Characterization of endothelin (ET) receptors in the isolated gall bladder of the guinea-pig: evidence for an additional ET receptor subtype

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1 We have characterized the receptors mediating contractions induced by endothelin-1 (ET-1), ET-2, ET-3 and the ET_B-selective receptor agonists, sarafotoxin 6c (SX6c), IRL 1620, BQ-3020, [Ala^{1,3,11,15}]ET-1 and ET (16-21) in strips of the isolated gall bladder of the guinea-pig (GPGB). We used as antagonists BQ-123 (ET_A receptor selective) and PD 145065 (ET_A/ET_B receptor non-selective).

2 ET-1, ET-2 and ET-3 (10^{-10} M to 3×10^{-7} M) caused similar slowly-developing concentration-dependent contractions of the GPGB. Contractile effects induced by ET-1, ET-2 or ET-3 (at 3×10^{-7} M) were also similar (230 ± 25 , 241 ± 7 and $287 \pm 37\%$ of that to histamine at 5×10^{-6} M, $n = 7, 6, 12$, respectively). However, the threshold concentration for ET-1 or ET-2 was 10^{-10} M whereas it was 3×10^{-9} M for ET-3.

3 SX6c (10^{-10} M to 3×10^{-7} M) also caused slowly-developing concentration-dependent contractions at a threshold concentration of 10^{-10} M ($n = 16$). However, the contraction caused by SX6c at 3×10^{-7} M was $116 \pm 9\%$ of that to histamine at 5×10^{-6} M, which was half of that induced by the same concentration of the ET isopeptides. The contraction induced by IRL 1620 at 3×10^{-7} M ($n = 9$) was $43 \pm 9\%$ of that to histamine at 5×10^{-6} M, which was one fifth of that produced by the same concentration of ET-1. Contractions induced by BQ-3020 or [Ala^{1,3,11,15}]ET-1 at 3×10^{-7} M were even less than those produced by IRL 1620. ET (16-21) was inactive up to 10^{-5} M. Addition of a concentration of 3×10^{-7} M of ET-1 to tissues with developed contractions induced by the bolus addition of 3×10^{-7} M SX6c caused a further contraction of the GPGB to the level observed with ET-1 alone at 3×10^{-7} M ($n = 8$).

4 BQ-123 (10^{-5} M) did not affect the concentration-response curve to ET-1 and the contraction induced by 3×10^{-7} M was also not affected ($n = 5$; $239 \pm 19\%$ of histamine at 5×10^{-6} M). PD 145065 (10^{-5} M) shifted the ET-1 concentration-response curve to the right and the contraction induced by ET-1 at 3×10^{-7} M was inhibited by 15% ($n = 6$; NS). A higher concentration of BQ-123 (10^{-4} M) caused a significant shift to the right of the ET-1 concentration-response curve similar to that caused by PD 145065 (10^{-5} M) and caused a 24% ($n = 6$; NS) inhibition of the contractions induced by ET-1 at 3×10^{-7} M. PD 145065 (10^{-4} M) abolished contractions induced by ET-1 (up to 10^{-7} M) and inhibited the response to ET-1 at 3×10^{-7} M by 52% ($n = 4$; $P < 0.05$).

5 Contractions induced by ET-3 were more sensitive to inhibition by the antagonists. BQ-123 (10^{-6} , 10^{-5} or 10^{-4} M) inhibited responses to 3×10^{-7} M ET-3 by 66, 71 and 83%, respectively ($n = 5, 5, 3$; $P < 0.05$). PD 145065 (10^{-6} , 10^{-5} or 10^{-4} M) attenuated more strongly than did BQ-123 the contractions induced by ET-3. For instance, the contractions caused by ET-3 at 3×10^{-7} M were decreased by 73 and 80% ($n = 5, 5$; $P < 0.05$) in the presence of PD 145065 (10^{-6} or 10^{-5} M, respectively). PD 145065 (10^{-4} M) completely abolished contractions to ET-3 ($n = 4$; up to 3×10^{-7} M).

