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Receptor and mechanism that mediate endothelin- and big endothelin-1-induced phosphoinositide hydrolysis in the rat spinal cord

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Abstract

In rat spinal cord slices, endothelin-1 and endothelin-3 enhanced [^{3}H]inositol phosphate production between 1 nM and 10 μM (endothelin-1 > endothelin-3) while sarafotoxin 6c and the endothelin ET_B receptor agonist IRL-1620 (Suc-[Glu^9,Ala^{11,15}]endothelin-1-(8–21)) were almost ineffective. BQ-123 (cyclo(D-Trp,D-Asp,L-Pro,D-Val,L-Leu), a selective endothelin ET_A receptor antagonist, reduced the endothelin-1- and endothelin-3-induced [^{3}H]inositol phosphate production, with similar inhibition constants (IC_{50}: 16.7 ± 3.4 and 8.0 ± 1.6 μM, respectively). The inhibition of endothelin-1 was enhanced when BQ-123 was preincubated for 30 min instead of 15 min. BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxy-carbonyltryptophanyl-D-Nle), a selective ET_B receptor antagonist, did not modify the endothelin-1-induced [^{3}H]inositol phosphate production. Big endothelin-1 (1 nM to 1 μM) was slightly less potent than endothelin-1 in enhancing [^{3}H]inositol phosphate production. This response was sensitive to phosphoramidon and [Phe^{22}]big endothelin-1-(19–37), two inhibitors of endothelin-converting enzyme. Pretreatment of slices with pertussis toxin, indomethacin or PN 200-110 ((−)-isradipine, a dual inhibitor of L- and R-type Ca^{2+} channels) did not alter the response to 1 μM endothelin-1 while this response was abolished by tetrodotoxin. Finally, endothelin-1 enhanced [^{3}H]inositol phosphate production with an identical EC_{50} (2.1 nM) in spinal cord slices of Wistar-Kyoto rats (WKY) and spontaneously hypertensive rats (SHR) although the maximal response was reduced in SHR. These data indicate that endothelins stimulated [^{3}H]inositol phosphate production in the rat spinal cord through the activation of an endothelin ET_A receptor that trigger the release of an unidentified neurotransmitter. This effect does not appear to be associated to activation of a G_i/G_o-type of G-protein, dihydropyridine-sensitive L-type Ca^{2+} channels or to the production of prostaglandins. Furthermore, the findings support the presence of a phosphoramidon-sensitive endothelin-converting enzyme in the spinal cord.

Keywords: Endothelin; Sarafotoxin; Big endothelin-1; Inositol phosphate; Spinal cord; Endothelin ET_A receptor

1. Introduction

Endothelins belong to a family of structurally related 21 amino acid isopeptides which includes endothelin-1, endothelin-2, endothelin-3 and sarafotoxins 6c. It was suggested that endothelins may act as neurotransmitters or neuromodulators (Gulati and Srimol, 1992) as supported by the presence of endothelin-1 and its mRNA in the brain, spinal cord and dorsal root ganglia (Giaid et al., 1989). Moreover, endothelin-1 binding sites were found in the brain and spinal cord of several species (Kar et al., 1991; Niwa et al., 1992). In conscious rats, intrathecal injections of endothelin-1 markedly increased mean arterial blood pressure via the activation of an endothelin ET_A receptor in the spinal cord (Poulat et al., 1994).

Two distinct endothelin receptor subtypes have been cloned in mammals: endothelin ET_A and endothelin ET_B. The endothelin ET_A subtype preferentially binds endothelin-1 and endothelin-2 while endothelin ET_B binds endothelin-1, endothelin-2 and endothelin-3 with equipotent affinity (Bax and Saxena, 1994). These two functional receptors have been further characterized with selective analogues. BQ-123 (cyclo(D-Trp,D-Asp,L-Pro,D-Val,L-Leu) and BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxy-carbonyltryptophanyl-D-Nle) are selective antagonists at endothelin ET_A and ET_B receptors, respectively, and IRL-1620 (Suc-[Glu^9,Ala^{11,15}]endothelin-1-(8–21)) is a selective agonist at the endothelin ET_B receptor (Bax and Saxena, 1994).

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Endothelin receptors belong to the superfamily of guanine nucleotide-binding regulatory protein (G-protein)-coupled receptors. In many tissues including the brain, these are mainly coupled to phospholipase C leading to phosphoinositide hydrolysis and increases of intracellular Ca^{2+} (Crawford et al., 1990; Sokolovsky, 1993; Rubanyi and Polokoff, 1994). In neural and cerebrovascular cells, endothelins also stimulate other pathways such as cAMP production, arachidonic acid production and Ca^{2+} entry through dihydropyridine-sensitive L-type Ca^{2+} channels (Yoshizawa et al., 1989; Zhang et al., 1990; Domae et al., 1994; Stanimirovic et al., 1994; Deschepper et al., 1995).

