5} M), (■); +PD 142893 (10^{-5} M), (◇). Results are calculated as the mean (± s.e. mean, vertical bars)($n \geq 6$).

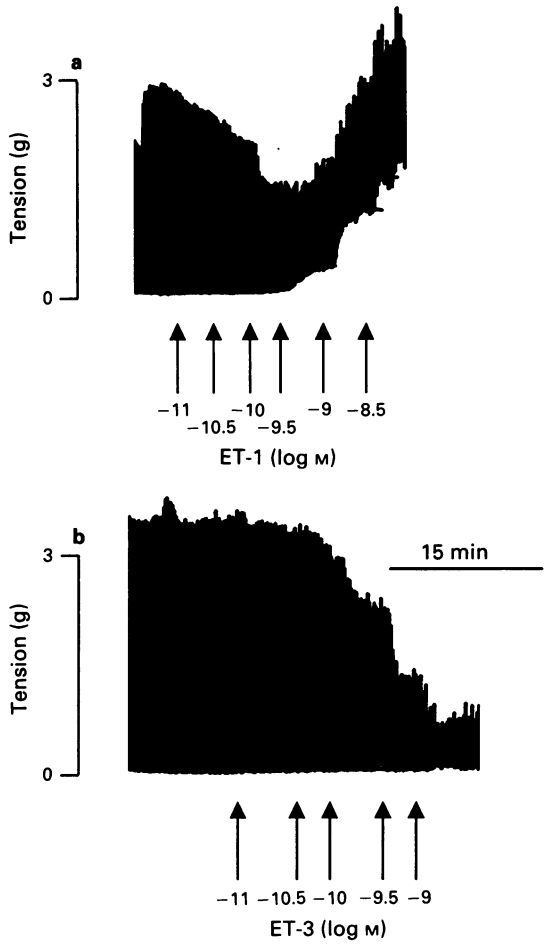

Figure 5 Effects of endothelin-1 (ET-1) and ET-3 on the responses of the transmurally stimulated guinea-pig ileum (GPI). (a) In the GPI cumulative concentrations of ET-1 (10^{-11} to 3×10^{-9} M) caused concentration-dependent reductions in twitch size and an elevation in the basal tone of the tissue. (b) In similar experiments ET-3 (10^{-11} to 10^{-9} M) caused only reductions in twitch size. Traces are from original experiments and are typical of 3-8 other preparations.

Discussion

We have compared the abilities of BQ-123 and PD 142893 to antagonize the effects of the endothelin/sarafotoxin peptides on autonomic nerve transmission in the RVD and GPI. Our data show that different endothelin-receptors mediate the effects of the endothelin/sarafotoxin peptides in these two preparations.

In the RVD the relative potencies of the peptides, SX6b $\geq$ ET-1 > ET-3, and the lack of effect of SX6c correspond with an action on the ET_A receptor. The strong antagonism by BQ-123 or PD 142893 of the potentiating effects of SX6b, ET-3 or ET-1 gives extra weight to this conclusion. This receptor would correlate with that present, for instance, in the rat thoracic aorta, on which ET-3 is less than one tenth as potent as ET-1, SX6c is inactive (Maggi *et al.*, 1989; Panek *et al.*, 1992; Warner *et al.*, 1993), and BQ-123 strongly antagonizes the effects of ET-1 (Sumner *et al.*, 1992). This conclusion is at odds with that of Télémaque & D'Orléans-Juste (1991), who suggested that ET_B receptors mediate the potentiation of twitches in the RVD. However, the observation that SX6c is without effect clearly points to mediation by non-ET_B receptors. On the other hand the relative resistance of the ET-1 response to BQ-123 and PD142893, and the closeness of the ET-1 and ET-3 concentration-response curves may suggest that this non-ET_B receptor is not identical to the ET_A receptor present on the