6 Contractions induced by SX6c, especially those observed at concentrations lower than 10^{-8} M, were attenuated by BQ-123 (up to 10^{-4} M). PD 145065 (10^{-5} M) shifted to the right the concentration-response curve to SX6c and inhibited by 38% ($P < 0.05$) the contractions induced by 3×10^{-7} M. However, the contractions induced by a bolus addition of a high concentration of SX6c (3×10^{-7} M) and the subsequent addition of an identical concentration of ET-1 on top of SX6c were not affected by BQ-123 (10^{-6} or 10^{-5} M).

7 These results suggest that ET_B receptors are involved in the contractions induced by endothelins in the GPGB. However, SX6c and other selective ET_B agonists produced only half or less than half of the contractile response induced by non-selective agonists. In addition, the responses to ET-1 but not to ET-3, were insensitive to the antagonist action of BQ-123 at 10^{-5} M whereas BQ-123 or PD 145065 at 10^{-5} M strongly antagonized contractions induced by ET-3. Finally, BQ-123 at 10^{-4} M inhibited contractions to ET-1 and SX6c. Thus, within the GPGB there may well be additional ET receptor(s) not conforming to the established ET_A/ET_B receptor subtype classification, as well as ET_B receptors.

Keywords: Endothelin; sarafotoxin; receptors; antagonists; gall bladder; guinea-pig

Introduction

Endothelin-1 (ET-1) is a 21 amino acid peptide, first isolated from the culture medium of aortic endothelial cells (Yana-

gisawa *et al.*, 1988). ET-1 and the related peptides ET-2, ET-3 and the sarafotoxins (SX6a, SX6b, SX6c, SX6d) cause vasoconstriction, vasodilatation, mitogenesis and various renal, cardiac and endocrine effects (see Huggins *et al.*, 1993).

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ET/SX peptides are also potent contractors of non-vascular smooth muscle preparations such as airways (Battistini *et al.*, 1992; Hay *et al.*, 1993), the urinary bladder (Maggi *et al.*, 1989b; Saenz de Tejada *et al.*, 1992; Traish *et al.*, 1992), the uterus (Bousso-Mittler *et al.*, 1989; Svane *et al.*, 1993; Rae *et al.*, 1993), the stomach fundus (Wood *et al.*, 1992; Warner 1993a), the ileum (Maggi *et al.*, 1989a; Wollberg *et al.*, 1991; Guimaraes & Rae, 1992; Miasiro *et al.*, 1993; Warner *et al.*, 1993b), the colon (Lin & Lee, 1990; Takahashi *et al.*, 1990) and the gall bladder (Maggi *et al.*, 1990; Moummi *et al.*, 1992). To date, two distinct ET receptors (ET_A and ET_B; Sakurai *et al.*, 1992) have been cloned from mammalian species. A third ET receptor, ET_C, has been cloned from dermal melanophores of *Xenopus laevis* (Karne *et al.*, 1993). The ET_A receptor displays greater affinity for ET-1 than ET-3 whereas the ET_B receptor has equal affinities to the two peptides. The ET_C receptor displays greater affinity for ET-3 than ET-1. In non-vascular tissues, either ET_A or ET_B receptors may mediate contractions to the endothelins. For instance, ET_A receptors mediate contractions of the rat uterus (Rae *et al.*, 1993) and the human urinary bladder (Maggi *et al.*, 1990; Sakurai *et al.*, 1992) while ET_B receptors mediate contractions of the guinea-pig bronchus (Battistini *et al.*, 1994b) and the rat stomach fundus (Warner *et al.*, 1993a). Here, using ET-1, ET-2, ET-3 and the ET_B selective agonists sarafotoxin 6c (Williams *et al.*, 1991), IRL 1620 (Takai *et al.*, 1992; Watakabe *et al.*, 1992), BQ-3020 (Ihara *et al.*, 1992c), [Ala^{1,3,11,15}]-ET-1 (Saeki *et al.*, 1991) and ET (16-21) (Maggi *et al.*, 1989a) together with the antagonists, BQ-123 (ET_A receptor selective; Ihara *et al.*, 1992a,b) and PD 145065 (ET_A/ET_B receptor non-selective; Cody *et al.*, 1993), we have attempted to characterize the receptors mediating the contractions of the endothelins in the isolated gall bladder of the guinea-pig.