Big endothelin-1-(1–38), the immediate precursor of endothelin-1, is converted to endothelin-1 by an endothelin-converting enzyme that is sensitive to phosphoramidon, a metalloproteinase inhibitor (Fukuroda et al., 1990). In the dog, the presence of endothelin-converting enzyme has been suggested in the central nervous system since pressor effects induced by intracisternal injection of big endothelin-1 were inhibited by phosphoramidon (Shinyama et al., 1991). In addition, we have previously shown in the conscious rat that intrathecal injection of big endothelin-1-induced cardiovascular responses which were similar to those evoked by endothelin-1 and were blocked by phosphoramidon and BQ-123 (Poulat et al., 1994).

The present study was designed to assess the effects of endothelins on phosphoinositide breakdown in relation to a previous study on the effects of intrathecal injection of endothelins on the cardiovascular responses in the conscious rat (Poulat et al., 1994). More specifically, the aims of this study were: (1) to assess the effects of endothelin-1, endothelin-3, sarafotoxin 6c, and IRL-1620 on phosphoinositide hydrolysis in rat spinal cord slices; (2) to further characterize the receptor involved in the response to endothelins using BQ-123 and BQ-788, selective antagonists at endothelin ET_A and ET_B receptors, respectively; (3) to examine the effect of big endothelin-1 in the absence and presence of inhibitors of endothelin-converting enzyme; and (4) to determine the effect of inhibitors of G_i/G_o G-protein, prostaglandin synthesis, dihydropyridine sensitive Ca^{2+} channels and of voltage-dependent Na^+ channels on the production of inositol phosphates induced by endothelin-1. Additionally, the effect of endothelin-1 on inositol production was compared in spinal cords isolated from normotensive (WKY) and spontaneously hypertensive rats (SHR).

2. Materials and methods

The care of animals and research protocols conformed to the guiding principles for animal experimentation as enunciated by the Canadian Council on animal care and approved by the committee responsible for animal care at the Université de Montréal.

2.1. Measurement of [^{3}H]inositol phosphates

Male Wistar rats weighing 270–350 g, 14–17 weeks old WKY and SHR (Charles River, St. Constant, Canada) were decapitated and the entire length of the spinal cord was rapidly ejected by pressure with a seringe and immersed in gassed (95% O_2/5% CO_2) and cold Krebs buffer of the following composition (mM): NaCl 118, KCl 3.5, $MgSO_4$ 2.4, KH_2PO_4 1.2, $CaCl_2$ 1.25, $NaHCO_3$ 25 and glucose 10 (pH 7.4). After removing the dura mater, the entire spinal cord was cross-chopped in slices (350 μm × 350 μm) with a Mc Ilwain tissue-chopper and processed essentially as described by Fisher et al. (1984). After three washes of 20 min each in gassed Krebs buffer maintained at 37°C under agitation in a water bath, 50 μl aliquots of packed slices (± 1 mg of protein) were preincubated for 60 min at 37°C in Krebs buffer containing 2 μCi of *myo*-[2-^{3}H]inositol (0.17 μM, specific activity 23 Ci/mmol) and 7 mM lithium chloride in a final volume of 475 μl. Then 25 μl of agonist were added and 60 min later the reaction was stopped by adding 1 ml choloroform/methanol (1:2, v/v). When necessary antagonists and inhibitors were added in a volume of 25 μl 15 or 30 min before adding agonist as indicated in the text. After 15 min, 0.4 ml of deionised water and 0.4 ml of chloroform were added to create two phases which were separated by centrifugation (1000 × *g*, 5 min). A sample of the upper aqueous phase (700 μl) was applied onto a anion-exchange chromatography column filled with 0.8 ml of Dowex AG1-X8 resin (200–400 mesh) in formate form. The columns were washed with 10 ml of deionised water and the [^{3}H]inositol phosphate pool (inositol phosphate, inositol bisphosphate, inositol trisphosphate) was eluted with 8 ml of 1 M ammonium formate/0.1 M formic acid. Fractions of 2 ml were collected and added to 12 ml of scintillating liquid (Universol, ICN, Montréal, Canada). A sample of the organic phase (600 μl) containing the [^{3}H]phosphatidylinositol pool was evaporated and 7 ml of Universol were added and the radioactivity was counted in a Beckman scintillation counter. Columns were regenerated with 5 ml formic acid 1 M, rinsed and stored with 0.1 M formic acid. Using this procedure the columns could be used up to 2–3 months.

2.2. Peptides and other compounds

Endothelin-1, endothelin-3 and sarafotoxin 6c were purchased from Bachem Bioscience (King of Prussia, PA, USA) and big endothelin-1 from Peninsula Labs. (Belmont, CA, USA). BQ-123 (cyclo(D-Trp,D-Asp,L-Pro,D-Val,L-Leu) and BQ-788 (*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxy-carbonyltryptophanyl-D-Nle) were generously given by Banyu Pharmaceuticals (Tsukuba, Japan). PN 200-110, also named (−)-isradipine (Sandoz, Canada), was generously given by Dr G. Bkaily (University of Sherbrooke, Sherbrooke, Canada).

