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ENDOTHELINS INHIBIT ADENYLATE CYCLASE IN BRAIN CAPILLARY ENDOTHELIAL CELLS

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The action of endothelins (Et) on cAMP formation was studied in endothelial cells from rat brain microvessels. Et-1 and Et-3 had no action by themselves. They both inhibited cholera toxin stimulated adenylate cyclase by about 50%. $K_{0.5}$ values were observed at 2 nM and 40 nM for Et-1 and Et-3 respectively, indicating an involvement of a low affinity Et-3 receptor. Coupling to adenylate cyclase was achieved by a pertussis toxin sensitive mechanism. Another action of endothelins in brain capillary endothelial cells was to stimulate phospholipase C. This action involved a low affinity Et-3 receptor and a pertussis toxin insensitive mechanism. It is concluded that in brain capillary endothelial cells, ET_A like receptors are coupled to phospholipase C and to adenylate cyclase via two different mechanisms.

Endothelins belong to a family of 21 aminoacid residue peptides with important cardiovascular actions [1]. The existence of several endothelin receptors was first hypothesized from pharmacological analyses [2,3] and then confirmed by the cloning and sequencing of two types of receptor molecules [4,5]. The ET_A receptor subtype has a high affinity for Et-1 and a low affinity for Et-3 [4]. It is coupled to phospholipase C. It is responsible for the actions of endothelins on vascular smooth muscle cells and on atrial cells [6,7]. The non selective ET_B receptor subtype is also coupled to phospholipase C [5] and is mainly expressed in neurons and in astrocytes [8,9].

Endothelial cells from rat brain microvessels (BCEC) express two types of receptor sites for endothelins: (i) a high capacity (Bmax=400 fmol/mg of protein), ET_A like, receptor that is positively coupled to phospholipase C [10] and (ii) a low capacity (Bmax=30 fmol/mg of protein), non selective, ET_B like, receptor that controls Na^+/H^+ exchange activity [11]. This

Abbreviations: BCEC; brain capillary endothelial cells, Et; endothelin, PTX; pertussis toxin, CT; cholera toxin.

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paper provides further informations about the intracellular actions of endothelins in BCEC. We show (i) that endothelins inhibit CT stimulated adenylate cyclase and (ii) that this action is mediated by ET_A like receptors.

MATERIALS AND METHODS

Et-1 and Et-3 were from Peninsula (St Helens, Merseyside, U.K.). *Bordetella pertussis* toxin and CT were from the Sigma Chemical Co.

Cloned endothelial cells from rat brain microvessels were used in this study. Cells from early passages stained with an anti-Factor VIII antibody but not with an anti-glial fibrillary protein antibody and grew as sheets with occasional domes. They stably express an amiloride sensitive cationic channel [12] and two types of receptor sites for endothelins [10,11]. BCEC were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 200 units/ml penicillin and 50 µg/ml of streptomycin at 37°C in a 95 % air, 5 % CO_2 humidified atmosphere. Passages <15 were used in this study.

Inhibition of adenylate cyclase was assayed by first inbubating BCEC at 37°C in a serum free Dulbecco's modified Eagle's medium buffered at pH 7.4 with 10 mM Hepes and supplemented with 100 ng/ml CT. Control experiments were performed using parallel incubations in the absence of CT. After 40 minutes, isobutylmethylxanthine (1 mM final) was added and the incubation was allowed to proceed for an additional 20 minute period. Endothelins were then added for 20 to 30 minutes. The incubation solution was aspirated off and cells extracted with 10 % (w/v) ice cold trichloroacetic acid. Supernatants were assayed for cAMP using the [^{3}H]cAMP assay kit from Amersham. In experiments using PTX, cells were treated for 4 hours with 100 ng/ml PTX prior to the experiments.