Methods

Experimental protocols

Male Hartley guinea-pigs (300–400 g) were killed by cervical dislocation. The gall bladder was rapidly excised, placed in warmed (37°C) and oxygenated (95% O₂:5% CO₂) Krebs-Henseleit buffer solution (composition, mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.18, NaHCO₃ 25, MgSO₄·7H₂O 1.18 and glucose 5.6, pH 7.4) and trimmed free of connective tissue and fat. Each gall bladder was opened along its longitudinal axis and two muscle strips prepared by cutting parallel to the long axis. The strips were mounted in 10 ml organ baths under tensions of 1 g. They were connected to Grass FT.03 force-displacement isometric transducers coupled to a Grass 79D pen recorder (Grass Instrument Co., Quincy, MA, U.S.A.). After equilibration for 60 min, tissues were contracted three times with 5×10^{-6} M histamine, with a 30 min washout period between each contraction. Tissues were then incubated with or without BQ-123 (10^{-5} or 10^{-4} M), PD 145065 (10^{-6} , 10^{-5} or 10^{-4} M) or vehicle for 20 min before addition of cumulative concentrations of the various agonists. The effects of each concentration of agonist were allowed to reach a plateau before addition of the next concentration. Only one cumulative concentration-response curve with or without antagonist was constructed with each preparation. Responses in the presence of antagonists and their respective vehicle controls were not compared in paired tissues. In a second set of experiments, one bolus addition of either ET-1, SX6c or SX6c followed by ET-1 at 3×10^{-7} M, with or without BQ-123 (10^{-6} or 10^{-5} M) given 20 min before, was given to equilibrated preparations. The responses in both sets of experiments are expressed as the percentage of the mean contraction evoked by histamine at 5×10^{-6} M.

Agonists and antagonists

Endothelin-1 (ET-1), ET (16-21) and IRL 1620 (Suc-[Glu⁹, Ala^{11,15}]-ET-1 (8-21)) were synthesized at the INRS-Santé

(Montréal, P.Q., Canada). Endothelin-3 (ET-3) and sarafotoxin 6c (SX6c) were obtained from Peptide Institute Inc. (Scientific Marketing Associates, Barnet, Herts.). BQ-3020 (as a non-acetylated analogue of [Ala^{11,15}]-ET-1 (6-21)) and [Ala^{1,3,11,15}]-ET-1 were obtained from Peninsula Laboratories Europe Ltd. (St. Helens, Merseyside). BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]), PD 145065 (Ac-D-Bhg-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp; Bhg = 5H-dibenzyl[a,d]cycloheptene-10, 11-dihydroglycine) and additional acetylated ET (16-21) were obtained as sodium salts from Parke-Davis Pharmaceuticals (Ann Arbor, MI, U.S.A.). All agonists and antagonists were dissolved in distilled water and stored at -20°C in aliquots of 10^{-3} to 5×10^{-5} M. Histamine hydrochloride was obtained from Sigma Chemical Co. (Poole, Dorset).

Statistical analysis

All values are expressed as mean $\pm$ s.e.mean. Statistical analysis of the data was performed by an unpaired, two-tailed, Mann-Whitney U test and $P < 0.05$ was considered significant.

Results

Effect of ET/SX peptides and other related analogues on the GPGB

ET-1, ET-2 and ET-3 caused slowly developing and long-lasting concentration-dependent contractions of the GPGB (Figure 1, 2). However, the threshold for ET-1 (10^{-10} M) was lower than that for ET-3 (3×10^{-9} M) (Figures 1, 2). SX6c or IRL 1620 also induced concentration-dependent contractions but caused only 50% and 19%, respectively, of the contractile responses induced by ET-1 at 3×10^{-7} M (Figures 1, 2).

