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# Signal transduction of a G protein-coupled receptor in caveolae: Colocalization of endothelin and its receptor with caveolin

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**ABSTRACT** **Caveolae are small invaginations of the plasma membrane 50–100 nm in diameter. Since calcium channels, inositol 1,4,5-trisphosphate receptors, and heterotrimeric GTP-binding proteins (G proteins) are localized in caveolae, they may participate in signal transduction by G protein-coupled receptors. Here we show that the G protein-coupled endothelin receptor subtype A ($ET_A$) and its bound endothelin ligand are found in plasma membrane caveolae. $ET_A$ and its bound ligand coimmunoprecipitate with caveolin, a structural component of caveolae, in extracts of cells expressing transfected $ET_A$ receptors. Confocal fluorescence microscopy shows colocalization of $ET_A$ receptors and caveolin in micropatches at or near the plasma membrane, in the absence of endothelin ligands. These observations demonstrate a functional role for plasma membrane caveolae in signal transduction by this G protein-coupled receptor.**

Most mammalian cells contain caveolae—nonclathrin-coated plasma membrane invaginations of 50–100 nm in diameter. Although they have been recognized for >40 years (1), their physiological functions are poorly understood. Caveolae are abundant in capillary endothelial cells, where they function in transcytosis of macromolecules such as albumin and low density lipoprotein (2–4). In many cells caveolae are involved in the uptake of small molecules, such as folate, from the extracellular space (5), the process termed potocytosis.

A role of caveolae in signal transduction by receptors for growth factors and hormones has been indicated by several recent studies. Immunoelectron microscopic studies showed that the plasma membrane $Ca^{2+}$ pump (6) and an inositol 1,4,5-trisphosphate (IP3) receptor-like protein (7) are localized to caveolae, as are many proteins involved in intracellular signaling: cell surface receptors coupled to heterotrimeric GTP-binding, signal-transducing proteins (G proteins) (8, 9), bacterial toxins that modify G proteins (10), adenylate cyclase (11), and heterotrimeric G proteins (12, 13). The plasma membrane muscarinic acetylcholine receptor is redistributed to plasmalemmal caveolae upon addition of agonists but not antagonists (8), suggesting the participation of caveolae in intracellular signal transduction by this G protein-coupled receptor. However, the involvement of caveolae in signal transduction by G protein-coupled receptors rests solely on microscopic observations. Here we show directly that the endothelin receptor subtype A ($ET_A$), together with its bound ligand, are localized in plasma membrane complexes that contain caveolin, a protein uniquely found in caveolae.

Endothelins (ETs) are a family of 21-amino acid peptides, containing two intramolecular disulfide bonds, with at least three distinct isoforms, ET-1, ET-2, and ET-3 (14). ET-1, first isolated from supernatants of cultured aortic endothelial cells, is the most potent vasopressor agent yet discovered (15). ETs have numerous effects on vascular endothelial and smooth muscle cells as well as on cardiac muscle, renal, and neuroendocrine cells; on several they exert mitogenic effects (16). The three cloned ET receptors, $ET_A$, $ET_B$, and $ET_C$ (17–19), span the plasma membrane seven times and are coupled to different G proteins: $ET_A$ is coupled to $G_q$ (E. Lobo and H. Ives, personal communication) and $ET_B$ is coupled to $G_s$ and $G_q$ (20). The receptors differ in their relative affinities for the three ET isoforms: $ET_A$ binds tightest to ET-1 and poorest to ET-3, whereas $ET_C$ binds tightest to ET-3 and poorest to ET-1 (17–19). $ET_B$ binds all three ETs with similar affinities (17). Binding of ET to ET receptors induces activation of phospholipase C (21), activation of protein kinase C (22), and the opening of $Ca^{2+}$ channels (23).

Our observations that the $ET_A$ receptor and its bound ligand can be coimmunoprecipitated with caveolin and that the $ET_A$ receptor and caveolin colocalize by confocal immunofluorescence microscopy are direct demonstrations of the colocalization of a G protein-coupled receptor and its ligand in caveolae. Together with immunoelectron microscopic studies showing that both calcium channels and IP3 receptors exist in caveolae (6, 7), our results indicate a functional role for caveolae in intracellular signal transduction by the ET receptor.

