Endothelin receptors mediating contraction in goat cerebral arteries

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1 The aim of the present study was to identify the subtype of receptor mediating contraction to endothelin-1 and sarafotoxin S6b in goat isolated middle cerebral arteries.

2 Endothelin-1, endothelin-2 and endothelin-3 contracted cerebral arteries in a concentration-dependent manner. Although the three peptides were full agonists, the order of potency was endothelin-1 = endothelin-2 > endothelin-3, with a relative potency of endothelin-1 and endothelin-2 *versus* endothelin-3 of ~280. Sarafotoxin S6b induced concentration-dependent contractions with lower potency than endothelin-1/endothelin-2, higher potency than endothelin-3 and a higher maximum response than the three endothelins.

3 The selective ET_A-receptor antagonist, BQ-123, did not induce changes in either the resting tension or in the active tone developed by depolarization. In contrast, BQ-123 produced concentration-dependent relaxations of endothelin-1-precontracted cerebral arteries, and to a greater extent of sarafotoxin S6b-precontracted arteries.

4 Concentration-response curves to endothelin-1 and sarafotoxin S6b were competitively antagonized by BQ-123 (pA₂ of 7.43 ± 0.12 and 8.41 ± 0.09, respectively). In contrast, BQ-123 had no effect on 5-hydroxytryptamine-elicited contractions even at 10⁻⁶ M.

5 It is concluded that both the order of potency of endothelin isopeptides and the antagonism of BQ-123 point to the existence of ET_A receptors mediating vasoconstriction to endothelin-1 and sarafotoxin S6b in the goat middle cerebral artery. The different antagonistic potency of BQ-123 against endothelin-1 and sarafotoxin S6b suggests the existence of subtypes of ET_A receptors.

Keywords: Endothelins; sarafotoxin S6b; BQ-123; cerebral arteries; ET_A receptors

Introduction

Endothelin-1 is a 21-amino acid endothelium-derived, vasoconstrictor peptide originally isolated from the culture supernatant of porcine aortic endothelial cells (Yanagisawa *et al.*, 1988). After the first report on endothelin-1, the existence of three distinct genes encoding for isopeptides with highly conserved sequence (endothelin-1, endothelin-2 and endothelin-3) was demonstrated (Inoue *et al.*, 1989). Furthermore, the amino acid sequences of sarafotoxins, found in the venom of the burrowing snake *Atractaspis engaddensis*, are very similar to those of endothelins (Kloog & Sokolovsky, 1989). All the endothelins and sarafotoxins are bioactive but differences exist in their pharmacological potencies and profiles (Kitazumi *et al.*, 1990; Cardell *et al.*, 1992).

Two distinct receptor subtypes for endothelins, namely ET_A (Arai *et al.*, 1990) and ET_B (Sakurai *et al.*, 1990) have been cloned and biochemically characterized. Early pharmacological characterization showed that vascular ET_A receptors were endothelin-selective and mediated vasoconstriction (Cardell *et al.*, 1992), while ET_B receptors were non-selective and linked to vasodilatation (Takayanagi *et al.*, 1991). The availability of BQ-123, a selective ET_A-receptor antagonist (Ihara *et al.*, 1992a), and of selective ET_B-receptor agonists (Williams *et al.*, 1991; Saeki *et al.*, 1991) have led to a reconsideration of early classification as there have been reports of venous ET_B receptors mediating vasoconstriction (Moreland *et al.*, 1992; Sumner *et al.*, 1992) and lung ET_A receptors mediating the release of prostacyclin (D'Orléans-Juste *et al.*, 1992).

Vasoconstrictor responses to endothelin-1 are greater in cerebral arteries than in peripheral arteries (Tanoi *et al.*, 1991), and the pathophysiological significance of endothelin-1

in cerebral vasospasm following subarachnoid hemorrhage is being investigated (Masaki *et al.*, 1992). We have previously reported the vasoconstrictor effects of endothelin-1 on the cerebrovascular bed of the goat (Salom *et al.*, 1991) and compared them with those of sarafotoxin S6b (Salom *et al.*, 1992). In the present study we have examined the agonist potency of endothelin isopeptides (endothelin-1, endothelin-2 and endothelin-3) and used the selective ET_A-receptor antagonist, BQ-123, to identify the receptors mediating endothelin-1- and sarafotoxin S6b-induced contraction in goat isolated middle cerebral artery.