Figure 1 Representative tracings of contractions of strips of the isolated gall bladder of the guinea-pig induced by endothelin-1 (ET-1, a), ET-3 (b) and sarafotoxin 6c (SX6c, c). The first arrow indicates the additions of histamine (5×10^{-6} M). The second arrow indicates addition of the first concentration (1×10^{-10} M) of the peptide followed by cumulative concentrations of 0.3, 1, 3, 10, 30, 100 and 300×10^{-9} M.

Figure 2 Contraction of strips of the isolated gall bladder of the guinea-pig induced by endothelin-1 (ET-1) (●), ET-2 (◆), ET-3 (▲), sarafotoxin 6c (SX6c) (□), IRL 1620 (◇), BQ-3020 (△), [Ala^{1,3,11,15}]ET-1 (▽) or ET (16-21) (▷); $n = 5-16$. Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are means with s.e.mean. * $P < 0.05$ between ET-1 and other agonists as compared by an unpaired, two-tailed, Mann-Whitney U test.

BQ-3020 and [Ala^{1,3,11,15}]ET-1 were much less active than SX6c and caused very weak contractions ($n = 14 \pm 3$ and $25 \pm 8\%$ of histamine at 5×10^{-6} M; Figure 2). ET (16-21) was without effect up to 10^{-5} M (Figure 2). Bolus addition of ET-1, ET-2, ET-3 or SX6c at (3×10^{-7} M) caused similar contractions to those following cumulative addition of agonist (data not shown; Figure 3). The contractions produced by the ET isopeptides were stable and long lasting while the contraction induced by SX6c (3×10^{-7} M) was not sustained. Addition of ET-1 (3×10^{-7} M) to tissues already precontracted by a bolus addition of SX6c (3×10^{-7} M) caused further contractions to the level observed with ET-1 alone at 3×10^{-7} M (Figure 3).

Effect of BQ-123 or PD 145065 on ET-1-induced contractions of the GPGB

BQ-123 (10^{-5} M) had no effect on contractions induced by ET-1 (Figure 4a) whereas BQ-123 (10^{-4} M) shifted the concentration-response curve 6 fold to the right and inhibited the contractions at 3×10^{-7} M by 24% (NS; Figure 4a). PD 145065 (10^{-5} M) caused a shift to the right of the ET-1 concentration-response curve and inhibited by 15% (NS) the contraction induced by ET-1 (3×10^{-7} M) (Figure 4b). PD 145065 (10^{-4} M) abolished contractions induced by ET-1 at concentrations $\leq 10^{-7}$ M and inhibited by 52% the response induced by ET-1 (3×10^{-7} M) ($P < 0.05$; Figure 4b). BQ-123 (10^{-6} M or 10^{-5} M) did not affect the contractions induced by a bolus addition of a high concentration of SX6c (3×10^{-7} M), nor the contractions caused by the subsequent addition of ET-1 (at 3×10^{-7} M; Figures 3, 5).

Effect of BQ-123 or PD 145065 on ET-3-induced contractions of the GPGB

BQ-123 (10^{-6} M, 10^{-5} M or 10^{-4} M) significantly inhibited contractions induced by ET-3 (Figure 6a). For instance, the

Figure 3 Representative tracings of contractions of strips of the isolated gall bladder of the guinea-pig induced by: a bolus addition of histamine (5×10^{-6} M); sarafotoxin 6c (SX6c) (3×10^{-7} M); ET-1 (3×10^{-7} M); SX6c + ET-1 (3×10^{-7} M); SX6c + ET-1 (3×10^{-7} M) in the presence of BQ-123 (10^{-6} M) and SX6c + ET-1 (3×10^{-7} M) in the presence of BQ-123 (10^{-5} M).

contractions induced by ET-3 (3×10^{-7} M) were reduced by 66, 71, and 83%, respectively ($P < 0.05$). Similarly, PD 145065 (10^{-6} M or 10^{-5} M) inhibited by 73 and 80%, respectively ($P < 0.05$), the contractions induced by ET-3 (3×10^{-7} M) (Figure 6b). PD 145065 (10^{-4} M) abolished contractions induced by ET-3 up to 3×10^{-7} M (Figure 6b).