[Phe^{22}]Big endothelin-1-(19–37) was synthesized at the Department of Pharmacology at Sherbrooke University. *myo*-[2-^{3}H]Inositol was purchased from New England Nuclear (Boston, MA, USA). Lithium chloride, indomethacin, tetrodotoxin, pertussis toxin and DL-thiorphan were purchased from Sigma Chemicals (St Louis, MO, USA), phosphoramidon and IRL 1620 (Suc-[Glu^9,Ala^{11,15}]endothelin-1-(8–21)) were from Peptide Institute (Louisville, KY, USA). Dowex AG1X8 resin was purchased from Bio-Rad (Mississauga, Canada).

2.3. Statistical analysis of data

The counts/min (cpm) contained in the aqueous phase were normalized according to those found in the organic phase and expressed as the ratio of [^{3}H]inositol phosphate pool over [^{3}H]*myo*-inositol incorporated into phosphatidylinositol lipid pool. This ratio allows for the corrections of intra-experimental variations. The data were expressed as means ± S.E.M. of at least 3 experiments each performed in triplicate. Statistical differences were evaluated with a one-way analysis of variance (ANOVA) followed by a post hoc Dunnett test for multiple comparisons with one control. Only probability values (P) smaller than 0.05 were considered to be statistically significant.

3. Results

Preliminary experiments were carried out to determine the optimal amount of tissue and incubation time for the remainder of the study. Since the ratio of [^{3}H]inositol phosphates over [^{3}H]phosphatidylinositol was similar from 50 to 100 μl of tissue in the absence (basal) and presence of 1 μM endothelin-1, the volume of 50 μl of packed slices (± 1 mg of proteins) was chosen to perform the maximum number of assays with the same pool of spinal cords (Fig. 1A). The ratio of [^{3}H]inositol phosphates over [^{3}H] phosphatidylinositol increased time dependently from 15 to 90 min in the presence of 1 μM endothelin-1 (Fig. 1B). The maximal change induced by endothelin-1 when compared to basal values was seen at 60 min ($P < 0.01$). Moreover, the basal values were not affected between 15–60 min while they were increased at 90 min. Hence, 60 min was considered the optimal incubation time under those conditions. In addition, as the cpm were normalized by the ratio of [^{3}H]inositol phosphates over [^{3}H]phosphatidylinositol, it was critical to determine whether or not the agonists interfere with *myo*-[2-^{3}H]inositol incorporation in slices. Thus, the effects of increasing concentrations of endothelin-1, endothelin-3, sarafotoxin 6c, IRL 1620 and big endothelin-1 were assessed on *myo*-[2-^{3}H]inositol incorporation. None of the agonists significantly altered the *myo*-[2-^{3}H]inositol incorporation in slices from 1 nM to 10 μM (values varie between 97.2 ± 3.0% and 115.3 ± 4.0% of the basal level, $n = 3$–5 for each agonist) except sarafotoxin 6c which inhibited the incorporation at 10 μM (58.8 ± 3.1% of the basal level, $n = 5$; $P < 0.01$).

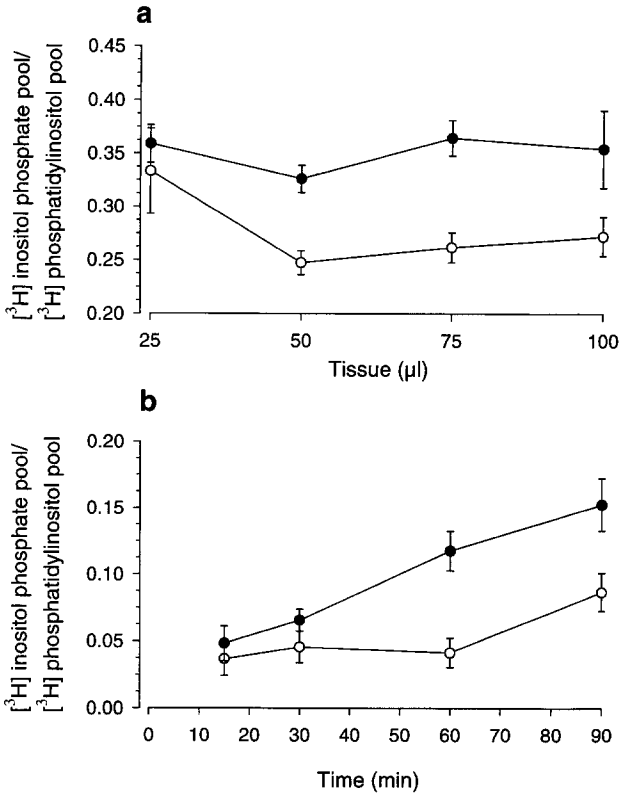

Fig. 1. (A) Effects of increasing volume of tissue on [^{3}H]inositol phosphate production in the absence (○) or presence of endothelin-1 (1 μM) for 1 h (●). (B) Time-dependent induction of [^{3}H]inositol phosphate production in the absence (○) or presence of endothelin-1 (1 μM) (●). Values represent means ± S.E.M. of three separate experiments.