Methods

Tissue preparation

Female goats (30–45 kg) previously sedated with 10 mg kg⁻¹ ketamine (Ketalar, Parke Davis, intramuscular) and anaesthetized with 2% sodium thiopentone (Tiobarbital, Palex, intravenous), were killed by intravenous injection of 30 mEq of KCl. The whole brain was rapidly removed, the middle cerebral arteries were dissected free and cut into cylindrical segments measuring 4 mm in length and about 1 mm in outer diameter. Each segment was prepared for isometric tension recording in an organ bath. Two stainless steel L-shaped pins (diameter of 125 µm) were introduced through the arterial lumen. One pin was fixed to the organ bath wall, while the other was connected to a strain gauge (Universal Transducing Cell, UC3, Gould Statham) for isometric tension recording (Omniscribe D 5237-5, Houston Instrument). The organ bath contained 5 ml of Ringer-Locke solution, which was bubbled continuously with 95% O₂ and 5% CO₂ to give a pH of 7.3–7.4. Temperature was kept at 37°C. A resting tension of 1 g was applied to the arterial segments and they

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were allowed to equilibrate for a period of 60–90 min before starting the experiments.

Procedure

The contractile capacity of the arterial segments was examined by exposure to a high-K⁺ (50 mM) Ringer-Locke solution. The effects of endothelin-1 (10^{-11} – 3×10^{-8} M), endothelin-2 (10^{-11} – 3×10^{-8} M), endothelin-3 (10^{-9} – 3×10^{-6} M) and sarafotoxin S6b (10^{-11} – 3×10^{-7} M) were studied by cumulative addition of the peptides to the organ bath. The effects of the selective ET_A-receptor antagonist BQ-123 (10^{-10} – 10^{-6} M) itself were examined in arteries at resting tone and in arteries precontracted with either high-K⁺ (50 mM) medium, endothelin-1 (10^{-9} M) or sarafotoxin S6b (10^{-8} M). To assess the selective antagonistic effects of BQ-123 against endothelin-1 and sarafotoxin S6b, concentration-response curves to endothelin-1 (10^{-11} – 3×10^{-7} M), sarafotoxin S6b (10^{-11} – 3×10^{-6} M) and 5-hydroxytryptamine (10^{-9} – 3×10^{-5} M) were obtained in control conditions and after incubation (30 min) with different concentrations of BQ-123 (10^{-8} M, 10^{-7} M, 10^{-6} M). Only one concentration-response curve was obtained from one arterial segment. In the experiments with BQ-123 pretreatment control tissues were run in parallel.

Statistics

Contractile responses to endothelin-1, endothelin-2, endothelin-3, sarafotoxin S6b and 5-hydroxytryptamine were expressed as a percentage of a previous contraction induced by 50 mM KCl. Relaxant responses to BQ-123 were expressed as a percentage of previously developed active tone. Concentration-ratios were calculated for the responses at the level of the EC₅₀ (half-maximal effective concentration). Maximum effects (E_{max}) were expressed as mean $\pm$ s.e.mean, and EC₅₀ as the geometric mean with its confidence limits (95%) for repeated experiments. Statistical comparisons of E_{max} and $-\log$ EC₅₀ (pD₂) values between endothelin-1, endothelin-2, endothelin-3 and sarafotoxin S6b, as well as for endothelin-1 and sarafotoxin S6b in the absence and the presence of BQ-123 at different concentrations were obtained by analysis of variance (ANOVA) and Newman-Keuls tests. Relative potencies of endothelin-1 *versus* endothelin-3 and sarafotoxin S6b were calculated. Values of EC₅₀ for endothelin-1 and sarafotoxin S6b obtained in the absence and in the presence of BQ-123 at different concentrations were subjected to Schild analysis to obtain pA₂ and slope values of antagonism. All the pharmacological and statistical calculations were performed according to Tallarida & Murray (1987). Probability values (*P*) of less than 5% were considered significant.

