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Involvement of ET_A and ET_B receptors in the activation of phospholipase D by endothelins in cultured rat cortical astrocytes

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1 This study was performed to characterize the receptor subtypes involved in the endothelin stimulation of phospholipase D (PLD) in rat cortical astrocytes in primary culture. PLD activity was determined by measuring the formation of [^{32}P]phosphatidylbutanol in [^{32}P]orthophosphate prelabelled cells stimulated in the presence of 25 mM butanol.

2 The agonists endothelin-1 (ET-1), endothelin-3 (ET-3), sarafotoxin 6c (S6c) and IRL 1620 elicited PLD activation in a concentration-dependent manner. The potencies of ET-1, ET-3 and S6c were similar. The maximal effects evoked by the ET_B-preferring agonists, ET-3, S6c and IRL 1620, were significantly lower than the maximal response to the non-selective agonist ET-1.

3 The response to 1 nM ET-1 was inhibited by increasing concentrations of the ET_A receptor antagonist BQ-123 in a biphasic manner. A high potency component of the inhibition curve (24.2 ± 3.5% of the ET-1 response) was defined at low (up to 1 μM) concentrations of BQ-123, yielding an estimated K_i value for BQ-123 of 21.3 ± 2.5 nM. In addition, the presence of 1 μM BQ-123 significantly reduced the maximal response to ET-1 but did not change the pD_2 value.

4 Increasing concentrations of the ET_B selective antagonist BQ-788 inhibited the S6c response with a K_i of 17.8 ± 0.8 nM. BQ-788 also inhibited the effect of ET-1, although, in this case, two components were defined, accounting for approximately 50% of the response, and showing K_i values of 20.9 ± 5.1 nM and 439 ± 110 nM, respectively. The ET-1 concentration-response curve was shifted to the right by 1 μM BQ-788, also revealing two components. Only one of them, corresponding to 69.8 ± 4.4% of the response, was sensitive to BQ-788 which showed a K_i value of 28.8 ± 8.9 nM.

5 Rapid desensitization was achieved by preincubation with ET-1 or S6c. In cells pretreated with S6c neither ET-3 nor S6c activated PLD, but ET-1 still induced approximately 40% of the response shown by non-desensitised cells. This remaining response was insensitive to BQ-788, but fully inhibited by BQ-123.

6 In conclusion, endothelins activate PLD in rat cortical astrocytes acting through both ET_A and ET_B receptors, and this response desensitizes rapidly in an apparently homologous fashion. The percentage contribution of ET_A and ET_B receptors to the ET-1 response was found to be approximately 20% and 80%, respectively, when ET_B receptors were not blocked, and 30–50% and 50–70%, respectively, when ET_B receptors were inhibited or desensitized. These results may be relevant to the study of a possible role of PLD in the proliferative effects shown by endothelins on cultured and reactive astrocytes.

Keywords: Endothelin; phospholipase D; astrocytes; ET_A receptor; ET_B receptor

Introduction

Endothelins are a family of three peptides named ET-1, ET-2 and ET-3 (Inoue *et al*., 1989) that were first identified in vascular tissue and recognized by their potent vasoconstrictor activity (Yanigisawa *et al*., 1988). Their actions are mediated by at least two receptors, ET_A and ET_B, that have been cloned (Arai *et al*., 1990; Sakurai *et al*., 1990) and characterized on the basis of their affinities for the different endothelins and their blockade by selective antagonists (Bax & Saxena, 1994). ET_A receptors show higher affinity for ET-1 than for ET-3 and can be blocked by the antagonist BQ-123, whereas ET_B receptors show similar affinities for ET-1 and ET-3, are stimulated by selective agonists such as the structurally related peptide sarafotoxin 6c (S6c) and the partial agonist IRL 1620 and are inhibited by the antagonist BQ-788.

ET-1 and ET-3 are also synthesized in the mammalian central nervous system where these peptides and their receptors are widely distributed, being present in various cell types including both neurons and glial cells (for a recent review see Kuwaki *et al*., 1997). In astrocytes, endothelins have been reported to induce DNA synthesis and cell proliferation (Supattapone *et al*., 1989; MacCumber *et al*., 1990; Stanimirovic *et al*., 1995; Lazarini *et al*., 1996), suggesting a role for endothelins in the development of reactive gliosis. In addition, several signal transduction pathways have been shown to be regulated by endothelins in astrocytes. These include activation of phosphoinositide phospholipase C (PLC; Lin *et al*., 1990; MacCumber *et al*., 1990; Marsault *et al*., 1990; Marin *et al*., 1991; Stanimirovic *et al*., 1995), increase of intracellular free Ca^{2+} concentration (Supattapone *et al*., 1989; Marsault *et al*., 1990; Goldman *et al*., 1991; Marin *et al*., 1991; Stanimirovic *et al*., 1995), stimulation of protein kinase C (PKC; Stanimirovic *et al*., 1995), activation of phospholipase A_2 (Tencé *et al*., 1992), inhibition of cyclic AMP accumulation (Marin *et al*., 1991; Lazarini *et al*., 1996), and phosphorylation and activation of mitogen-activated protein kinase (Cazaubon *et al*., 1993; Kasuya *et al*., 1994; Lazarini *et al*., 1996).