Endothelin stimulated phospholipase C activity was measured as the production of total inositol phosphates. Confluent monolayers of BCEC grown in 6-well tissue culture dishes were labelled with 1 µCi/ml of myo-[2-^{3}H]inositol (18.9 Ci/mmol, Amersham) for 24 hours in Dulbecco's modified Eagle's medium supplemented with 0.1% fetal bovine serum. Cells were equilibrated at 37°C in a serum free Dulbecco's modified Eagle's medium buffered at pH 7.4 with 10 mM Hepes, exposed to 20 mM LiCl for 20 minutes and then to endothelins. After 30 minutes, cells were extracted with 1 ml of ice cold 10 mM formic acid. The supernatants were neutralized with Tris, diluted to 4 ml with distilled water and loaded onto AG 1X8 columns (200-400 mesh formate form, Bio Rad) with a bed volume of 500 µl. After eluting the column with 3 ml water and 4 ml 40 mM ammonium formate total inositol phosphates were eluted with 3 x 2 ml of 900 mM ammonium formate (pH 5).

Cell proteins were assayed according to Hartree [13] after solubilization of the cell layers into 0.1 N NaOH.

Means ± SEM are indicated.

RESULTS AND DISCUSSION

An exposure of BCEC to 100 ng/ml CT increased cAMP levels about 30 fold from 91 ± 6 pmol/mg of protein (n=48) to 2.85 ± 0.15 nmol/mg of protein (n=60). Figure 1 shows that Et-1 and Et-3 reduced CT stimulated cAMP formation. Mean inhibitions achieved were 50 % (n=29) and 33 % (n=12) for 100 nM Et-1 and 100 nM Et-3 respectively. The action of endothelin on CT activated cAMP formation was already detected 1 minute after the addition of the peptide. Maximum inhibition was reached after 10 minutes and remained stable for at least an additional 20 minute period (not shown). BCEC express high levels of β–adrenoceptors that are functionally coupled to adenylate cyclase [14]. Isoproterenol (10 µM) stimulated

adenylate cyclase was also reduced by 46 % in the presence of 100 nM Et-1. Finally, the right panel of Figure 1 shows that when BCEC had been treated with 100 ng/ml PTX prior to the experiments, the inhibitory actions of Et-1 and Et-3 on adenylate cyclase were completely abolished. We checked that PTX and endothelins had no action on cAMP formation in the absence of CT treatment (Figure 1). ADP ribosylation experiments indicated the presence in BCEC of three substrates for PTX: a major 40 kDa substrate and two minor 41-42 kDa substrates (not shown).

The left panel of Figure 1 presents the dose response curves for Et-1 and Et-3 inhibition of CT stimulated adenylate cyclase. $K_{0.5}$ values were 2 nM and 40 nM for Et-1 and Et-3 respectively. These values clearly indicated an involvement of a receptor that had a low affinity for Et-3, i.e. that had properties of an ET_A receptor. The action of endothelins on cAMP formation has been investigated in a few cell types. In rat atrial cells, Et-1 does not reduce isoproterenol stimulated cAMP formation [15]. In rat mesangial cells, that express ET_B like receptors, Et-1 potentiates the action of isoproterenol on adenylate cyclase via an indirect mechanism involving prostaglandin E2 [16]. In mouse brain astrocytes [17] and in C6