3.1. Effects of agonists on [^{3}H]inositol phosphate production

Endothelin-1, endothelin-3, big endothelin-1, sarafotoxin 6c and IRL 1620, an endothelin ET_B receptor agonist, produced increases of [^{3}H]inositol phosphate production in rat spinal cord slices with the following rank order of potency (EC_{50} nM): endothelin-1 (14) > endothelin-3 (28) > big endothelin-1 (55) ≫ sarafotoxin 6c (298) = IRL 1620 (470) (Fig. 2). Whereas endothelin-1 and big endothelin-1 induced concentration-dependent increases of [^{3}H]inositol phosphate production from 1 nM to 1 μM, the concentration-dependent increases induced by endothelin-3 reached a maximum at 100 nM. Higher concentrations were somewhat less efficacious. Sarafotoxin 6c and IRL-1620 were almost inactive up to concentrations of 10 μM. Because 10 μM of sarafotoxin 6c reduced by 42% the incorporation of *myo*-[2-^{3}H]inositol, the ratio of [^{3}H]inositol phosphates over [^{3}H]phosphatidylinositol was normalized by using mean incorporation of the samples used for concentrations lower than 10 μM in the same experi-

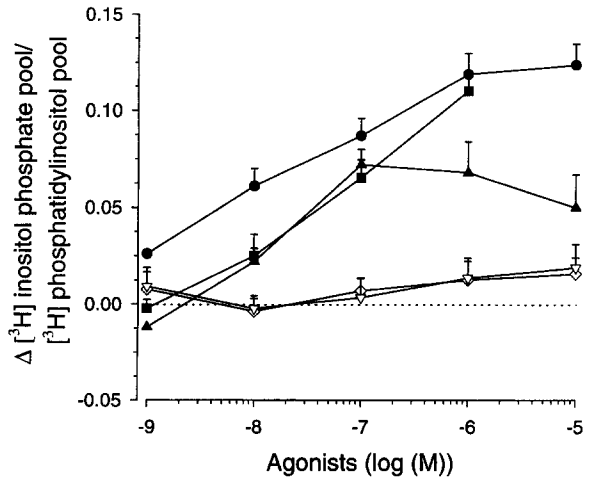

Fig. 2. Concentration-dependent effects of endothelin-1 (●), endothelin-3 (▲), sarafotoxin 6c (◇), IRL-1620 (▽), and big endothelin-1 (■) on changes of [^{3}H]inositol phosphate production in rat spinal cord slices. Values are means ± S.E.M. of 3–5 separate experiments.

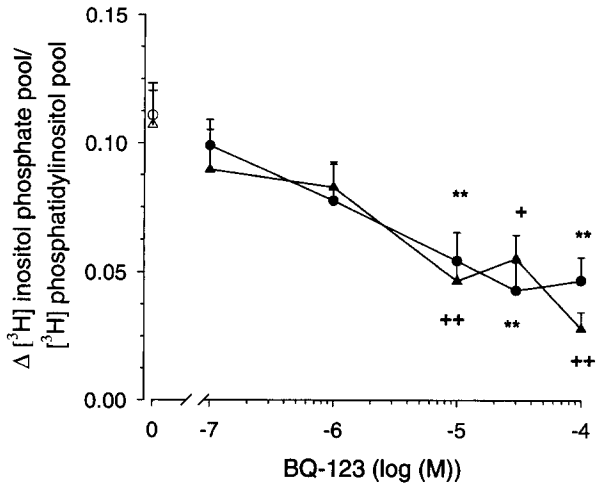

Fig. 3. Concentration-dependent effects of BQ-123 on changes of [^{3}H]inositol phosphate production induced by 100 nM endothelin-1 (●) or 100 nM endothelin-3 (▲). [^{3}H]Inositol phosphate production without BQ-123 incubation is shown as open symbols. Values are means ± S.E.M. of 3 separate experiments. Statistical comparison to control values (no antagonist) is indicated by ** $P < 0.01$ for endothelin-1 and $^{+}$ $P < 0.05$; $^{++}$ $P < 0.01$ for endothelin-3.

ments. The maximal effect for each agonist showed the following rank order of intrinsic activity (α^E): endothelin-1 (1) > endothelin-3 (0.58) ≫ sarafotoxin 6c (0.15) = IRL 1620 (0.14). Big endothelin-1 has not been tested at 10 μM but its effect at 1 μM was similar to that of endothelin-1. Among the total inositol phosphate production induced by 1 μM endothelin-1, inositol monophosphates, inositol bisphosphates and inositol trisphosphates represent 80, 16 and 4%, respectively.