Various of these works provide data suggesting that these responses are mediated by ET_B receptors (Marsault *et al*.,

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1990; Kasuya *et al*., 1994; Lazarini *et al*., 1996). This is consistent with results showing that, in the intact tissue, brain astrocytes express exclusively the ET_B receptor mRNA (Hori *et al*., 1992). However, in cultured astrocytes, where the above responses have been studied, Northern blot analysis and radioligand binding studies indicate that both ET_A and ET_B receptors are expressed (Lazarini *et al*., 1996; Sasaki *et al*., 1997). Moreover, Stanimirovic *et al*. (1995) have reported that endothelin activation of PLC, PKC and DNA synthesis in cultured astrocytes is inhibited by the ET_A antagonist BQ-123 and Sasaki *et al*. (1997) have shown that the endothelin induced glutamate efflux in cultured astrocytes is partially resistant to ET_B receptor antagonists and completely inhibited by the simultaneous blockade of the two receptor subtypes, suggesting a role for ET_A receptors in this response.

It has been recently described that, in mouse striatal astrocytes, endothelins also activate phospholipase D (PLD; Desagher *et al*., 1997). This enzyme hydrolyzes membrane phospholipids, mainly phosphatidylcholine, yielding phosphatidate and the polar head group. PLD can be stimulated by a wide variety of receptor agonists as well as by stimulators of PKC and its activation has been related to the regulation of cell proliferation and vesicular trafficking (Boarder, 1994; Exton, 1997). Previous studies carried out in rat hippocampal slices showed that PLD was activated by ET-1 and ET-3 with similar potencies as well as by the selective ET_B agonist S6c (Sarri *et al*., 1995). Similarly, in mouse striatal astrocytes, ET-1 and ET-3 displayed the same potency (Desagher *et al*., 1997). Although these results suggest that endothelin activation of PLD in astrocytes is mediated by ET_B receptors, a more complete pharmacological characterization is required to verify the role of ET_A receptors, also present in these cells, in the stimulation of PLD. In this work we have characterized the receptors involved in PLD activation by endothelins in primary cultures of astrocytes from rat brain cortex by using non-selective (ET-1) and selective (ET-3, S6c, IRL 1620) agonists as well as selective antagonists (BQ-123, BQ-788). We have also characterized the response after specific ET_B receptor desensitization.

Methods

Cell culture

Primary cultures of cerebral cortical astrocytes were prepared from newborn (<24 h) Sprague-Dawley rats by the method of Hamprecht & Löffler (1985) with minor modifications. Animals were decapitated and their cerebral cortices were immediately isolated. After removing meninges and blood vessels, the tissue was minced and incubated in Ca^{2+}-free buffer containing 0.025% trypsin for 15 min at 37°C. Cells were then dissociated by gentle trituration through a fire-polished Pasteur pipette in the presence of 0.52 mg/ml soybean trypsin inhibitor (SBTI) and 170 units/ml DNAseI. The cell suspension was then filtered through a 40-μM nylon mesh. Cells were collected by centrifugation at 500 *g* and plated into 24-well plates at a density of 0.5×10^6 viable cells/ml in Basal Eagle's Medium (BEM) supplemented with 10% fetal calf serum (FCS), 33 mM glucose, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2/95% air. After 24 h, the medium was changed to remove non-adhered cells and then changed every 3–4 days. Cells were used for experiments after 20–30 days *in vitro*. At this time immunocytochemical staining for glial fibrillary acidic protein (GFAP) performed as detailed previously (Castellano *et al*., 1991) revealed that cultures consisted of 90–95% astrocytes. Each independent experiment was performed using a separate culture.

