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HIGH REACTIVITY OF AORTIC FIBROBLASTS TO VASOACTIVE AGENTS: ENDOTHELINS, BRADYKININ AND NUCLEOTIDES

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Cultured aortic fibroblasts express high affinity Et-1 binding sites that poorly discriminate between Et-1 and Et-3. Both endothelins activate phospholipase C hence indicating the presence of ETB receptors. Fibroblasts respond to bradykinin by large activations of phospholipase C and increases in $[Ca^{2+}]i$ in a manner that was abolished by D-Arg, [Hyp^3,Thi^{5,8},D-Phe^7]-bradykinin, thus indicating the presence of B2 kinin receptors. Finally, ATP, UTP and ADP increases $[Ca^{2+}]i$ in aortic fibroblasts *via* a nucleotide receptor that has a higher affinity for ATP and UTP (3 μM) than for ADP (50 μM) and that is distinct from P2x and P2y purinoceptors.

Complex intercellular relationships are present within vessel walls. Vascular endothelial cells process important vasoactive peptides such as angiotensin I and BK. They release Et-1, NO and prostacyclin that have major actions on the contractility of underlying smooth muscle cells (1-4). Other cell types, such as fibroblasts and nerve terminals are also present in vessel walls and their reactivities to vasoactive substances has not yet been evaluated. In this paper we show that fibroblasts prepared from rat aortic adventice respond to Et-1, Et-3, BK, ATP, UTP and ADP. The receptors involved are characterized.

MATERIAL AND METHODS

Et-1, Et-3, BK, D-Arg, [Hyp^3,Thi^{5,8},D-Phe^7]-BK, nucleotides and indo-1/AM were from the Sigma Chemical Co. [^{125}I]Et-1 (2000 Ci/mmol) and *myo*-[2-^{3}H]inositol were from Amersham. 2-methyl thio ATP was from RBI.

Thoracic aortae were isolated from 6 to 8 week old Wistar-Kyoto rats and suspended into a Ca^{2+} and Mg^{2+} free phosphate buffered saline supplemented with 0.1 % collagenase (type II, Worthington) for 1 hour at 37°C. The adventice was then mechanically removed and further digested for 30 minutes in the same medium. Cells were collected by centrifugation at low speed for 5 minutes and seeded into M199 culture medium supplemented with 20 % fetal calf serum (Dutscher, Strasbourg, France), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Confluency was reached within one week and cells were passaged after dissociation with 0.1 % trypsin. Cells at passages < 5 were used in this study.

For intracellular Ca^{2+} measurements, cells were loaded with 5 μM indo-1/AM in complete culture medium for 2 h at 37°C. After dissociation of the cell layer, the indo-1 fluorescence ratio was measured by flow cytometry as previously described (5).

Abbreviations used are: Et-1: endothelin-1, Et-3: endothelin-3, BK: bradykinin.

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For measuring the production of inositol phosphates, monolayer of aortic fibroblasts seeded into 6-well tissue culture clusters were first labelled to equilibrium (24 hours) with 2 µCi/ml of [^{3}H]inositol. After washing with an Earle's salt solution (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5 mM glucose buffered at pH 7.5 with 25 mM Hepes-Tris), cells were incubated for 15 minutes in the same medium supplemented with 40 mM LiCl and then stimulated with Et-1, Et-3 or BK for 5 minutes. The radioactivity incorporated into total inositol phosphates was then measured as previously described (6).

[^{125}I]Et-1 binding experiments were performed as previously described using intact cell monolayers (5). Time of association was 60 minutes at 37°C. [^{125}I]Et-1 was used at a concentration of 28 pM. Acid resistant and acid soluble binding were determined as previously described (7). Binding data were analyzed using the LIGAND program.