## METHODS

**Preparation of Anti-Peptide Antibodies Specific for $ET_A$ and $ET_B$.** A peptide (SSHVEDFTPFPGTEF) corresponding to the amino terminus of the rat $ET_A$ protein (18) and a peptide (KKAANDHGYDNFRSSNN) corresponding to the carboxyl terminus of the rat $ET_B$ protein (17) were coupled to keyhole limpet hemocyanin and used to immunize rabbits using the protocol detailed in ref. 24.

**COS Cell Transfection and Binding of Radiolabeled ET.** COS cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated (1 hr, 56°C) bovine calf serum, 2 mM L-glutamine, and 2 mM L-proline. Transfection of COS cells was performed by the DEAE-dextran/chloroquine procedure as described (18). The binding of $^{125}$I-labeled ET-1 ($^{125}$I-ET-1) to transfected cells was performed exactly as described (18).

**Immunoprecipitations.** Immunoprecipitations using the $ET_A$ antibody (see Fig. 1) were performed as follows. Nontransfected COS cells and COS cells transfected with the $ET_A$ cDNA, in 10-cm dishes, were labeled with [$^{35}$S]cysteine (1 mCi/3 ml of culture medium; 1 Ci = 37 GBq) for 1 hr at 37°C

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Abbreviations: ET, endothelin; $ET_A$, ET receptor subtype A; $ET_B$, ET receptor subtype B; $ET_C$, ET receptor subtype C; IP3, inositol 1,4,5-trisphosphate; G proteins, heterotrimeric GTP-binding, signal-transducing proteins; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate.

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in DMEM lacking cysteine and containing 5% dialyzed fetal calf serum. The cells were chilled to 4°C and briefly washed three times with phosphate-buffered saline (PBS). While still attached to the culture dish, the cells were extracted for 30 min on ice without agitation with 1 ml of MBST [25 mM Mes, pH 6.5/0.15 M NaCl/1% Triton X-100/2 mM phenylmethylsulfonyl fluoride (PMSF)] and this Triton extract was collected. The labeled cells still attached to the dish were reextracted for 30 min on ice with 1 ml of TBST (10 mM Tris·HCl, pH 8.0/0.15 M NaCl/1% Triton X-100/2 mM PMSF) containing 60 mM octyl glucoside. After centrifugation to remove insoluble material, both detergent extracts were incubated with a 1:200 dilution of an $ET_A$ antibody for 2 hr at 4°C. The immune complexes were incubated for 2 hr to overnight at 4°C with 75 μl of a 1:1 slurry of protein A-Sepharose (Pharmacia). The immunoprecipitates were washed six times with 1 ml of TBST, resolved by SDS/PAGE through a 10% acrylamide gel, and visualized by fluorography.

For the immunoprecipitation experiment using caveolin antibody (see Table 2), $ET_A$-transfected COS cells were incubated for 3 hr at 4°C with 50 pM of $^{125}$I-ET-1 in binding buffer [PBS, pH 7.4/11 mM glucose/0.5% bovine serum albumin (BSA)]. The cells were washed with PBS three times and then lysed in TBST buffer containing 60 mM octyl glucoside. The total amount of $^{125}$I-ET-1 in an aliquot of the lysate was measured using a liquid scintillation counter. The remainder of the lysate was incubated with various amounts of anti-caveolin antibody (Transduction Laboratories, Lexington, KY) or anti-$ET_B$ antibody, as indicated in the figure legends, for 10–30 min at 4°C, and then with protein A-Sepharose for 20 min at 4°C. The immunoprecipitates were collected by centrifugation and briefly washed three times in TBS buffer containing 1% Triton X-100, and the amount of $^{125}$I was measured as above.

**Western Blot Analysis.** All reactions were performed at room temperature. Proteins were transferred from an SDS gel to a nitrocellulose filter with 0.22-μm pores (Schleicher & Schuell). The filter was incubated in PBS containing 5% nonfat powdered milk for 1 hr at 4°C and then for 1 hr in the same solution containing a 1:500 dilution of the anti-caveolin antibody. The filter was then washed briefly three times with PBS buffer containing 5% nonfat powdered milk. Bound antibody was visualized with the ECL system as described by the manufacturer (Renaissance kit, NEN).