Determination of PLD activity

PLD was assayed by measuring the formation of [^{32}P]phosphatidylbutanol ([^{32}P]PtdBut) from prelabelled phospholipids by the PLD catalyzed transphosphatidylation reaction in the presence of butanol, essentially as described by Sarri *et al*. (1995). Phospholipids were labelled by incubating astrocytes with [^{32}P]orthophosphoric acid ([^{32}P]P_i; 1.5 μCi/well) in 300 μl of Krebs-Henseleit buffer (KHB) without added P_i (in mM: NaCl 116.0, KCl 4.7, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, $CaCl_2$ 1.3, and glucose 11.0) for 4 h at 37°C in an atmosphere of 5% CO_2/95% air. This procedure was selected to avoid the heterogeneous labelling of different molecular species of phosphatidylcholine found when using preincubation with precursor ^{3}H-fatty acids (Sarri *et al*., 1996). After the labelling period, cells were rinsed twice with KHB to remove non-incorporated [^{32}P]P_i and cells were incubated for the indicated times with agonists in the presence of butanol (final concentration 25 mM). When appropriate, astrocytes were preincubated for 30 min with antagonists before the addition of agonists and butanol. In desensitization experiments, astrocytes were preincubated for the indicated times with agonists and then were rinsed extensively with KHB before rechallenging with agonists and butanol. The reaction was stopped by adding 800 μl of ice cold methanol/HCl (98 : 2, v/v) and cells were scrapped and transferred to test tubes. Two phases were generated by adding 900 μl of chloroform and 750 μl of water. After 5-min centrifugation at 2,000 *g*, the upper phases were aspirated, and the lower (organic) phases containing ^{32}P-lipids were washed with 1.55 ml methanol/water (1 : 1, v/v). Organic phases were centrifuged under vacuum to evaporate the solvent. Lipids were resuspended in 10 μl chloroform/methanol (4 : 1, v/v) and spotted onto silica gel-60 HPTLC plates with concentrating zones that were developed with chloroform/methanol/acetic acid (65 : 15 : 2, by vol). [^{32}P]-PtdBut (R_f 0.45) and the rest of ^{32}P-phospholipids (R_f 0.05–0.30) were quantified in a GS-525 Molecular Imager System (Bio-Rad). To correct for sample size and interexperimental variations of [^{32}P]P_i labelling, accumulation of [^{32}P]PtdBut was expressed as the percentage of total radioactivity incorporated into the lipids present in the organic phase. Triplicate samples were always included in which butanol was omitted. The radioactivity present at the PtdBut position in butanol-free controls was considered to be background radioactivity and was subtracted from the value determined for each butanol-containing sample.

Data analysis

Concentration-response and inhibition curves were analysed by non-linear regression with the program GRAPHPAD PRISM (GraphPad Software Inc.). Each concentration response curve was fitted to a logistic function of the form: $E = E_{max}[A]^n/([A]^n + (EC_{50})^n)$ where [A] is the agonist concentration, E is the agonist effect, E_{max} the maximal response, n the Hill coefficient and EC_{50} the agonist concentration giving half the maximal response. Similarly, individual inhibition curves were fitted to a logistic function of the form: Percent inhibition $= 100[B]^n/([B]^n + (IC_{50})^n)$ where [B] is the antagonist concentration and IC_{50} the antagonist concentration causing 50% inhibition of the agonist response. Both EC_{50} and IC_{50}

values were actually estimated as logarithms and EC_{50} was expressed as $pD_2(-\log(EC_{50}))$. The computed parameter estimates for each group of *n*-replicated experiments were expressed as mean ± s.e.mean and, for display purposes, they were used to generate a logistic curve that was drawn through the experimental data.

Curves yielding Hill coefficients significantly lower than unity were fitted to models of both one and two components. The best fit was taken as the one showing the lowest residual sum of squares and the significance of the difference between the two models was assessed by the Fisher's *F* test. Antagonist K_i values were calculated from either the IC_{50} values found in the inhibition curves or from the dose-ratio values estimated from the displacement by the antagonist of the concentration-response curves. Statistical significance of differences between values was evaluated by performing analysis of variance (ANOVA) followed by Scheffé's test for multiple comparisons. Significance was taken at $P<0.05$.

Materials

ET-1, ET-3, S6c, BQ-123, BQ-788, IRL 1620 were purchased from Alexis (France). BEM, FCS, penicillin, streptomycin, glutamine were obtained from Gibco. Phorbol myristate acetate, noradrenaline, trypsin, SBTI, DNAseI were purchased from Sigma. Silica gel-60 HPTLC plates with concentrating zones were from Merck. [^{32}P]orthophosphate acid (carrier free) was purchased from Amersham. Other chemicals used were of analytical grade.

Results

PLD activation by ET receptor agonists

Preliminary experiments showed that ET-1 induced formation of [^{32}P]PtdBut in astrocytes increased with time reaching a maximum at 15 min with a slight decrease thereafter (Figure 1). Subsequent experiments were performed using 10-min incubation with agonists.

Concentration-response curves were generated for ET-1, ET-3, S6c and IRL 1620 (Figure 2) and their analysis by non-linear regression allowed the estimation of EC_{50} and E_{max} values as well as Hill coefficients, the latter being not significantly different from unity in all cases (1.10 ± 0.10, 0.99 ± 0.08, 1.05 ± 0.09, 1.01 ± 0.11 for ET-1, ET-3, S6c and IRL 1620, respectively; $n=3$). The four agonists stimulated PLD with potencies in the nanomolar range (pD_2 values: 9.69 ± 0.23, 9.74 ± 0.27, 9.22 ± 0.20, 8.55 ± 0.16 for ET-1, ET-3, S6c and IRL 1620, respectively), but ET-1 showed a significantly higher E_{max} than the other agonists. The E_{max} values for ET-3, S6c and IRL 1620 were, respectively, $82.7\pm2.7\%$, $80.1\pm1.4\%$ and $60.9\pm2.4\%$ of the maximal accumulation of [^{32}P]PtdBut elicited by ET-1 ($5.84\pm0.13\%$ of ^{32}P-lipids; $P<0.05$).