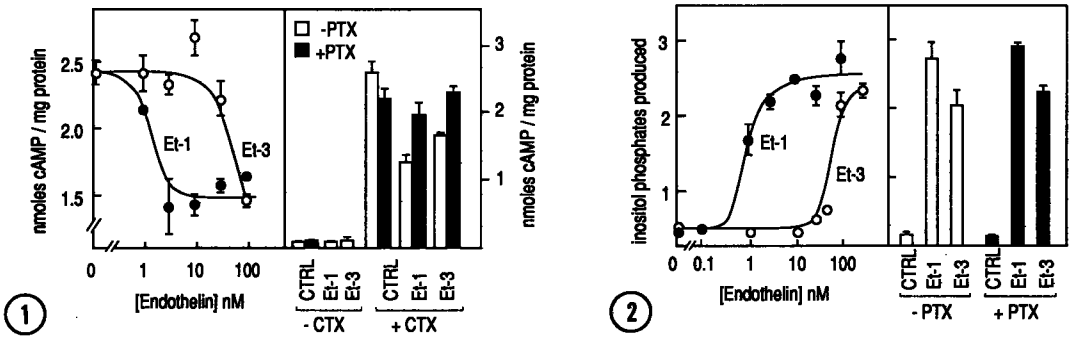

Figure 1. Endothelins inhibit CT stimulated adenylate cyclase in BCEC *via* a PTX sensitive mechanism.
Left panel: Dose response curves for Et-1 (●) and Et-3 (○) inhibition of CT stimulated adenylate cyclase activity. Means ± SEM (n=3) are shown. Identical results were obtained in 3 other independent experiments. cAMP levels in control cells that were not exposed to CT (CTRL) were 0.8 nmol/mg of protein (n=3). Right panel: PTX reversed the actions of Et-1 and Et-3 (100 nM) on CT stimulated adenylate cyclase. Means ± SEM (n>6) are shown.

Figure 2. Endothelins activate phospholipase C in BCEC *via* a PTX insensitive mechanism.
Left panel: Dose response curves for Et-1 (●) and Et-3 (○) activations of phospholipase C. Right panel: PTX did not reverse the stimulating actions of Et-1 and Et-3 (100 nM) on phospholipase C. Black bars represent experiments performed in the presence of PTX. CTRL: control experiments performed in the absence of endothelins. Means ± SEM (n=3) are shown. When no error bar is shown, it was smaller than the size of the points. The production of inositol phosphates is expressed in $cpm.10^{-4}$/well.

astrocytoma cells [18], endothelins inhibit adenylate cyclase but the receptor involved is a high affinity Et-3 receptor. Data presented here are the first evidence of a negative coupling of ET_A like receptors to adenylate cyclase.

Another action of endothelins in BCEC is to stimulate phospholipase C [10]. The left panel of Figure 2 presents the dose response curves for Et-1 and Et-3 stimulation of the production of inositol phosphates. $K_{0.5}$ values were 1 nM and 60 nM for Et-1 and Et-3 respectively, indicating an involvement of an ET_A like receptor. The right panel of Figure 2 further shows that PTX did not prevent the actions of Et-1 or of Et-3 on the formation of inositol phosphates. PTX insensitive activations of phospholipase C by endothelins have been observed in rat-1 fibroblasts, A10 and A7r5 smooth muscle cells [19], rat atrial cells [7] and aortic myocytes [20] that all have ET_A like receptors. PTX sensitive activations of phospholipase C have been reported in rat mesangial cells [21] and in mouse astrocytes [17] that express ET_B like receptors.

Taken together the results presented here thus indicated that ET_A like receptors of BCEC mediated the intracellular actions of endothelins *via* two distinct mechanisms (and probably via two distinct G proteins). A PTX insensitive mechanism coupled the receptor to phospholipase C. A PTX sensitive mechanism mediated the inhibitory action of endothelins on adenylate cyclase. In mouse brain astrocytes, ET_B like receptors mediate both an activation of phospholipase C and an inhibition of adenylate cyclase. In that case however, the two couplings are sensitive to PTX [17].

In many cell types, receptors that are functionally coupled to adenylate cyclase also activate Na^+/H^+ exchange [22]. In BCEC, Et-1 and Et-3 stimulate Na^+/H^+ exchange. This action involves ET_B like receptors and a PTX insensitive mechanism [11]. At a concentration that fully activates Na^+/H^+ exchange (10 nM), Et-3 had no action on adenylate cyclase (Figure 1) or on phospholipase C (Figure 2) activities. These indicated that the mechanism of activation of Na^+/H^+ exchange by endothelins was independent of phospholipase C and of adenylate cyclase activities.

The vascular actions of Et-3 and Et-3 differ. Et-3 is more active as a depressor than as a pressor agent [3] and this action is dependent on the presence of an intact vascular endothelium. The inhibition of adenylate cyclase by endothelins being mediated by a low affinity Et-3 receptor, it is unlikely that this action is involved in the depressor action of

endothelins. Brain microvessels are innervated by noradrenergic fibres [23]. BCEC which express high levels of β–adrenoceptors may be involved in noradrenergic control of the vascular tone [14]. The inhibition by endothelins of adenylate cyclase may antagonize the vasodilating action of noradrenaline and thus contribute to the prominent constrictor action of endothelins on brain vessels [24].

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