Drugs and solutions

Endothelin-1 (Peptide Institute Inc.) was dissolved in twice distilled water. Endothelin-2 and endothelin-3 (Peptide Institute Inc.) were dissolved in 0.1% aqueous acetic acid. Sarafotoxin S6b and BQ-123 (cyclo(D-Asp-Pro-D-Val-Leu-D-Trp)) (Nova-Biochem) were dissolved in twice distilled water. All the peptides were diluted in phosphate buffered saline with 0.05% bovine serum albumin (fraction V, Sigma). 5-Hydroxytryptamine (creatinine sulphate complex, Sigma) was dissolved and diluted in 0.9% saline solution. The Ringer-Locke solution had the following composition (mM): NaCl 120, KCl 5.4, CaCl₂ 2.2, MgCl₂ 1.0, NaHCO₃ 25 and glucose 5.6. In KCl-depolarizing solution, NaCl was replaced by an equimolar amount of KCl. The phosphate buffered saline had the following composition (mM): NaCl 150, NaH₂PO₄ 10.0.

Results

Endothelin-1 (10^{-11} – 3×10^{-8} M), endothelin-2 (10^{-11} – 3×10^{-8} M) and endothelin-3 (10^{-9} – 3×10^{-6} M) produced slowly

developing, concentration-dependent contractions of the goat middle cerebral artery (Figure 1). As shown in Table 1, maximum responses to the three peptides were not significantly different. Values of EC₅₀ for endothelin-1 and endothelin-2 were not significantly different from each other, but both of them were significantly lower (*P* < 0.01) than that for endothelin-3. The relative potency of endothelin-1 *versus* endothelin-3 was 280 (range 135–604). Sarafotoxin S6b (10^{-11} – 3×10^{-7} M) induced concentration-dependent contractions (Figure 3) with an EC₅₀ value between those of endothelin-1 and endothelin-3, and a maximum response significantly higher (*P* < 0.01) than those of the three endothelins (Table 1). The relative potency of endothelin-1 *versus* sarafotoxin S6b was 3.3 (range 1.5–7.2).

BQ-123 (10^{-10} – 10^{-6} M) did not induce changes in resting tension or in active tone developed with high-K⁺ (50 mM) medium. However, BQ-123 induced concentration-dependent relaxations of arteries precontracted with endothelin-1 (10^{-9} M) or sarafotoxin S6b (10^{-8} M). Complete relaxation was induced by BQ-123 in sarafotoxin S6b-precontracted arteries, with a mean EC₅₀ of 6.2×10^{-9} M. Since concentrations of BQ-123 higher than 10^{-6} M could not be used, a true maximum relaxant effect could not be obtained in endothelin-1-precontracted arteries (Figure 2).

Figure 1 Concentration-response curves to endothelin-1 (○, *n* = 8), endothelin-2 (●, *n* = 8) and endothelin-3 (□, *n* = 8) in goat isolated middle cerebral arteries. Symbols represent mean values $\pm$ s.e.mean.

Figure 2 Concentration-response curves to BQ-123 in goat isolated middle cerebral arteries precontracted with 10^{-9} M endothelin-1 (●, *n* = 8) and 10^{-8} M sarafotoxin S6b (○, *n* = 9). Symbols represent mean values $\pm$ s.e.mean.

BQ-123 (10^{-8} – 10^{-6} M) produced concentration-dependent, rightward parallel displacement of the concentration-response curves to endothelin-1 and sarafotoxin S6b (Figure 3a and b, respectively). Table 1 summarizes EC_{50} and maximum effect

Figure 3 Concentration-response curves to (a) endothelin-1 and (b) sarafotoxin S6b in goat isolated middle cerebral arteries. (○) Control arteries, $n = 8$ in (a) and $n = 6$ in (b); (●) arteries incubated with 10^{-8} M BQ-123, $n = 10$ (a) and $n = 6$ in (b); (□) 10^{-7} M BQ-123, $n = 9$ in (a) and $n = 5$ in (b); (■) 10^{-6} M BQ-123, $n = 7$ in (a) and $n = 6$ in (b). Symbols represent mean values $\pm$ s.e.mean.