**Immunofluorescence.** All reactions were performed at room temperature. Transfected COS cells were fixed for 10 min in PBS containing 2% paraformaldehyde and then extensively washed with PBS. The cells were washed once with PBS containing 0.5% Triton X-100 (Boehringer Mannheim) and then incubated for 30 min with 50 μg of normal IgG per ml (The Jackson Laboratory) in PBS containing 2% BSA. The cells were then incubated for 1 hr with both a protein A-purified IgG fraction of the rabbit anti-$ET_A$ polyclonal antibody (20 μg/ml) and the mouse anti-caveolin monoclonal antibody (1:500 dilution). Then the cells were washed three times for 5 min each with PBS containing 2% BSA. The cells were incubated for 1 hr in PBS containing 2% BSA and both fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (5 μg/ml) (Cappel or The Jackson Laboratory) and Cy3-conjugated donkey anti-mouse antibody (3 μg/ml) (The Jackson Laboratory). (These secondary antibodies had been preabsorbed to serum proteins bound to agarose gels to remove any cross-reacting antibody species.) The cells were washed extensively with PBS, six times 10 min each, and then mounted with Slow-Fade antifade reagent (Molecular Probes). Samples were viewed under a Bio-Rad MRC600 confocal fluorescence microscope (Bio-Rad) and photographed using Fujichrome Velvia film (Fuji, Tokyo).

## RESULTS

**$ET_A$ Resides in Plasma Membrane Caveolae.** Caveolae, as well as their principal protein caveolin, are insoluble in buffers containing Triton X-100, but are soluble in ones containing octyl glucoside together with Triton X-100 (12). Fig. 1*A* shows that, in COS cells transfected with $ET_A$ cDNAs and labeled with [$^{35}$S]cysteine, none of the radiolabeled $ET_A$ is soluble in a buffer containing Triton X-100 (lane 2), but all is soluble in a buffer containing both octyl glucoside and Triton X-100 (lane 4, large arrow). A radiolabeled ≈22-kDa protein (small arrow) coimmunoprecipitates with $ET_A$ from the octyl glucoside/Triton X-100 extract (lane 4) but not from the Triton X-100 extract (lane 2). This protein is caveolin (Fig. 1*B*, lane 4, small arrow). To show this, the immunoprecipitated samples from Fig. 1*A* were blotted and allowed to immunoreact with anti-caveolin IgG; only the $ET_A$ immunoprecipitates from the cell extract in the octyl glucoside/Triton X-100 buffer contain caveolin (lane 4, compare with lane 2). The species of ≈55 kDa, immunoreactive with the anti-caveolin antibody in Fig. 1*B*, lane 4 (arrowheads),