Figure 4 Effects of (a) BQ-123 (10^{-5} M, ●; 10^{-4} M, ■) or (b) PD 145065 (10^{-5} M, ●; 10^{-4} M, ■) on contractions induced by endothelin-1 (ET-1, ○) of strips of the isolated gall bladder of the guinea-pig ($n = 4-7$). Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are the means with s.e.mean. * $P < 0.05$ as compared to ET-1 by an unpaired, two-tailed, Mann-Whitney U test.

Figure 5 Contractions of strips of the isolated gall bladder of the guinea-pig induced by bolus concentrations of sarafotoxin 6c (SX6c, $n = 14$), endothelin-1 (ET-1, $n = 5$) or SX6c + ET-1 ($n = 7$) at 3×10^{-7} M and the effects of BQ-123 (10^{-6} M or 10^{-5} M) on contractions induced by SX6c + ET-1 ($n = 6$). Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are means with s.e.mean. NS, non-significant, as compared to ET-1 or SX6c by an unpaired, two-tailed Mann-Whitney U test.

Effect of BQ-123 or PD 145065 on SX6c-induced contractions of the GPGB

BQ-123 (10^{-5} M or 10^{-4} M) attenuated contractions induced by low concentrations ($< 10^{-8}$ M) of SX6c in the isolated GPGB without affecting the responses induced by SX6c at concentrations $> 3 \times 10^{-8}$ M (Figure 7). PD 145065 (10^{-5} M)

concentrations $> 3 \times 10^{-8}$ M (Figure 7). PD 145065 (10^{-5} M) shifted the concentration-response curve to the right 11 fold and caused a 38% ($P < 0.05$) inhibition of the response to SX6c at 3×10^{-7} M (Figure 7).

The two antagonists used (up to 10^{-4} M) affected neither the basal tone of the GPGB, nor the contractions induced by histamine (5×10^{-6} M). The latter concentration of histamine induced an increase in tension of 1.58 ± 0.09 g ($n = 116$).

Discussion

The present study illustrates that all the members of the ET family of peptides potentially contract the GPGB as has been shown for ET-1 (Moummi *et al.*, 1992). ET-1, ET-2 or ET-3 produced clearly similar effects, although financial restraints prevented the construction of complete concentration-response curves. It suggests that these contractile effects are mediated by an ET_B receptor subtype. This conclusion may be supported by the observation that PD 145065, a mixed ET_A/ET_B receptor antagonist, inhibited contractions induced by either ET-1, ET-3 or SX6c whereas $10 \mu\text{M}$ BQ-123 which strongly inhibits contractions to ET-1 in the rat thoracic aorta (Sumner *et al.*, 1992), was inactive against ET-1 in the GPGB. However, this conclusion is not fully supported by our other data. Firstly, SX6c and the other ET_B selective agonists did not cause contractile responses as great as those produced by the non-selective ET receptor agonists. Secondly, addition of ET-1 on top of the constriction induced by SX6c further contracted the tissue, a response which was unaffected by BQ-123 (up to 10^{-5} M). Thirdly, both ET receptor antagonists were more active against ET-3 than against ET-1. Fourthly, ET-1 and ET-2 produced contractions at lower concentrations ($\leq 10^{-8}$ M) than did ET-3. These observations suggest mediation by an additional non- ET_B non- ET_A receptor.

Interestingly, 10^{-5} M of either BQ-123 or PD 145065 was more effective in antagonizing contractions induced by ET-3 than by ET-1. Similar differences in the effectiveness of antagonists against various agonists have been reported previously in experiments on the human umbilical artery (Bodelsson & Stjernquist, 1993), the human saphenous vein (Bax *et al.*, 1993), the goat cerebral artery (Salom *et al.*,

Figure 6 Effects of (a) BQ-123 (10^{-6} M, ●; 10^{-5} M, ■; or 10^{-4} M, ▲) or (b) PD 145065 (10^{-6} M, ●; 10^{-5} M, ■; or 10^{-4} M, ▲) on contractions induced by endothelin-3 (ET-3, ○) of strips of the isolated gall bladder of the guinea-pig ($n = 3-12$). Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are the means with s.e.mean. * $P < 0.05$ as compared to ET-1 by an unpaired, two-tailed, Mann-Whitney U test.