3.2. Effects of BQ-123 and BQ-788 versus endothelin-1 or endothelin-3

The inhibitory effects of increasing concentrations of BQ-123, an antagonist of endothelin ET_A receptor, were determined on [^{3}H]inositol phosphate production induced by 100 nM endothelin-1 or endothelin-3 in rat spinal cord slices (Fig. 3). BQ-123 (pre-incubated 15 min earlier) reduced the effects of endothelin-1 and endothelin-3 in a concentration-dependent manner with IC_{50} of 16.7 ± 3.4 and 8.0 ± 1.6 μM, respectively. In the presence of 100 μM of BQ-123, [^{3}H]inositol phosphate production induced by 100 nM endothelin-1 or endothelin-3 was reduced respectively to 44% and 26% of the initial [^{3}H]inositol phosphate production (Table 1). When BQ-123 was preincubated 30 min instead of 15 min at 100 μM, the inhibition was more pronounced and [^{3}H]inositol phosphate production induced by 100 nM endothelin-1 was reduced to 23% of the control (Table 1). BQ-123 itself had no significant effect on [^{3}H]inositol phosphate production (data not shown). BQ-788 (100 μM), an antagonist of endothelin ET_B receptor, incubated 30 min before 100 nM endothelin-1 did not alter the response to endothelin-1 (Table 1).

3.3. Effects of big endothelin-1 and of endothelin-converting enzyme inhibitors

The effects of phosphoramidon (300 μM), DL-thiorphan (300 μM) and [Phe^{22}]big endothelin-1-(19–37) (10 μM) were examined on [^{3}H]inositol phosphate production induced by increasing concentrations of big endothelin-1 (30 nM to 1 μM) (Fig. 4). Although the three inhibitors reduced the response to 100 nM big endothelin-1, only phosphoramidon and [Phe^{22}]big endothelin-1-(19–37) significantly reduced the responses to 1 μM big-endothelin-1. It should be noted, however, that [Phe^{22}]big endothelin-1-(19–37) reduced the response to big endothelin-1 only in the presence of DL-thiorphan (0.5 μM) that was used to prevent the degradation of the peptide inhibitor in the incubation medium (Claing et al., 1995). DL-Thiorphan (300 μM for 3 separate experiments) incubated 30 min before endothelin-1 did not significantly alter the [^{3}H]inositol phosphate production (Δ[^{3}H]inositol

Table 1
Maximal effects of BQ-123 and BQ-788 on [^{3}H]inositol phosphates production induced by endothelin-1 or endothelin-3

Antagonist	Agonist	ΔIPP/PIP (%)
None	ET-1 100 nM	100.0 ± 7.0
None	ET-3 100 nM	100.0 ± 12.5
BQ-123 100 μM, 15 min	ET-1 100 nM	44.3 ± 8.5 [b]
BQ-123 100 μM, 15 min	ET-3 100 nM	26.2 ± 6.0 [a]
BQ-123 100 μM, 30 min	ET-1 100 nM	23.2 ± 4.2 [b]
BQ-788 100 μM, 30 min	ET-1 100 nM	116.0 ± 8.2

Data are expressed as percentages of the control response without antagonist (changes of [^{3}H]inositol phosphate pool/[^{3}H]phosphatidyl-inositol pool). Values are means ± S.E.M. of 3 separate experiments. Statistical comparison to the agonist alone is indicated by [a] $P < 0.01$; [b] $P < 0.001$.

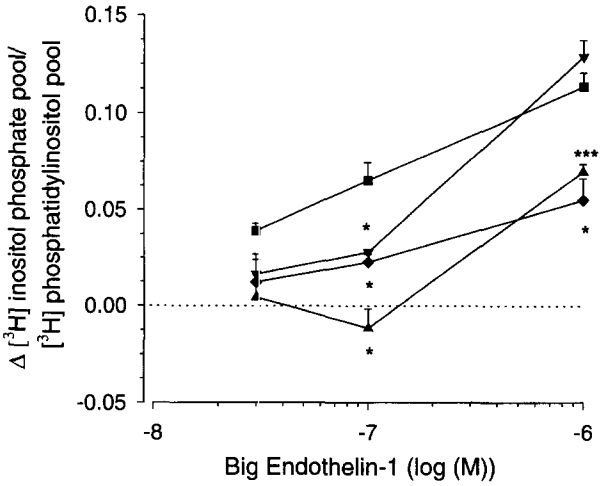

Fig. 4. Concentration-dependent effects of big endothelin-1 on changes of [^{3}H]inositol phosphate production in the absence (■) or presence of phosphoramidon (◆; 300 μM, 30 min), DL-thiorphan (▼; 300 μM, 30 min), [Phe^{22}]big endothelin-1-(19–37) plus 0.5 μM DL-thiorphan (▲; 10 μM, 30 min). Values are means ± S.E.M. of 3 separate experiments. Statistical comparisons to big endothelin-1 without inhibitor are indicated by * $P < 0.05$; *** $P < 0.001$.