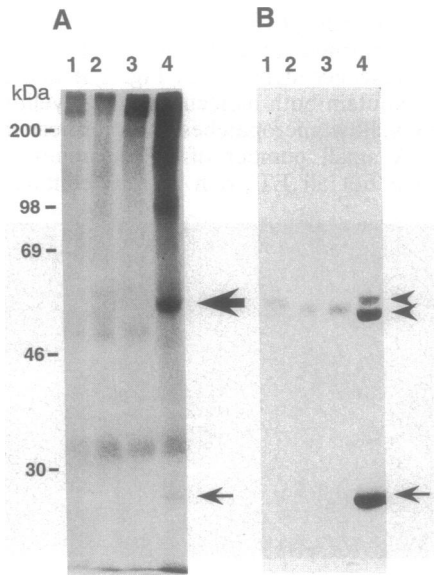


FIG. 1. Coimmunoprecipitation of $ET_A$ and caveolin in COS cells transfected with $ET_A$. (*A*) Immunoprecipitation of $ET_A$ using anti-$ET_A$ antibody. Nontransfected COS cells (lanes 1 and 3) and cells transfected with $ET_A$ cDNA (lanes 2 and 4) were labeled with 300 μCi of [$^{35}$S]cysteine per ml for 1 hr at 37°C, chilled to 4°C, and washed three times with PBS. The labeled cells were extracted in 1 ml of MBST for 30 min at 4°C; the pellet was removed by centrifugation and then further extracted for 30 min at 4°C in 1 ml of TBST containing 60 mM octyl glucoside. The Triton extracts (lanes 1 and 3) and the Triton/octyl glucoside extracts (lanes 2 and 4) were allowed to react with a 1:200 dilution of a polyclonal antibody specific for the N terminus of $ET_A$. After incubation for 2 hr at 4°C, antibody was bound to protein A-Sepharose for 2 hr at 4°C. The immunoprecipitates were washed with TBST buffer and labeled proteins were visualized by fluorography after SDS/PAGE. Note that only the Triton-insoluble, Triton/octyl glucoside-soluble complexes (lane 4) contain labeled $ET_A$ (large arrow); a species of ≈22-kDa protein (small arrow) is coimmunoprecipitated. (*B*) Western blot analysis using anti-caveolin antibodies of samples from *A* that were immunoprecipitated by anti-$ET_A$. Proteins were transferred from the SDS gel to a nitrocellulose filter and allowed to react with a 1:500 dilution of the anti-caveolin antibody for 1 hr at room temperature. Bound antibodies were visualized by chemiluminescence reagents (Renaissance kit, NEN), so that the radioactive proteins do not interfere with the signal. Only the Triton-insoluble, Triton/octyl glucoside-soluble complexes (lane 4) contain caveolin (small arrow) that coprecipitates with the anti-$ET_A$ (*A*, lane 4, small arrow).

may be a caveolin homodimer stable to SDS denaturation; such species have been detected (M.P.L., unpublished data).

The coimmunoprecipitation of $ET_A$ and caveolin strongly suggests a physical interaction between the two proteins. Furthermore, since caveolin is a structural component of caveolae, these results establish that $ET_A$ resides in caveolae.

As shown by confocal laser immunofluorescence microscopy, $ET_A$ and caveolin colocalize at or near the plasma membrane (Fig. 2). COS cells cotransfected with cDNAs encoding $ET_A$ and caveolin were fixed and incubated with IgGs derived from rabbit anti-$ET_A$ and mouse anti-caveolin antibodies and then with FITC-conjugated goat anti-rabbit IgG antibody (for $ET_A$) and Cy3-conjugated donkey anti-mouse IgG (for caveolin). In Fig. 2 *A–C*, the focal plane is close to the bottom of the cells, near the nuclei. Staining with the anti-$ET_A$ IgG reveals many small micropatches scattered in the periphery of the cell; there is an intense accumulation of micropatches at the cell surface (Fig. 2*A*, arrows). Staining with anti-caveolin antibody reveals many micropatches scattered throughout the cell (Fig. 2*B*, arrows). Note that the cell in the center of the field, expressing the transfected $ET_A$, contains more caveolin than the neighboring, presumably untransfected, cells. Most of the plasma membrane micropatches contain both $ET_A$ and caveolin (arrows in Fig. 2 *A* and *B*), as is confirmed in Fig. 2*C*, an overlay of the $ET_A$ and caveolin fluorescence depicted in Fig. 2 *A* and *B*. Micropatches that contain both molecules have a yellow fluorescence; many yellow micropatches are at or near the plasma membrane. A small number of micropatches are green, indicating that not all $ET_A$-rich regions contain caveolin. Similarly, the many red micropatches indicate that not all caveolin-containing complexes also contain $ET_A$.

Fig. 2 *D–F* show the same cells as shown in Fig. 2 *A–C* but viewed at a focal plane in the middle of the nuclei. $ET_A$ is largely localized to the perinuclear area (Fig. 2*D*, open arrows) and to the cell surface (closed arrows). Micropatches containing caveolin (Fig. 2*E*) were also enriched in the perinuclear area (open arrows) and the cell surface (closed arrows). The apparent immunostaining of nuclei with the anti-caveolin antibodies (Fig. 2*F*, curved arrows) is due to background staining by the Cy3-conjugated secondary antibody, since cells labeled only with the Cy3-conjugated donkey anti-mouse IgG exhibited staining over the nuclei but not over any part of the cytoplasm (data not shown). Thus, the staining of the cytoplasm by the anti-caveolin antibodies (Fig. 2 *B* and *E*) indeed represents caveolin. As in Fig. 2*C*, the yellow fluorescence in Fig. 2*F* indicates colocalization of $ET_A$ and caveolin. Together with the coimmunoprecipitation results in Fig. 1, the immunofluorescence results in Fig. 2 establish that most of the $ET_A$ receptor resides in caveolae at or near the plasma membrane.