Figure 7 Effects of BQ-123 (10^{-5} M, ●; 10^{-4} M, ▲) or PD 145065 (10^{-5} M, ■) on contractions induced by sarafotoxin 6c (SX6c, ○; $n = 3-16$) of strips of the isolated gall bladder of the guinea-pig. Results are expressed as the percentage of the response to histamine (5×10^{-6} M), and are the means with s.e.mean. * $P < 0.05$ as compared to ET-1 by an unpaired, two-tailed, Mann-Whitney U test.

1993), the rat vas deferens (Eglezos *et al.*, 1993) and the rat aorta (Sumner *et al.*, 1992). These data have been taken by some to suggest that different receptors are being activated by the agonists, although this may be incorrect. It may rather be that the effectiveness of e.g. BQ-123, as an antagonist, varies with different agonists at the same receptor, possibly because ET-3 does not bind to ET receptors in the same irreversible manner as ET-1 (Hirata *et al.*, 1988; Waggoner *et al.*, 1992; Marsault *et al.*, 1993). For example, we have recently reported that the effectiveness of two ET_A/ET_B non-selective antagonists, Ro 46-2005 and PD 145065, against contractions mediated by ET_B receptors in the guinea-pig upper bronchus vary with the agonist used (Battistini *et al.*,

1994c). In this tissue, both antagonists produced greater inhibition of contractions induced by IRL 1620 than they did of contractions induced by ET-1. This variability in antagonist potency is a very important consideration since many of the pA₂ values reported for antagonists of the ET_B receptor have relied upon other ET-related peptides as agonists (Clozel *et al.*, 1993). We also found that a high concentration of BQ-123 (10^{-4} M) reduced contractions induced by either ET-1 or SX6c. However, at this concentration, BQ-123 has been reported to inhibit the binding of ET-1 to ET_B receptors (Williams *et al.*, 1993) and may no longer be a selective ET_A receptor antagonist.

In conclusion, the data presented in this study suggests that ET-1 activates at least two ET receptor subtypes, an ET_B receptor and an additional, atypical, ET receptor subtype. Until recently, the classification of endothelin receptor subtypes was based on the relative potency of two non-selective ET receptor agonists: ET-1 and ET-3 (Masaki *et al.*, 1994). The present study, like several others (see Battistini *et al.*, 1994a), suggests the existence of additional types of ET receptors and also that the classification of endothelin receptors needs reconsideration. Functionally distinct receptors cannot readily be differentiated on the basis of the relative potencies of the ET-1 and ET-3 isopeptides alone but requires the use of additional ligands such as SX6c or IRL 1620 and antagonists of ET_A and/or ET_A/ET_B receptors. Finally, as ET-1 may be an endogenous regulator of the gall bladder tone and is secreted from the epithelial cells (Housset *et al.*, 1993), endothelins may regulate gall bladder motility via these receptor subtypes.

This work was supported by the Parke-Davis Pharmaceutical Division of Warner-Lambert Co (Ann Arbor, MI, U.S.A.), the Wellcome Trust and the Frances and Augustus Newman Trust. We thank Drs A.M. Doherty and W. Cody of the Cardiovascular and Peptides Chemistry Division of Parke-Davis for generously providing the BQ-123 and PD 145065. We also thank Drs A.M. Doherty, R. Botting and R. Corder for helpful discussions. B.B. is a recipient of Research Fellowship Awards from the National Heart and Stroke Foundation of Canada and Le Fond pour les Chercheurs et l'Aide à la Recherche (FCAR) du Québec. A.F. is a Scientist of The Fonds de la Recherche en Santé du Québec (FRSQ).

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(Received March 4, 1994

Revised April 19, 1994

Accepted April 27, 1994)