values for endothelin-1 and sarafotoxin S6b in the absence and in the presence of BQ-123. Schild analysis of BQ-123 antagonism yielded a pA_2 value of 7.43 ± 0.12 (slope of 1.31 ± 0.17) against endothelin-1, and a pA_2 value of 8.41 ± 0.09 (slope of 1.26 ± 0.12) against sarafotoxin S6b. Analysis of the regression lines showed that slope values were not significantly different from unity. In contrast, the concentration-response curve to 5-hydroxytryptamine (10^{-9} – 3×10^{-5} M) in goat middle cerebral artery was not significantly modified by BQ-123 (Figure 4), even at the highest concentration tested (10^{-6} M).

Figure 4 Concentration-response curves to 5-hydroxytryptamine in goat isolated middle cerebral arteries in control conditions (○, $n = 6$) and after incubation with BQ-123 (●, 10^{-7} M, $n = 5$; □ 10^{-6} M, $n = 6$). Symbols represent mean values $\pm$ s.e.mean.

Discussion

The cloned receptors for endothelins are classified into two subtypes, ET_A which has a high affinity for endothelin-1 and endothelin-2, but low affinity for endothelin-3 (Arai *et al.*, 1990), and ET_B which has an equal affinity for all three endothelins (Sakurai *et al.*, 1990). Our results show that endothelin-1, endothelin-2 and endothelin-3 were full agonists on goat middle cerebral artery. The order of potency of endothelin isopeptides was endothelin-1 = endothelin-2 > endothelin-3, which is consistent with the existence of ET_A

Table 1 EC_{50} and maximum effect (E_{max}) values for endothelin-1 (control and after BQ-123), endothelin-2, endothelin-3 and sarafotoxin S6b (control and after BQ-123) in goat isolated middle cerebral arteries

Vasoconstrictor peptide	Pretreatment	EC_{50} (M)	E_{max} (% KCl)	n
Endothelin-1	None (control)	$2.9 (1.9-4.4) \times 10^{-10}$	96 ± 3	8
	BQ-123, 10^{-8} M	$3.3 (1.8-6.0) \times 10^{-10}$	102 ± 4	10
	BQ-123, 10^{-7} M	$2.0 (1.4-2.8) \times 10^{-9a}$	104 ± 6	9
	BQ-123, 10^{-6} M	$1.8 (1.1-2.8) \times 10^{-8a}$	92 ± 8	7
Endothelin-2	None	$2.5 (2.0-3.2) \times 10^{-10}$	84 ± 4	8
Endothelin-3	None	$8.1 (5.5-12.0) \times 10^{-8b}$	92 ± 9	8
Sarafotoxin S6b	None	$2.5 (1.2-5.1) \times 10^{-9c}$	128 ± 7^c	6
	BQ-123, 10^{-8} M	$1.1 (0.7-1.6) \times 10^{-8d}$	134 ± 6	6
	BQ-123, 10^{-7} M	$1.5 (1.1-2.0) \times 10^{-7d}$	136 ± 12	5

EC_{50} values are geometric mean with 95% confidence interval and E_{max} values, expressed as a percentage of previous response to 50 mM KCl, are mean $\pm$ s.e.mean from n arterial segments.

^aSignificantly different from control for endothelin-1, $P < 0.01$.

^bSignificantly different from controls for both endothelin-1 and endothelin-2, $P < 0.01$.

^cSignificantly different from controls for endothelin-1, endothelin-2, and endothelin-3, $P < 0.01$.

^dSignificantly different from control for sarafotoxin S6b, $P < 0.01$.

receptors mediating vasoconstriction. Therefore, cerebral arteries resemble other arterial tissues, like rat thoracic aorta (Sumner *et al.*, 1992), guinea-pig pulmonary artery (Cardell *et al.*, 1992), porcine coronary artery (Harrison *et al.*, 1992), and iliac arteries from several species (Moreland *et al.*, 1992), in which the existence of ET_A receptors has been proposed according to endothelin-1/endothelin-2 selectivity. In contrast, endothelin-induced contraction is mediated by non-selective ET_B receptors in venous (Moreland *et al.*, 1992; Sumner *et al.*, 1992), tracheal (Cardell *et al.*, 1992) and intestinal (Yoshinaga *et al.*, 1992) smooth muscles.