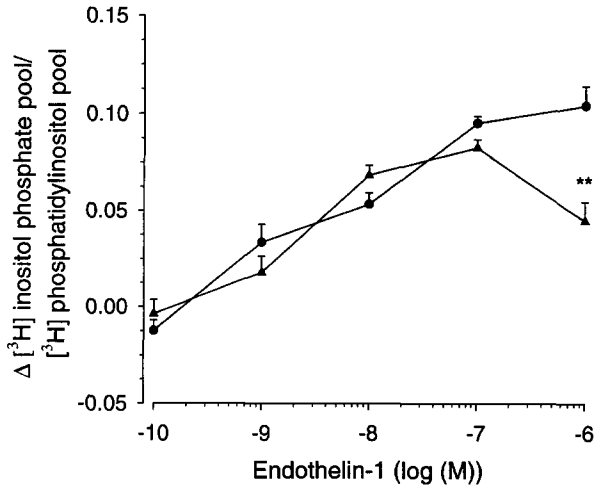

Fig. 6. Concentration-dependent effects of endothelin-1 on changes of [^{3}H]inositol phosphate production in spinal cord slices of WKY (●) or SHR (▲). Values are means ± S.E.M. of 5 separate experiments. Statistical difference betwen WKY and SHR is indicated by ** $P < 0.01$.

phosphates/[^{3}H]phosphatidyl inositol) induced by endothelin-1 at 10 nM (0.060 ± 0.009), 100 nM (0.069 ± 0.013) and 1 μM (0.100 ± 0.011) when compared to their respective controls (0.061 ± 0.009, 0.087 ± 0.009 and 0.117 ± 0.011).

3.4. Effects of various inhibitors versus endothelin-1

The effects of several inhibitors were also examined on endothelin-1-induced [^{3}H]inositol phosphate production (Fig. 5). Pertussis toxin (2.5 μg/ml, 1 h at 37°C according to Sokolovsky, 1993), indomethacin (1 μM, 30 min) and PN 200–110, a dual L- and R-type Ca^{2+} channel blocker (100 nM, 30 min), did not alter the [^{3}H]inositol phosphates response to 1 μM endothelin-1. However, tetrodotoxin (1 μM, 30 min), a Na^+ channel blocker, completely blocked the response to 1 μM endothelin-1.

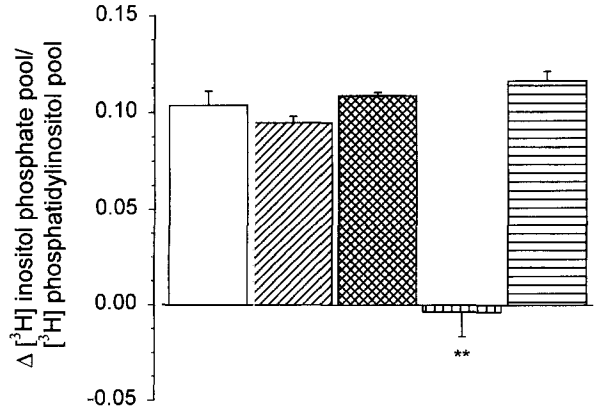

Fig. 5. Effects of 1 μM endothelin-1 in the absence (blank column) or presence of pertussis toxin (hatched column; 2.5 μg/ml, 1 h), PN 200-110 (cross-hatched column; 100 nM, 30 min), tetrodotoxin (vertically striped column; 1 μM, 30 min) or indomethacin (horizontally striped column; 1 μM, 30 min) on changes of [^{3}H]inositol phosphate production. Values are means ± S.E.M. of 3 separate experiments. Statistical comparison to endothelin-1 in the absence of inhibitor is indicated by ** $P < 0.01$

3.5. Effects of endothelin-1 on spinal cord slices of WKY and SHR rats

Fig. 6 represents concentration-dependent increases of [^{3}H]inositol phosphate production evoked by endothelin-1 in spinal cord slices of 14–17 weeks old WKY and SHR. The EC_{50} was identical in both cases (2.1 nM) but the maximal effect was blunted in the SHR (α^E WKY = 1, α^E SHR = 0.79). At 1 μM endothelin-1, the [^{3}H]inositol phosphate production in SHR rats was 43% of that of the WKY rats.