RESULTS AND DISCUSSION

Endothelins produce a profound vasoconstriction by acting on ETA receptors localized in aortic myocytes. They also produce a transient depressor effect after *in vivo* injection. This action is mediated by ETB receptors localized in vascular endothelial cells and involves the release of NO and of prostacyclin (3,4). Figure 1 shows that [^{125}I]Et-1 binding to aortic fibroblasts was prevented by unlabelled Et-1 (IC_{50}=2 nM) and Et-3 (IC_{50}=5 nM). A Scatchard plot for the specific [^{125}I]Et-1 binding showed a Kd value of 1.5 nM and a maximum binding capacity of 100 ± 10 fmol/mg of protein (data not shown). Binding of [^{125}I]Et-1 to its receptor in aortic fibroblasts was rapidly followed by its internalization. At any time of an association of [^{125}I]Et-1 with cells > 85 % of the label could not be released by an acidic treatment (Fig. 1), hence suggesting that fibroblasts may participate to the clearing off the peptide. Both Et-1 and Et-3 induced the production of inositol phosphates by aortic fibroblasts with very similar potencies (Fig. 2). Et-3 (Fig. 3) and Et-1 (not shown) increased [Ca^{2+}]i in aortic fibroblasts as evidenced by flow cytometric analysis of indo-1 loaded cells. Taken together these results thus indicated that aortic fibroblasts expressed functional receptor sites for endothelins that have a high affinity for both Et-1 and Et-3. These are properties of ETB receptors (8) similar to the one found in vascular

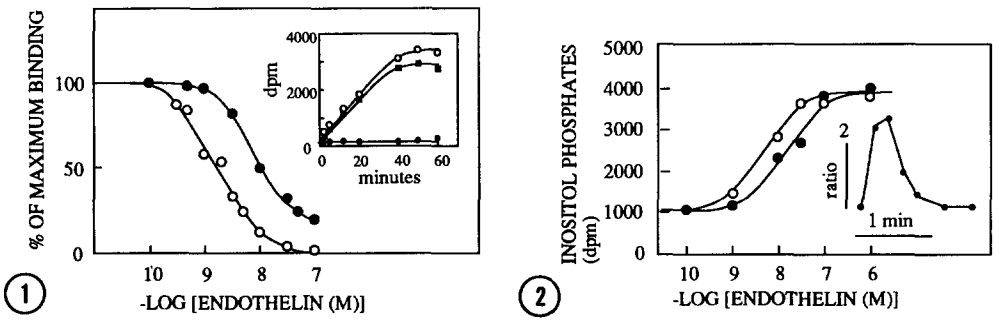

Figure 1. [^{125}I]Et-1 binding to aortic fibroblasts.
Inset: Time course of [^{125}I]Et-1 binding. Total binding component (○), non specific binding component (●) and acid resistant (i.e. internalized) binding component (■). Main panel: Inhibition by unlabelled Et-1 (○) and unlabelled Et-3 (●) of the specific [^{125}I]Et-1 binding.

Figure 2. Endothelins activate phospholipase C in aortic fibroblasts
Main panel: Dose response curves for Et-1 (○) and Et-3 (●) induced production of total inositol phosphates. Inset: Typical change in indo-1 fluorescence ratio induced by 100 nM Et-3.