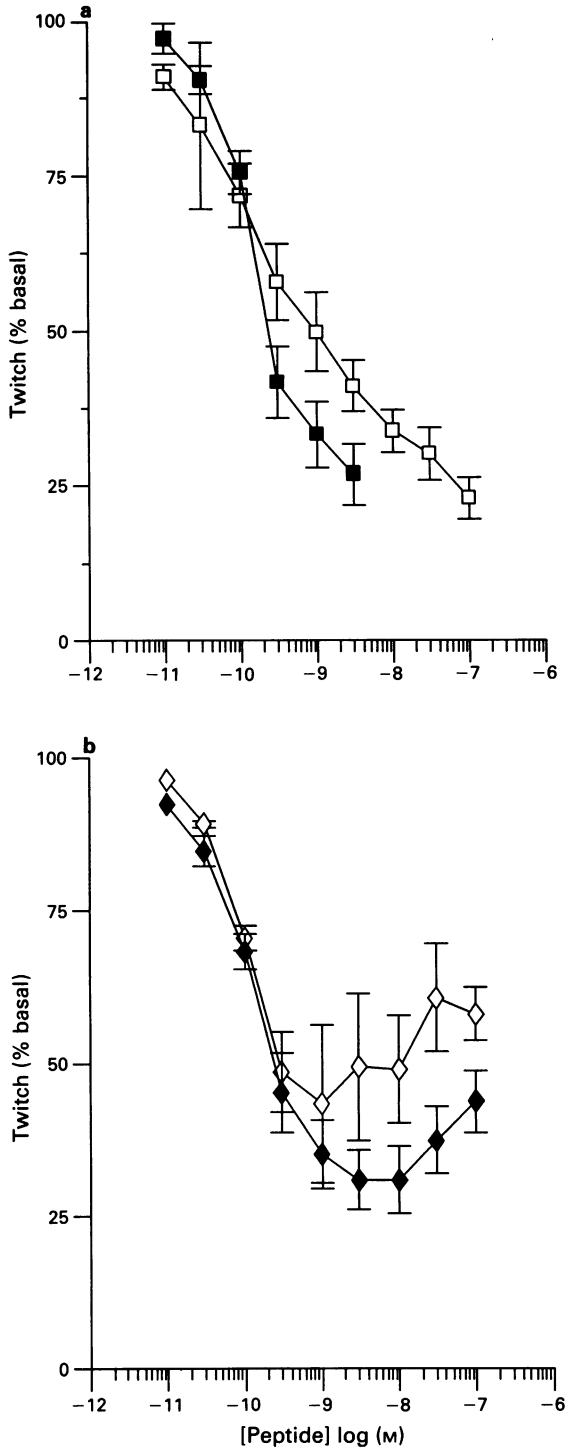

Figure 6 Comparison of the inhibitory effects of the endothelin/sarafotoxin peptides on the twitch responses of the guinea-pig ileum (GPI) to electrical field stimulation. (a) Comparison of the inhibitory effects of endothelin (ET-1) and ET-3 on the twitch responses of the GPI to electrical field stimulation. ET-1, (□); ET-3, (■). (b) Comparison of the inhibitory effects of sarafotoxin 6b (SX6b) and SX6c in the same tissue. SX6b, (◇); SX6c, (◆). Results are calculated as the mean (± s.e. mean, vertical bars) ($n = 3-9$).