4. Discussion

Endothelin-1 and endothelin-3 caused concentration-dependent increases of [^{3}H]inositol phosphate production although endothelin-1 had a greater potency and intrinsic activity. Kloog et al. (1989) reported that 1 μM sarafotoxin 6c induces a 2.5-fold increase in [^{3}H]inositol phosphate production in rat spinal cord slices which is in agreement with our results. Endothelin-induced [^{3}H]inositol phosphate production was shown in various regions of the central nervous system (Kloog et al., 1989; Crawford et al., 1990; Sokolovsky, 1993). The EC_{50} for endothelin-1-induced [^{3}H]inositol phosphate production in spinal cord slices (14 nM in Wistar and 2.1 nM in WKY) is in the same range than those observed for endothelin-1

in rat cerebral cortex (7 ± 2 nM; Crawford et al., 1990) and rat cerebellum (50 ± 5 nM; Sokolovsky, 1993).

4.1. Receptor subtype mediating [^{3}H]inositol phosphate production by endothelins

From the present results, it can be concluded that the effects of endothelins on [^{3}H]inositol phosphate production are mediated by an endothelin ET_A receptor on the basis of the following considerations: (1) the order of potency of the agonists: endothelin-1 > endothelin-3 in terms of potency and intrinsic activity while sarafotoxin 6c and IRL-1620, two endothelin ET_B receptor agonists, were found almost inactive; (2) BQ-788, a selective endothelin ET_B receptor antagonist, did not affect the endothelin-1-induced [^{3}H]inositol phosphate production; (3) BQ-123, a selective endothelin ET_A receptor antagonist, reduced in a concentration-dependent manner the endothelin-1- and endothelin-3-induced [^{3}H]inositol phosphate production. The efficiency of BQ-123 was enhanced when incubated for a longer period with the tissue (30 versus 15 min prior to endothelin-1). This could be explained by the fact that antagonist binding is more reversible than endothelin-1 binding to endothelin ET_A receptors as suggested by Wu-Wong et al. (1994). Previous binding studies have suggested the presence of endothelin ET_A-like receptors in the rat spinal cord (Gulati, 1991; Bertelsen et al., 1992). Moreover, this is in accordance with our previous functional study showing increases in mean arterial blood pressure after intrathecal injection of endothelins in conscious rats (Poulat et al., 1994). In the latter study, endothelin-1 was more potent than endothelin-3 and the effect of endothelin-1 was blocked by intrathecal pretreatment with BQ-123. Since the cellular locus of this receptor has not yet been studied, its presence on vessels, astrocytes and neurons has to be considered. It was reported that cerebrovascular smooth muscle cells and brain capillary endothelial cells are endowed with endothelin ET_A receptors which are coupled to inositol phosphate production (Vigne et al., 1990; Stanimirovic et al., 1994; Yu et al., 1995). Cerebellar granular cells and hypothalamic neurons in culture exhibit an increase of [^{3}H]inositol phosphate production in the presence of endothelins with a rank order of potency compatible with an endothelin ET_A receptor (Chuang et al., 1991; Krsmanovic et al., 1991). Conversely, endothelin ET_B receptors are found on neurons, astrocytes and microglial cells (Hama et al., 1992; Yamashita et al., 1994; Wu-Wong et al., 1995; Horie et al., 1995). Thus, we suggest that endothelin ET_A receptors in the spinal cord are located either on vessels or on neurons rather than on astrocytes. Further anatomical localization studies are required to address this issue.

The reduction of *myo*-[2-^{3}H]inositol incorporation observed with sarafotoxin 6c (10 μM) but not with IRL-1620 suggests that the former peptide interacts with a non-ET_A/non-ET_B binding site. Interestingly, different signalling pathways for endothelins and sarafotoxins, involving cGMP, nitric oxide and carbon monoxide have been recently demonstrated in rat cerebellar slices (Shraga-Levine et al., 1994).

4.2. Mediating factors of endothelin response

In rat spinal cord slices, tetrodotoxin completely blocked the [^{3}H]inositol phosphate production induced by endothelin-1, suggesting an indirect action through the release of other neurotransmitter(s). In various models, endothelins are found to stimulate the release of various transmitters which could be mediators for the activation of signalling pathways. In the pituitary, a prostaglandin-mediated effect was observed in endothelin-1-induced cAMP production (Domae et al., 1994). In brain capillary endothelial cells, endothelins release numerous prostanoids through the endothelin ET_A receptor activation (Stanimirovic et al., 1993). In our case, we can rule out this possibility since indomethacin did not alter the endothelin-1-induced [^{3}H]inositol phosphate production. Based on the observation that endothelin-1 enhances the release of [^{3}H]D-aspartate in cerebellar granule cells (Chuang et al., 1991) which was shown to increase [^{3}H] inositol phosphate production in striatal neurons (Manzoni et al., 1991), one cannot exclude amino acids as putative neurotransmitters of the indirect effect of endothelin on phosphoinositide hydrolysis.