With regard to sarafotoxin S6b, this endothelin-related peptide of reptilian origin (Kloog & Sokolovsky, 1989), showed a vasoconstrictor potency lower than endothelin-1 and endothelin-2, but higher than endothelin-3 in goat cerebral arteries. Such an intermediate potency has also been reported for sarafotoxin S6b in pig coronary artery (Harrison *et al.*, 1992). The finding is in agreement with the mediation by ET_A receptors of the sarafotoxin S6b-induced vasoconstriction, since the ability of sarafotoxin S6b to prevent endothelin-1 from binding to the cloned ET_A receptor has been reported to be lower than that of endothelin-1 or endothelin-2, but higher than that of endothelin-3 (Arai *et al.*, 1990).

The synthetic cyclic pentapeptide, BQ-123, is a potent, selective antagonist of ET_A receptors (Ihara *et al.*, 1992a). The use of this antagonist has permitted us to confirm the existence of ET_A receptors mediating vasoconstriction in goat cerebral arteries. First, BQ-123 relaxed cerebral arteries previously contracted with endothelin-1 and sarafotoxin S6b, but did not relax arteries precontracted by depolarization. Second, BQ-123 competitively antagonized the endothelin-1 and sarafotoxin S6b-induced contraction of cerebral arteries. In confirmation of the specificity of BQ-123, 5-hydroxy-tryptamine-induced contractions remained unchanged. Arterial receptors mediating contraction to endothelins have been usually identified as ET_A-type according to endothelin-selectivity (Cardell *et al.*, 1992; Harrison *et al.*, 1992; Moreland *et al.*, 1992) or by using the selective antagonist BQ-123 (Ihara *et al.*, 1992a; Sumner *et al.*, 1992). Since our results demonstrate the presence of ET_A receptors in cerebral arteries, the

report that ET_B receptors mediate vasoconstriction in rabbit pulmonary artery (Ihara *et al.*, 1992b) seems to be an exception among arterial tissues.

The antagonistic potency of BQ-123 against endothelin-1-induced contraction of goat cerebral arteries (pA₂ of 7.43) is similar to the potency originally reported in porcine coronary artery (Ihara *et al.*, 1992a) and comparable to that found in rat thoracic aorta (Sumner *et al.*, 1992). In contrast, the antagonist potency of BQ-123 against sarafotoxin S6b-induced contraction of cerebral arteries (pA₂ of 8.41) was approximately ten times higher than the value generated against endothelin-1-induced contractions. This is consistent with the greater potency of BQ-123 in relaxing sarafotoxin S6b-precontracted arteries than endothelin-1-precontracted arteries, and suggests the existence of a heterogeneous receptor population on goat cerebral arteries (Kenakin, 1987). It is possible that different subtypes of ET_A receptors mediate endothelin-1- and sarafotoxin S6b-elicited responses. A similar differentiation of ET_A receptor subtypes has been proposed for endothelin-1- and endothelin-3-induced contractions in rat thoracic aorta (Sumner *et al.*, 1992). In a previous report (Salom *et al.*, 1992) we have shown that the nature of the extracellular Ca²⁺ source mobilized by endothelin-1 and sarafotoxin S6b in goat cerebral arteries is also different since endothelin-1 depends on free extracellular Ca²⁺ and sarafotoxin S6b depends on externally membrane-bound Ca²⁺. The Ca²⁺ source could be related to the receptor subtype involved in the response to each of the peptides.

In conclusion, both the order of potency of endothelin isopeptides and antagonism of endothelin-1- and sarafotoxin S6b-elicited responses by BQ-123 demonstrate the existence of ET_A receptors mediating vasoconstriction in the goat middle cerebral artery. The difference in the antagonist potency of BQ-123, which is higher against sarafotoxin S6b than against endothelin-1, suggests the existence of subtypes of ET_A receptors.

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