Effect of ET receptor antagonists

The effect of antagonists was first studied by determining the PLD activation elicited by either 1 nM ET-1 or 3 nM S6c in the presence of increasing concentrations of either BQ-123 or BQ-788. The ET-1 response was inhibited by BQ-123 in a biphasic manner (Figure 3a). One component was defined by BQ-123 concentrations up to 1 μM and non-linear regression analysis allowed estimation of a K_i value of 21.3 ± 2.5 nM and a percent contribution to the total response of $24.2\pm3.5\%$ ($n=3$). The same BQ-123 concentrations did not antagonize the S6c response (not shown). On the other hand, BQ-788 inhibited the response induced by both S6c and ET-1 (Figure 3b). In the case of S6c the curve showed a single component, which defined a K_i value of 17.8 ± 0.8 nM ($n=3$). In contrast, the inhibition of ET-1 effect by BQ-788 was best fitted to two components (Fisher's *F* test, $P<0.005$ in each individual curve) that accounted for $50.5\pm9.7\%$ and $49.5\pm9.7\%$ of the

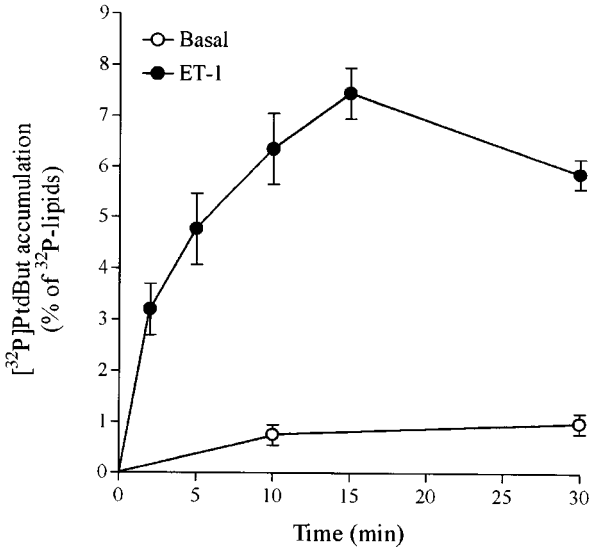

Figure 1 Time-course of [^{32}P]PtdBut accumulation in ET-1 stimulated rat astrocytes. [^{32}P]P_i-labelled cells were incubated for the indicated times under basal conditions or with 10 nM ET-1 in the presence of 25 mM butanol. [^{32}P]PtdBut levels were determined as described in Methods and expressed as per cent of total ^{32}P-lipids. Values are mean ± s.e.mean of two independent experiments performed in triplicate.

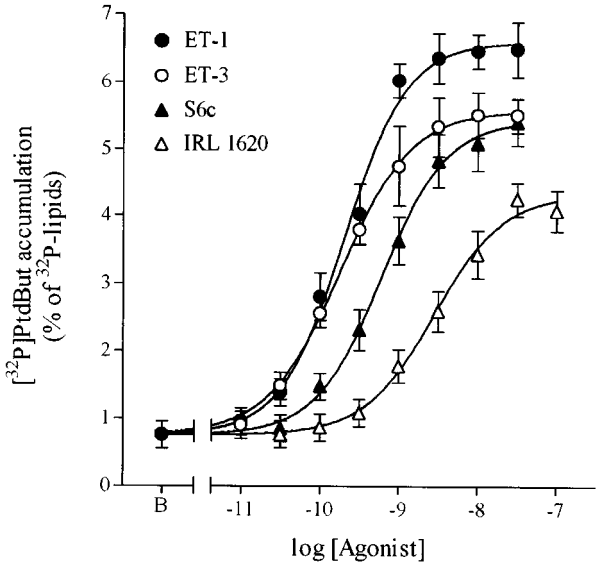

Figure 2 Concentration-response curves for the stimulation of PLD by endothelin-receptor agonists. [^{32}P]P_i-labelled astrocytes were incubated for 10 min with the indicated concentrations of ET-1, ET-3, S6c, and IRL 1620 in the presence of 25 mM butanol. B:PLD activity in the absence of agonists. PLD activity was determined by measuring the accumulation of [^{32}P]PtdBut, which was expressed as per cent of ^{32}P-lipids. Values are mean ± s.e.mean of three independent experiments performed in triplicate.