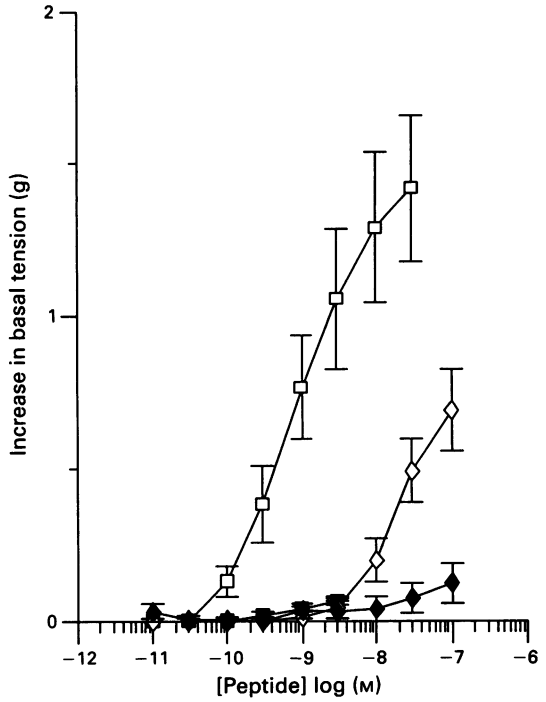

Figure 7 Comparison of the increases in basal tone of the guinea-pig ileum induced by the endothelin/sarafotoxin peptides. Endothelin-1 (ET-1; □); ET-3, (■); sarafotoxin 6b (SX6b; ◇); SX6c, (◆). Results are calculated as the mean (± s.e. mean, vertical bars) ($n \geqslant 6$).

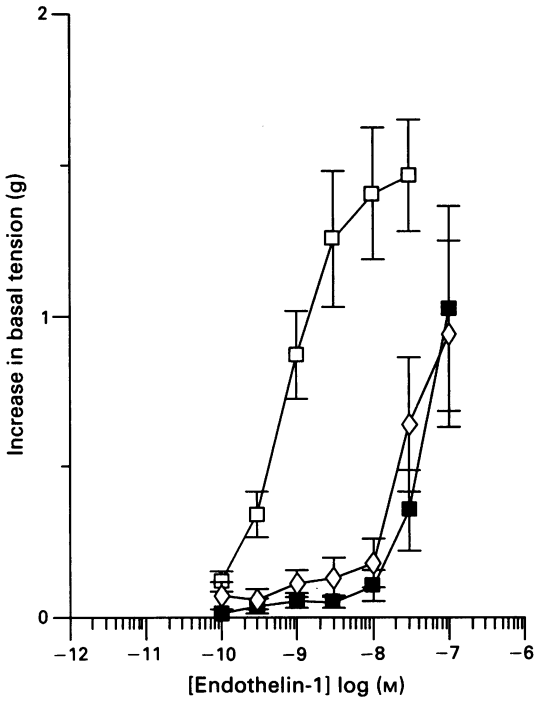

Figure 8 Effects of BQ-123 and PD 142893 on elevations of the basal tone of the guinea-pig ileum induced by endothelin-1 (ET-1). Control, (□); +BQ-123 (10^{-5} M), (■); +PD 142893 (10^{-5} M), (◇). Results are calculated as the mean (± s.e. mean, vertical bars) ($n \geqslant 6$).

rat thoracic aorta. We also found that at the highest concentrations used ET-3 produced a greater potentiation in the twitch response than ET-1 (Télémaque & D'Orléans-Juste, 1991). One explanation for this lack of parallelism between the concentration-response curves for ET-1 and ET-3/SX6b is provided by the presence of endothelin receptors both pre- and postsynaptically in the RVD. Activation of the presynaptic receptors inhibits noradrenaline release, whereas the postsynaptic ones mediate an increased responsiveness to nerve stimulation (Wiklund *et al.*, 1991). Thus, at low concentrations ET-1 potentiates transmission by a postsynaptic effect that becomes limited at higher concentrations due to presynaptic inhibition. The net result is a flattened ET-1 concentration-response curve. The subtype of this presynap-