On the other hand, [^{3}H]inositol phosphate production induced by endothelin-1 was not affected by a G_i/G_o-like G-protein inhibitor (pertussis toxin), suggesting that endothelin-1-induced [^{3}H]inositol phosphate production is rather mediated by a $G_q/11$ type of G-protein that is found in the spinal cord (Milligan, 1993). Similarly, pertussis toxin does not alter the endothelin-1-induced [^{3}H]inositol phosphate production in cultured cerebellar granule cells (Chuang et al., 1991). In rat cerebellar slices, phospholipase C activation is transduced specifically by a pertussis toxin-insensitive $G_q/11$ type of G-protein (Sokolovsky, 1993). However, the potentiation of maximal stimulation of phosphoinositide hydrolysis by endothelin-1 in the presence of pertussis toxin in rat cerebellum which was ascribed to an inhibitory G_i-like or G_o-like protein that may normally act to diminish phospholipase C activity (Sokolovsky, 1993) was not observed in spinal cord slices.

Endothelin-1-induced [^{3}H]inositol phosphate production is unlikely mediated by Ca^{2+} influx through voltage-dependent L- and R-type Ca^{2+} channels since PN 200-110, a new selective inhibitor of these channels (Bkaily et al., 1993), did not affect the response to endothelin-1.

4.3. Spinal cord endothelin-converting enzyme

Big endothelin-1 induced concentration-dependent increases of [^{3}H]inositol phosphate production similar to those induced by endothelin-1 but with a lower potency. From the present results, it may be postulated that big

endothelin-1 is partly cleaved by an endothelin-converting enzyme to yield endothelin-1 in the rat spinal cord. This is supported by the fact that preincubation with phosphoramidon, a neutral endopeptidase inhibitor thought to interfere with endothelin-converting enzyme activity (Fukuroda et al., 1990), or with [Phe^{22}]big endothelin-1–(19–37), a selective peptide inhibitor of endothelin-converting enzyme (Claing et al., 1995), reduced the [^{3}H]inositol phosphate production induced by big endothelin-1. [Phe^{22}]big endothelin-1-(19–37) was more potent than phosphoramidon since a similar inhibition could be achieved with 10 μM of the former and 300 μM of the latter. DL-thiorphan, another neutral endopeptidase inhibitor, also reduced the effect of big endothelin-1 albeit with a lesser efficacy than phosphoramidon. Likewise, it was shown in guinea pig lung and rat aortic smooth muscle cells that DL-thiorphan is less potent than phosphoramidon in reducing big endothelin-1-induced effects (Trapani et al., 1993; Battistini et al., 1995). However, in our study, DL-thiorphan did not increase the endothelin-1 response suggesting the absence of endothelinase neutral endopeptidase 24.11 in the spinal cord as reported in the lung (Battistini et al., 1995). Since residual responses to big endothelin-1 were still measured in the presence of endothelin-converting enzyme inhibitors, it is possible that both neutral endopeptidases and endothelin-converting enzyme cleave big endothelin-1 in the rat spinal cord. Again, this is in good agreement with our previous functional study showing a phosphoramidon-sensitive increase of mean arterial pressure following intrathecal injection of big endothelin-1 in the conscious rat (Poulat et al., 1994). Furthermore, these new data support the presence of endothelin-converting enzyme in the spinal cord, as previously suggested by Shinyama et al. (1991). Recently, it has been shown that a membrane-bound endothelin-converting enzyme is also present on cultured astrocytes (Deschepper et al., 1995).

4.4. Endothelin-1 response in WKY and SHR

It was observed that endothelin-1-induced [^{3}H]inositol phosphate production in the spinal cord of WKY and SHR was similar in terms of affinity (EC_{50}) but that the maximal effect (1 μM endothelin-1) was reduced in SHR. This is in agreement with binding studies showing no differences in the K_d value (0.22 ± 0.04 nM) for [^{125}I]endothelin-1 in the spinal cord of WKY and SHR (Gulati, 1991). Moreover, displacement studies showed that in rat spinal cord the affinity of endothelin-1 for endothelin-1 binding sites was similar in WKY and SHR (Gulati, 1991). The alteration of endothelin-1-induced maximal [^{3}H]inositol phosphate production in the SHR spinal cord is unlikely to be due to a decrease in the number of receptors since the B_{max} value (534.5 ± 30.7 fmol/mg protein in WKY) was similar in SHR (Gulati, 1991). The exact mechanism underlying the reduced maximal response to endothelin-1 on [^{3}H]inositol phosphate production remains to be elucidated. Its significance can be questioned since this phenomenon was seen at the highest concentration of 1 μM.

In conclusion, the stimulation by endothelins of [^{3}H]inositol phosphate production in rat spinal cord slices appears to be mediated by the activation of an endothelin ET_A receptor. While G_i/G_o G-protein, voltage-sensitive Ca^{2+} channels and prostaglandins are not involved in the endothelin-1 response, tetrodotoxin abolished it suggesting that this effect could be mediated through the release of another neurotransmitter. The results also highlight the presence of a phosphoramidon sensitive endothelin-converting enzyme in the rat spinal cord.

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