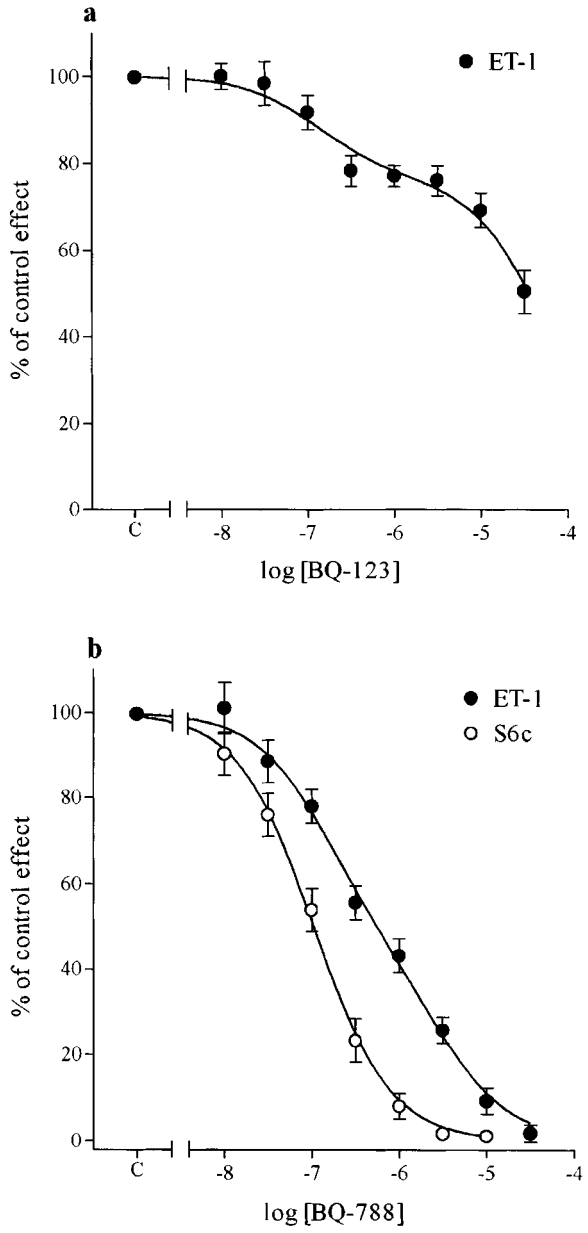

Figure 3 Inhibition of ET-1 and S6c induced PLD stimulation by increasing concentrations of antagonists. [^{32}P]P_i-labelled astrocytes were incubated for 30 min with the indicated concentrations of BQ-123 (a) or BQ-788 (b) before the addition of either 1 nM ET-1 or 3 nM S6c. After subtracting basal PLD activity, each value was normalized to the effect of the corresponding agonist in the absence of antagonists (control, C; ET-1: 5.18±0.26%; S6c: 4.40±0.22%). Values are mean±s.e.mean of three experiments performed in triplicate.

response and showed K_i values of 20.9±5.1 nM and 439±110 nM, respectively ($n=3$).

The experiments shown in Figure 3 indicate that 1 μM BQ-123 blocks the first component of the ET-1 response and 1 μM BQ-788 inhibits more than 90% of the effect of the ET_B selective agonist S6c. Thus, these concentrations of antagonists were selected to determine the effect of both compounds on the response to single concentrations of agonists. As shown in Table 1, the presence of either 1 μM BQ-123 or 1 μM BQ-788 reduced the response to 1 nM ET-1 to 78.1±0.6% or 42.4±1.5% ($P<0.05$), respectively, of its value in the absence of antagonists, and complete inhibition was observed when both antagonists were present. In contrast, the response to 3 nM ET-3 remained unchanged in the presence of 1 μM BQ-123 and the effect of either 3 nM ET-3, 3 nM S6c or 100 nM IRL 1620 was fully inhibited by 1 μM BQ-788.

To further investigate the involvement of endothelin receptor subtypes in PLD activation we determined the effect of the selective antagonists on the ET-1 concentration-response curve (Figure 4). In the presence of 1 μM BQ-123 the E_{max} was significantly lower than in the absence of antagonist (82.1±2.1%; $P<0.05$; $n=3$) with no significant change in the agonist potency (pD_2 in the absence and presence of BQ-123; 9.75±0.06 and 9.69±0.09 respectively). In contrast, 1 μM BQ-788 shifted the concentration-response curve to the right, although its effect was more prominent at higher agonist concentrations (Figure 4). In the presence of BQ-788, the curve was best fitted to a two-component model (Fisher's F test, $P<0.001$ in each individual curve; $n=3$). The first component accounted for 30.2±4.4% of the E_{max} and showed a pD_2 value of 9.90±0.15, which was not significantly different from that shown by ET-1 alone. The second component was antagonized by 1 μM BQ-788 showing a pD_2 value of 8.35±0.12. Dose-ratio values were calculated from the pD_2's for the second component and those shown by ET-1 alone in each independent experiment, allowing estimation of a K_i value for BQ-788 of 28.8±8.9 nM.