**Receptor-Bound ET-1 Resides in Caveolae.** The ligand ET-1, bound to cell surface $ET_A$ receptor in transfected COS cells, is, like caveolin and the $ET_A$ receptor, insoluble in a buffer containing Triton X-100, but soluble in a buffer containing both octyl glucoside and Triton X-100 (Table 1). In this experiment, nontransfected and $ET_A$-transfected cells were incubated with $^{125}$I-ET-1 for 3 hr at 4°C to allow binding but not internalization of ligand. The cells were then subjected to successive extractions in a buffer containing 1%

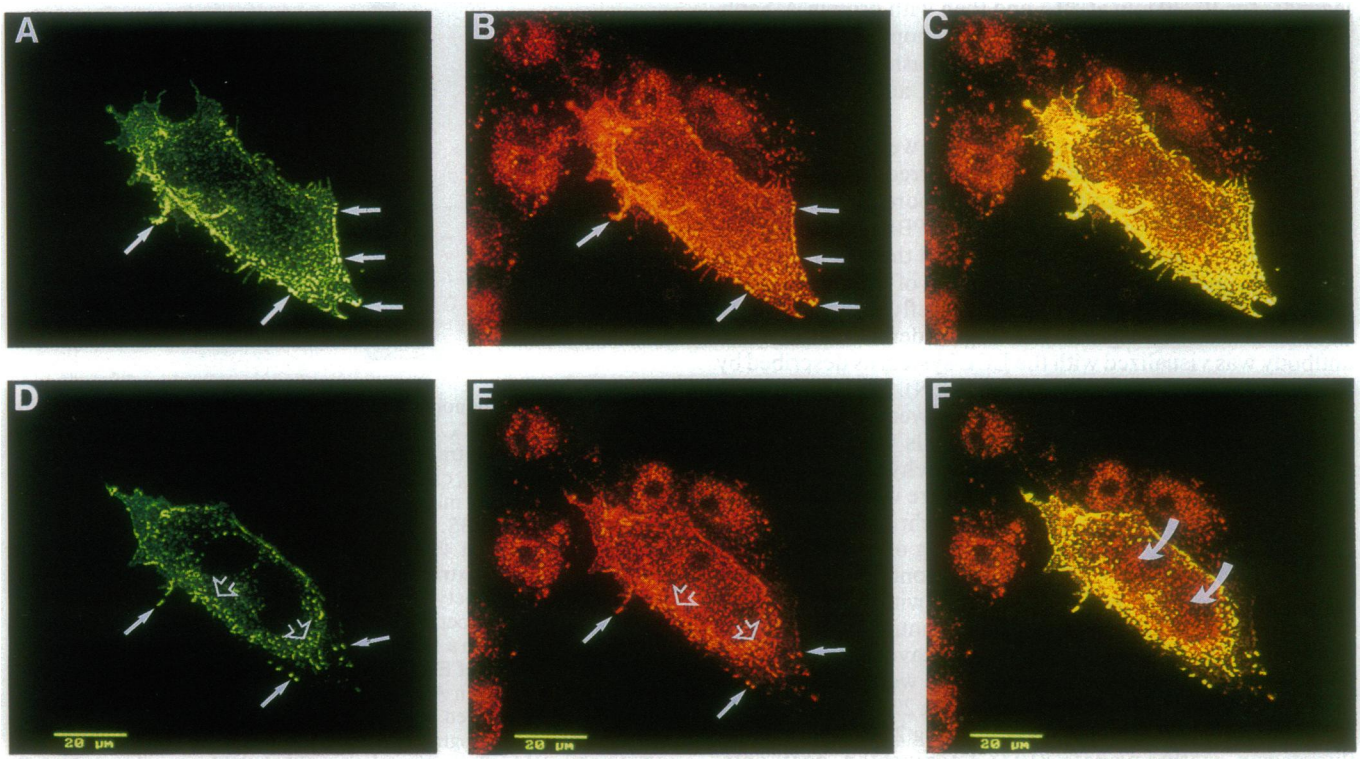


FIG. 2. $ET_A$ and caveolin colocalize in COS cells cotransfected with $ET_A$ and caveolin cDNAs. (*A*) Confocal fluorescent microscopic image of fixed cells, immunostained for 1 hr at room temperature with a 1:200 dilution of a rabbit anti-$ET_A$ antibody and then with an FITC-conjugated goat anti-rabbit antibody. The green fluorescent dots are found only in the central cell, one presumably expressing the transfected $ET_A$ receptor. (*B*) The same field of cells immunostained for 1 hr at room temperature with a 1:500 dilution of a mouse anti-caveolin antibody and then with Cy3-conjugated donkey anti-mouse antibody. The fluorescent image was recorded using the filter normally employed for rhodamine fluorescence. As detected by the red fluorescence, all cells express caveolin; the cell in the middle shows higher expression probably due to transfection with the caveolin cDNA. Many red dots, representing caveolin-rich complexes, are scattered throughout the cells. (*C*) Superposition of the fluorescent images in *A* and *B*. Colocalization of $ET_A$ and caveolin generates a yellow fluorescence. Note that the two primary and two secondary antibodies employed are from different animal species, to reduce the background staining and eliminate cross-reaction of the primary antibodies with the secondary ones. (*D–F*) The same field as in *A–C* but viewed in a different focal plane. (*D*) Immunostaining with anti-$ET_A$ antibody. (*E*) Immunostaining with anti-caveolin antibody. (*F*) Superposition of the fluorescent images in *D* and *E*.

Table 1. Triton X-100 insolubility of ET-1 bound to $ET_A$ receptors in transfected COS cells

| COS cells | $^{125}$I-ET-1, cpm | |
|---|---|---|
| | Triton-soluble | Triton-insoluble, Triton/octyl glucoside-soluble |