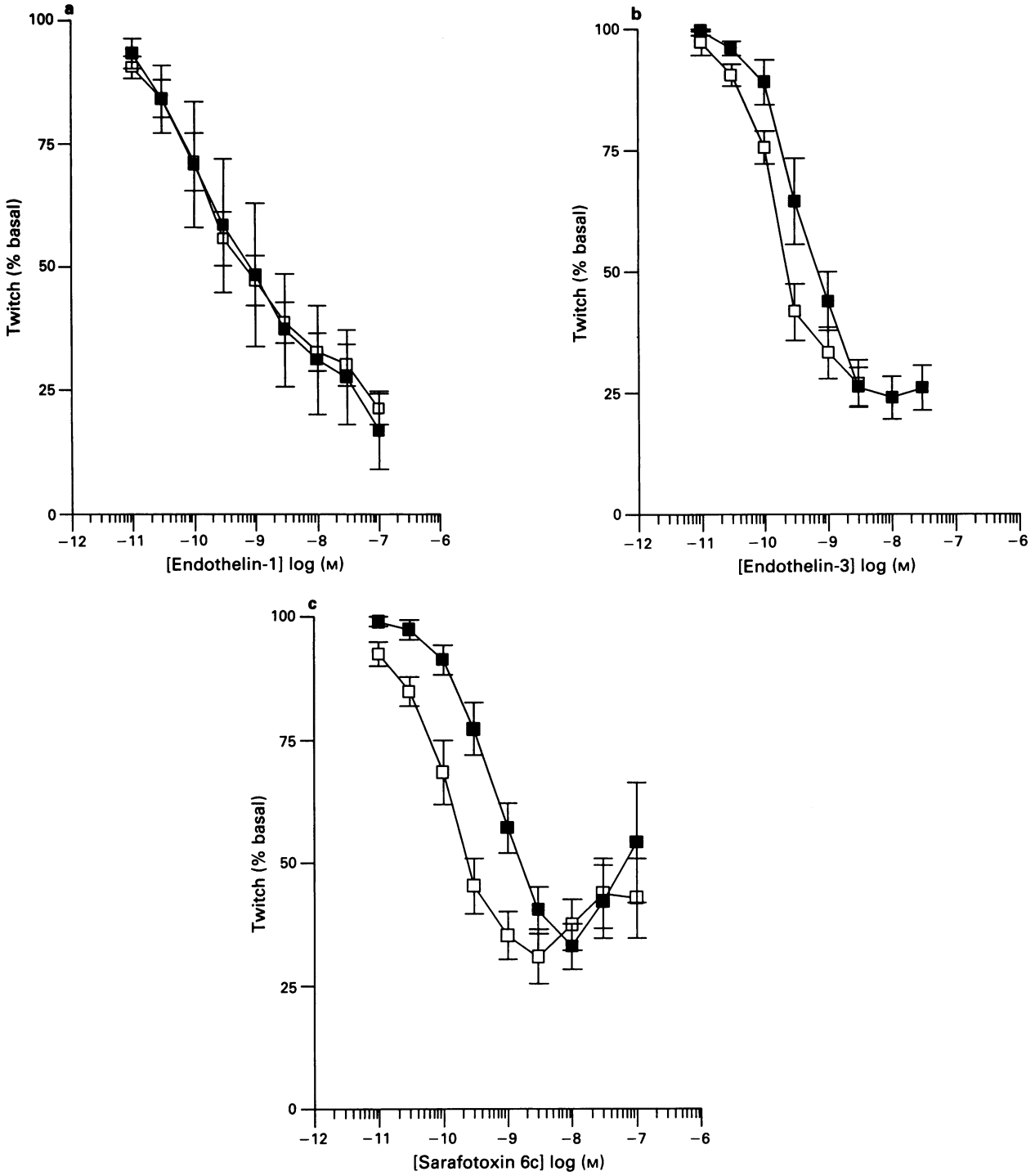

Figure 9 Effect of BQ-123 or PD 142893 on the inhibitory effects of the endothelin/sarafotoxin peptides on the twitch responses of the guinea-pig ileum to electrical field stimulation. (a) Endothelin-1 (ET-1): control, (□); +PD 142893 (10^{-5} M), (■). (b) ET-3: control, (□), + BQ-123 (10^{-5} M) (■). (c) Sarafotoxin 6c: control, (□); + BQ-123 (10^{-5}), (■) Results are calculated as the mean (± s.e. mean, vertical bars) ($n \geqslant 6$).

tic receptor is unclear, but may most closely resemble the ET_A receptor. For instance, ET-1 in relatively high concentrations, reduces transmitter release, whereas ET-3 does not (Wiklund *et al.*, 1991). On the other hand PD 142893, but not BQ-123 appears to antagonize the effects of ET-1 on this receptor, producing a potentiation of the responses to higher concentrations of ET-1. In addition, SX6b appears to be without effect, for its concentration-response curve is parallel to that of ET-3. Thus, this receptor may represent a subtype of the ET_A receptor. An alternative explanation of the non-parallelism between the concentration-response curves for ET-1 and ET-3/SX6b may be that different receptors mediate the effects of the former and latter peptides. The receptor mediating the effects of ET-3/SX6b may also be more sensitive to antagonism by BQ-123 and PD 142893, explaining why the two antagonists were less effective against ET-1. However, this model does not include a role for those receptors that influence neurotransmitter release presynaptically (Wiklund *et al.*, 1991). The true reasons for these disparities in the endothelin/sarafotoxin peptide con-centration-response curves may finally be provided by experiments examining the effects of a full range of endothelin/sarafotoxin receptor agonists and antagonists on neurotransmitter release in conjunction with studies of mechanical responsiveness. However,