Desensitization of ET receptors

Preincubation of astrocytes with 50 nM ET-1 resulted in a time-dependent desensitization of the endothelin induced PLD activation with an almost complete loss of the response after 30 min of incubation (Figure 5). In contrast, approx. 40% of the response remained unaltered after 1 h preincubation with 50 nM S6c. Desensitization was rapid in both cases with half of the response loss being achieved after 5 and 7 min of incubation with ET-1 and S6c respectively. The effect seemed to be restricted to endothelin receptors since 1 h preincubation with 50 nM ET-1 decreased the activation of PLD by other agents such as noradrenaline and the PKC activator phorbol myristate acetate by 14.0±5.8% and 12.3±4.6%, respectively

Table 1 Effect of endothelin-receptor antagonists on PLD stimulation by agonists in cortical astrocytes

	Control	*1 μM BQ-123*	*1 μM BQ-788*	*1 μM BQ-123+1 μM BQ-788*
ET-1 (1 nM)	100	78.1±0.6 (6)*	42.4±1.5 (6)*	5.3±2.5 (3)*†
ET-3 (1 nM)	84.0±3.0 (3)	82.9±3.2 (3)	6.6±2.0 (3)*†	nd
S6c (3 nM)	82.1±3.5 (3)	81.0±2.0 (3)	8.1±3.1 (3)*†	nd
IRL 1620 (10 nM)	55.5±4.0 (3)	nd	1.9±1.7 (3)*†	nd

[^{32}P]P_i-labelled astrocytes were incubated in the absence (control) or presence of 1 μM BQ-123, 1 μM BQ-788 or both for 30 min before the addition of the indicated agonists. Accumulations of [^{32}P]PtdBut were normalized as per cent of the ET-1 induced increase of PLD activity (5.41±0.13%, $n=9$). Values are mean±s.e.mean of the number of experiments given in parenthesis performed in triplicate. nd, not determined. *$P<0.05$ compared to the respective control. †$P>0.05$ compared to basal.

($n=3$). Preincubation with 50 nM S6c for 30 min resulted in a 58.2±3.6% decrease of the maximal ET-1 response without a change in its potency (pD_2 before and after S6c preincubation: 9.71±0.08 and 9.75±0.10 respectively, $n=3$; Figure 6).

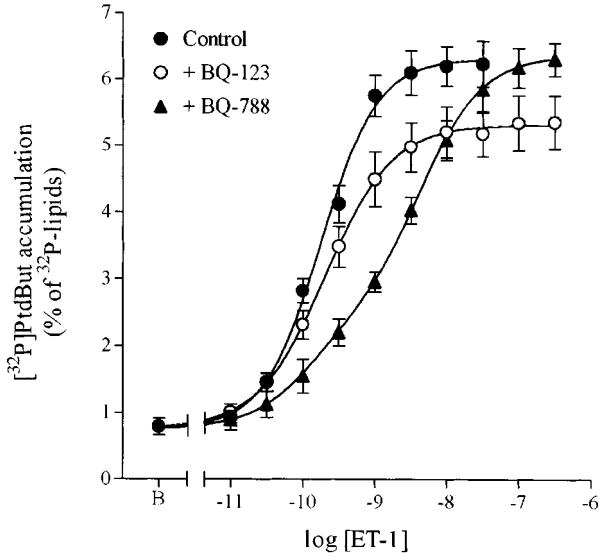

Figure 4 Effect of BQ-123 and BQ-788 on the ET-1 concentration-response curve for PLD stimulation. Concentration-response curves were generated in [^{32}P]-P_i-labelled astrocytes incubated for 30 min in the absence or presence of either 1 μM BQ-123 or 1 μM BQ-788 and then exposed for 10 additional min to the indicated concentrations of ET-1. B: PLD activity in the absence of agonists. Values are mean±s.e.mean of three experiments performed in triplicate.

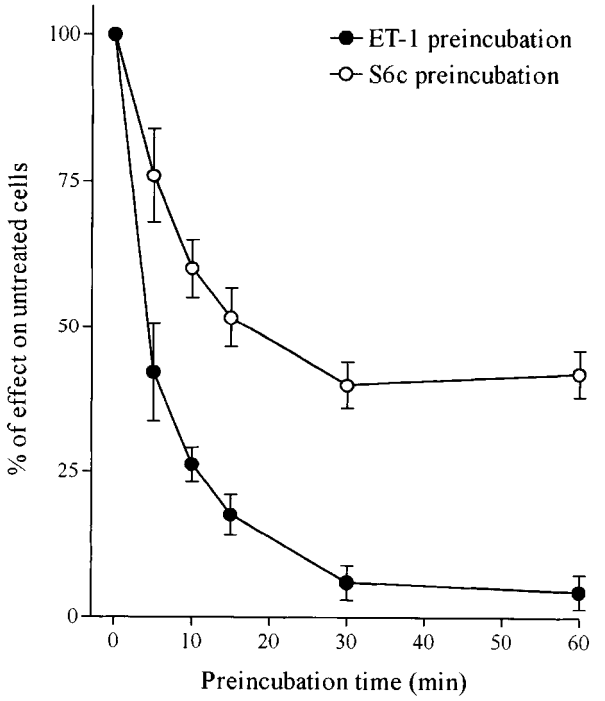

Figure 5 Time-course of desensitization of ET-1 and S6c activation of PLD. [^{32}P]P_i-labelled astrocytes were pretreated for the indicated times with 30 nM ET-1 or 30 nM S6c, extensively washed, and then challenged for 10 min with 10 nM ET-1. After subtracting basal PLD activity, each value was expressed as per cent of the effect of ET-1 in untreated cells (5.05±0.20%). Values are mean±s.e.mean of three experiments performed in triplicate.