| Control | 141 | 204 |
| Transfected with $ET_A$ cDNA | 8867 | 369,482 |

Nontransfected and $ET_A$-transfected COS cells were incubated with $^{125}$I-ET-1 for 3 hr at 4°C, washed with PBS, and then, as in Fig. 1, extracted with MBST buffer for 30 min at 4°C. The extract was collected and the amount of $^{125}$I-ET-1 was measured. Then the Triton-insoluble fraction was extracted with TBST buffer containing 60 mM octyl glucoside; the extracted $^{125}$I-ET-1 was quantified. Results are expressed as the means of triplicate determinations. Variations among triplicate samples were <5%.

Triton-X-100, followed by one containing 1% Triton X-100 and 60 mM octyl glucoside. Over 95% of the radiolabeled ET-1 bound to the transfected cells was extracted only with Triton X-100 and 60 mM octyl glucoside. Nontransfected cells bound, as expected, insignificant amounts of radiolabeled ET-1.

Table 2 shows that most of the radiolabeled ET-1, bound to the $ET_A$ receptor on the surface of transfected cells, immunoprecipitates with an antibody specific to caveolin. In this study, $ET_A$-transfected and nontransfected COS cells were incubated with $^{125}$I-ET-1 for 3 hr at 4°C. The cells were then lysed in a buffer containing both 1% Triton X-100 and 60 mM octyl glucoside. The lysates were immunoprecipitated with differing amounts of anti-caveolin antibody or, as a control, of an antibody to the $ET_B$ receptor, which does not crossreact with the $ET_A$ receptor. We employed relatively large amounts of antibodies and short immunoprecipitation times to prevent dissociation of $^{125}$I-ET-1 from the receptor during the immunoprecipitation reactions. Over 70% of the cell-bound $^{125}$I-ET-1 is coimmunoprecipitated by the anti-caveolin antibody; regardless how much control $ET_B$ antibody was added, insignificant amounts of $^{125}$I-ET-1 were immunoprecipitated.

## DISCUSSION

Here we showed that, in the absence of ET-1 ligand, caveolin and the $ET_A$ receptor are in a complex in the plasma membrane; after ET-1 addition this complex also contains receptor-bound ET-1 ligand. Taken together, all of our experiments show that the $ET_A$ receptor and its ligand reside in caveolae. Our findings provide a direct demonstration of the colocalization of a G protein-coupled receptor and its ligand in caveolae.