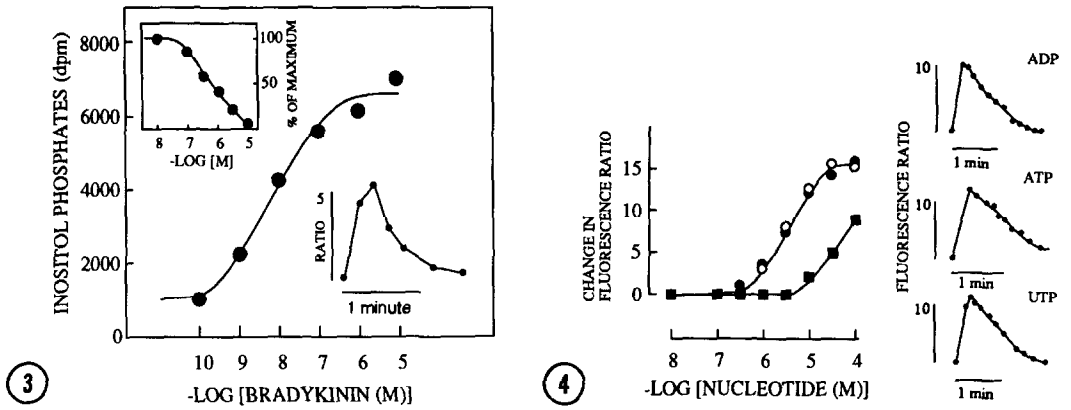

Figure 3. Bradykinin activates phospholipase C in aortic fibroblasts *via* B2 kinin receptors
Main panel: Dose response curve for BK stimulation of the production of inositol phosphates. Lower panel: Typical changes in indo-1 fluorescence ratio after the application of 100 nM BK. Upper panel: Dose response curve for D-Arg, [Hyp^3,Thi^{5,8},D-Phe^7]-BK inhibition of BK (100 nM) induced changes in [Ca^{2+}]i.

Figure 4. The actions of nucleotides on aortic fibroblasts.
Right panels: Typical changes in intracellular [Ca^{2+}] observed after the application of 10 μM ATP, 10 μM UTP and 0.1 mM ADP. Left panel: Dose response curves for ATP (●), ADP (■) and UTP (○) actions on [Ca^{2+}]i measured 30 seconds after the addition of the nucleotides.

endothelial cells (9). The ETA receptor of vascular myocytes which is responsible for the vasoconstrictor action of endothelins has a low affinity for Et-3 (10). It has often been observed (11,12), that in cultures of rat aortic myocytes, ETA and ETB receptors coexists and that after serial passaging *in vitro*, ETB receptors progressively increase in density and outnumber ETA receptors. The shift from ETA to ETB receptors observed in culture could be accounted for by an overgrowth of the cultures by fibroblasts that predominantly express ETB receptors.

BK is a potent vasorelaxing agent that acts on vascular endothelial cells *via* B2 receptors coupled to phospholipase C and NO production (13,14). Figure 3 shows that BK was a potent activator of phospholipase C and a Ca^{2+} mobilizing agent in fibroblasts (EC50 = 10 nM). It further shows that D-Arg, [Hyp^3,Thi^{5,8},D-Phe^7]-BK, a selective B2 receptor antagonist (13,14) prevented the action of BK (100 nM) on [Ca^{2+}]i with an IC50 value of 1 μM. Thus aortic fibroblasts express funtional B2 kinin receptors.

Adenine nucleotides induce an endothelium dependent vasorelaxation *via* P2y receptors and an endothelium independent vasoconstriction *via* P2x receptors (15). ATP, ADP but also UTP increased [Ca^{2+}]i in aortic fibroblasts. EC50 values were 3 μM for ATP and UTP and 50 μM for ADP (Fig. 4). ATP responses were mimicked by ATPγS but not by α,β methylene ATP, a selective agonist of P2x receptors (15), or by 2-methylthioATP, a selective agonist of P2y and P2t receptors (15,16). Neither α,β methylene ATP nor 2-methylthio ATP prevented cells to respond to an application of ATP by a large increase in [Ca^{2+}]i. Thus aortic fibroblasts express a receptor site for nucleotides that is pharmacologically distinct from the P2y receptor of vascular endothelial cells and of the P2x receptor of vascular myocytes. The observation that UTP had an action similar to ATP suggests the presence of a "nucleotide" receptor (16).

The large reactivity of aortic fibroblasts to Et-1, Et-3, BK and to nucleotides suggests that the action of vasoactive agents is not restricted to the inner part of the vascular wall and extends beyond the media. In preliminary experiments we observed that aortic rings whose adventice has been enzymatically removed, showed normal responses to vasoactive agents, thus indicating that unlike the intima, the adventice does not control contractility in a direct manner. Other roles of the adventice, such as in vascular remodelling, remain to be established.

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