Finally, in S6c preincubated cells, neither ET-3 nor S6c at 10 nM stimulated PLD and the response to 10 nM ET-1 was insensitive to 1 μM BQ-788 and fully inhibited by 1 μM BQ-123 (Table 2).

Discussion

The results presented in this work provide evidence for the involvement of both ET_A and ET_B receptors in the activation of PLD by endothelins in rat cortical astrocytes in primary culture. The similar pD_2 values of ET-1 and ET-3 and the stimulation of PLD by S6c and IRL 1620 indicate the involvement of ET_B receptors in the response. However, the ET_B selective agonists, ET-3 and S6c, elicited only part of the full effect induced by the non-selective agonist ET-1, raising the possibility that other receptor subtypes may have a role. The E_{max} of ET-3 and S6c most likely represents the ET_B contribution to the total ET-1 response, since the ET-3 effect

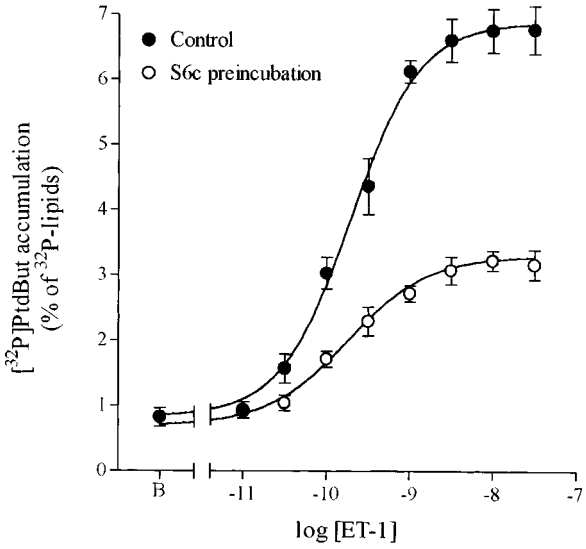

Figure 6 Effect of S6c preincubation on the ET-1 concentration-response curve. [^{32}P]P_i-labelled astrocytes were preincubated for 60 min without or with 30 nM S6c, extensively washed, and then challenged with the indicated concentrations of ET-1 for 10 min. B: PLD activity in the absence of agonists. Values are mean±s.e.mean of three experiments performed in triplicate.

Table 2 Characteristics of the PLD activation by endothelin-receptor agonists after desensitization with S6c

	Control	*S6c preincubation*	n
ET-1 (1 nM)	100	41.7±0.5*	5
+BQ-123 (1 μM)	78.2±2.5	0.7±2.0*†	3
+BQ-788 (1 μM)	41.1±4.1	36.0±3.0	3
ET-3 (1 nM)	84.0±3.0	2.5±1.7*†	3
S6c (3 nM)	83.5±2.1	0.6±1.5*†	5

[^{32}P]P_i-labelled astrocytes were incubated for 60 min in the absence (control) or presence of 50 nM S6c. After extensive washing the cells were stimulated with the indicated agonists for 10 min. When present, antagonists were added 30 min before the agonist. All PLD activities were normalized as per cent of the activation achieved by ET-1 in control cells (5.25±0.25%, $n=5$). Values are mean±s.e.mean of n experiments performed in triplicate. *$P<0.05$ compared to the respective control. †$P>0.05$ compared to basal.

was not prevented by 1 μM of the selective ET_A antagonist BQ-123 but the same concentration of the ET_B antagonist BQ-788 inhibited PLD activation by ET-3 and S6c. On the other hand, the ET-1 effect was only partially blocked by 1 μM of each of the antagonists and fully inhibited only when both were present, suggesting that in this case both ET_A and ET_B receptors are involved in the response.

The inhibitory effect of BQ-123 was apparently non-competitive, inducing a decrease of the E_{max} of ET-1 with no change of its pD_2. Similar results have been shown for the increase of intracellular free Ca^{2+} concentration induced by ET-1 in human neuroblastoma (Hiley *et al*., 1992) and rat brain capillary endothelial cells (Vigne *et al*., 1993). A possible explanation is given in this latter report suggesting a low dissociation rate for the interaction of BQ-123 with ET_A receptors, which would result in a quasi-irreversible inhibition when the antagonist is added to the tissue some time before the agonist, as in the present work. The inhibition constant for BQ-123 was estimated from the inhibition of the response to 1 nM ET-1 by increasing concentrations of the antagonist. A high affinity component was defined at BQ-123 concentrations up to 1 μM, with an estimated K_i value similar to those reported for the affinity of ET_A receptors for BQ-123 (Bax & Saxena, 1994). The size of this component ($24.2 \pm 3.5\%$) was not significantly different to the decrease elicited in the E_{max} of ET-1 by micromolar concentrations of BQ-123 ($17.9 \pm 2.1\%$) neither to the difference between the maximal effect of ET-1 and those of ET-3 and S6c ($17.3 \pm 2.7\%$ and $19.9 \pm 1.4\%$ respectively). Thus, the above results indicate that the ET_A receptors contributed approx. 20% to the total ET-1 response.