the results presented here suggest that multiple receptor types may be involved in mediating the effects of the endothelin/sarafotoxin peptides within this tissue.

In the GPI the four endothelin/sarafotoxin peptides were approximately equipotent at inhibiting the twitches induced by field stimulation, as has been partly demonstrated before (Maggi *et al.*, 1989; Guimarães & Rae, 1992) whereas ET-1 and SX6b were considerably more active than ET-3 or SX6c in directly contracting the preparations. Coupled together with our observations that both BQ-123 and PD 142893 strongly antagonized the contractions induced by the endothelin/sarafotoxin peptides, as is the case in the rat thoracic aorta (Warner *et al.*, 1993), this clearly suggests that this latter effect is mediated by ET_A receptors. Conversely, the inhibitory effects of ET-1 on twitches were not influenced by either antagonist. Taken together with the similar potencies of the endothelin/sarafotoxin peptides this suggests mediation by ET_B receptors, but different from those present on the endothelium, which are antagonized by PD 142893 (Warner *et al.*, 1993). However, we did observe that BQ-123 caused small, approximately three fold rightward shifts in the inhibitory concentration-response curves to ET-3 or SX6c. Thus, there may possibly be an involvement of a receptor mediating these responses that is not identical to that ET_B receptor present, for instance, on the guinea-pig bronchus (Hay, 1992) or rabbit jugular vein (Sumner *et al.*, 1992) which is insensitive to the effects of BQ-123.

Thus, at least two receptor populations must be present in the GPI, one mediating the neuromodulatory effects of the endothelin/sarafotoxin peptides and one their direct effects on the intestinal smooth muscle. Interestingly, binding studies (Wollberg *et al.*, 1991) have indicated only one population of non-discriminating, i.e. ET_B receptors, that presumably represents those mediating the effects of the endothelin/sarafotoxin peptides on neurotransmission. Possibly, therefore, the ET-1/SX6b-selective receptors represent only a fraction of those present within the tissue. However, as ET-1 was approximately 10 fold more potent at inhibiting the twitch response than increasing the basal tone, and ET-3, which is abundantly expressed in the intestine (Matsumoto *et al.*, 1989), selectively inhibited the twitch responses, the non-selective ET receptors mediating the neuromodulatory effects of the endothelins are likely to be the more important in regard to ileum motility.

Thus, the effects of the endothelins on neurotransmission are mediated by both pre- and postsynaptic receptors. In the RVD and GPI the postsynaptic ET receptor appears to be an ET_A receptor, which produces potentiation of the twitch response of the RVD and an increase in the basal tone of the GPI. Activation of the presynaptic receptors leads to a reduction in transmitter release (Wiklund *et al.*, 1991). In the RVD this presynaptic receptor is acted upon by ET-1, but apparently not by ET-3. However, it appears to be antagonised by PD 142893. In the GPI the presynaptic receptor is more clearly of the ET_B subtype. However, the complexity of the nervous system in this latter preparation may be the explanation for the presence of additional receptors, as indicated by the effects of BQ-123 on the responses to ET-3 and SX6c.

This work was supported by the Parke-Davis Pharmaceutical Research Division of Warner-Lambert Co. We thank Drs A. Doherty and W. Cody of the Chemistry Department for providing the endothelin antagonists BQ-123 and PD 142893 (Ac-(3,3-d-diphenylalanyl)-L-Leu-L-Asp-L-Ile-L-Trp trifluoroacetate) for these studies.

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(Received April 2, 1993
Revised June 8, 1993
Accepted June 10, 1993)