Two components of the ET-1 effect were revealed by BQ-788 both in the displacement by the antagonist of the ET-1 concentration response curves and in the curves for inhibition of the ET-1 response by increasing concentrations of BQ-788. One of these components, accounting for 50–70% of the total effect, most probably corresponds to the contribution of ET_B receptors, since it was antagonized by BQ-788 with low K_i values (20.9–28.8 nM). These values were similar to that shown by BQ-788 when the S6c response was blocked by increasing concentrations of this antagonist and to the value reported for the inhibition by BQ-788 of the ET_B receptor mediated contraction in human bronchi (Fukuroda *et al*., 1996).

Additional evidence on the participation of both receptor subtypes in ET-1 activation of PLD in astrocytes was provided by results from desensitization experiments. Desensitization was rapid and its extent depended on the agonist used in the preincubation. Whereas 60 min preincubation with ET-1 caused the total loss of response to a subsequent ET-1 challenge, approx. 40% of the response remained after S6c preincubation. This difference suggests that the non-selective agonist ET-1 desensitizes the response mediated by both receptor subtypes, whereas pretreatment with the ET_B selective agonist S6c only removes the response mediated by ET_B receptors. This interpretatioan was confirmed by results showing that, after S6c preincubation, PLD was not activatd by ET-3 or S6c and the effect of ET-1 was inhibited by BQ-123 and not by BQ-788, thus indicating that the remaining response is mediated by ET_A receptors.

Our results show some discrepancy regarding the percentage contribution of each receptor subtype to the ET-1 response. In experiments where the ET_B mediated effect was not inhibited, the participation of ET_A and ET_B receptors accounted for approximately 20% and 80% respectively. On the other hand, when ET_B receptors were antagonized or desensitized, the estimated contributions of ET_A and ET_B receptors ranged between 30–50% and 50–70% respectively. In rat striatal astrocytes, ET_A and ET_B receptors represent approx. 20% and 80% of total [^{125}I]ET-1 specific binding sites (Lazarini *et al*., 1996), and Northern blot analysis has shown that the ratio of ET_A and ET_B receptor mRNAs in rat cortical astrocytes is approx. 1/3 (Sasaki *et al*., 1997). We do not know whether these values actually represent the densities of ET receptor subtypes in rat cortical astrocytes. Nevertheless, the higher ET_A response found after inhibition of the ET_B mediated effect might represent either a compensatory enhanced efficacy of ET-1 acting through ET_A receptors or an unmasking of the true extent of the ET_A effect, which would be only partially expressed when ET_B receptors are acting.

The presence of functionally active ET_A and ET_B receptors in rat cortical astrocytes in culture shown here is in contrast with data indicating that quiescent astrocytes in the intact brain express only ET_B receptors (Hori *et al*., 1992). However, reactive astrocytes proliferating after lesions involving cerebrovascular damage also express ET_A receptors and show increased ET-1 immunoreactivity (Sakurai-Yamasita *et al*., 1997). Moreover, endothelin receptor antagonists inhibit astrocyte proliferation induced by spinal cord lesions, strongly suggesting a proliferative role for endogenous endothelins in reactive gliosis (Uesugi *et al*., 1996), which is consistent with the reported mitogenic action of endothelins in proliferative astrocytes in culture (Supattapone *et al*., 1989; MacCumber *et al*., 1990; Stanimirovic *et al*., 1995; Lazarini *et al*., 1996). In this context, the possible functional significance of our results lies in the proposed involvement of PLD activation in the mitogenic actions of endothelins and other agonists (Boarder, 1994). A role for PLD in these mitogenic effects has been suggested in the case of the proliferative actions of ET-1 and angiotensin II in vascular smooth muscle cells (Wilkes *et al*., 1993; Wilkie *et al*., 1995) and those of acetylcholine in rat cortical astrocytes (Guizzetti & Costa, 1996), where exogenously added phosphatidate increases DNA synthesis (Pearce *et al*., 1994). In addition, a good correlation between the time-courses of agonist activation of PLD and cell proliferation has been reported in chicken astrocytes (Mangoura *et al*., 1995). Taken together, these data raise the possibility that the activation of DNA synthesis by endothelins and other agonists in cultured astrocytes as well as in astrocytes accumulating after lesions of central nervous system might require the stimulation of PLD as part of the mechanism involved.

In conclusion, we have shown that endothelins activate PLD in rat cortical astrocytes acting through both ET_A and ET_B receptors and that this response desensitizes rapidly in an apparently homologous fashion. This action could play some role in the proliferative effects shown by endothelins on cultured astrocytes and on reactive glia proliferating after brain lesions.

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