Table 2. Fraction of $^{125}$I-ET-1 immunoprecipitated with anti-caveolin antibody

| Antibody | Amount used, μg | Fraction recovered |
|---|---|---|
| Caveolin | 1.75 | 0.40 |
| | 5.0 | 0.71 |
| | 10.0 | 0.70 |
| $ET_B$ | 15. | 0.01 |
| | 60. | 0.01 |
| | 120. | 0.01 |

$ET_A$-transfected COS cells were incubated with 50 pM of $^{125}$I-ET-1 for 3 hr at 4°C, washed with PBS, and lysed in TBST buffer containing 60 mM octyl glucoside. The lysate (containing 159,000 cpm of $^{125}$I-ET-1) was incubated with various amounts of either anti-caveolin antibody (1.75, 5, and 10 μg) or anti-$ET_B$ antibody (15, 60, and 120 μg) for 30 min at 4°C and then with protein A-Sepharose for 20 min, at 4°C. The immunoprecipitates were collected and briefly washed three times in TBS buffer containing 1% Triton, and the amount of $^{125}$I radioactivity was counted (cpm). Shown is the fraction of $^{125}$I-ET-1 recovered in the immunoprecipitates.

In capillary endothelial cells and visceral smooth muscle cells, two signal-transducing proteins activated by binding of ET-1 to the $ET_A$ receptor, a calcium channel, and an IP3 receptor are enriched in plasma membrane caveolae (6, 7). Caveolae isolated from tissues and cultured cells are enriched in both $G_\alpha$ and $G_\beta$ subunits of heterotrimeric GTP-binding proteins (25). In particular, $G_{\alpha q}$, the subunit of the heterotrimeric G protein that is coupled to $ET_A$, is also present in caveolae (13). Taken together, these results strongly indicate that the ET receptor binds its ligand and generates an intracellular signal while localized in plasma membrane caveolae.

We showed by coimmunoprecipitation that the $ET_A$ receptor and caveolin form a complex stable in a mixture of Triton X-100 and octyl glucoside detergents. However, we do not know whether the two proteins physically interact or whether they are part of a complex containing other proteins. Since only a few other [$^{35}$S]cysteine-labeled proteins also coimmunoprecipitate with the $ET_A$ receptor (Fig. 1), we suspect there is a physical interaction between the two proteins.

Our immunofluorescence data also indicate that $ET_A$ and caveolin are colocalized and thus that $ET_A$ is a resident protein in caveolae. As judged by similar immunofluorescence experiments, glycosylphosphatidylinositol (GPI)-anchored proteins were also reported to reside in caveolae (5). A recent paper, however, suggests that GPI-anchored proteins are not normally concentrated in caveolae but are induced to enter caveolae by cross-linking with polyclonal secondary antibodies (26). In our experiments we used cells fixed for 10 min in 2% paraformaldehyde, and it is unlikely that that cross-linking by the secondary antibody caused $ET_A$ to localize in caveolae. Importantly, the coimmunoprecipitation of $ET_A$ and caveolin (Fig. 1) provides independent evidence that $ET_A$ localizes in caveolae in the absence of ligand.

Other G protein-coupled receptors such as the muscarinic acetylcholine receptor and $\beta_2$-adrenergic receptor, however, are uniformly distributed throughout the plasma membrane and are redistributed to plasmalemmal caveolae upon ligand binding (8, 9, 27). In the case of the muscarinic acetylcholine receptor, this process is reversible; when cells are incubated without agonist for 4 hr, the receptors separate from caveolae.

This difference may relate to other aspects of signal transduction by these receptors. β-Adrenergic receptors, and most other G protein-coupled receptors, transmit signals for only a few minutes before they are desensitized. Continued exposure of cells to $\beta_2$-adrenergic agonists causes an attenuation of signal transduction due to the phosphorylation of the $\beta_2$-adrenergic receptor by both the cAMP-dependent protein kinase (28) and the β-adrenergic receptor kinase (29); this desensitization is complete within minutes. In contrast, activation of the ET receptor produces a long-lasting intracellular signal; a single small injection (1 nmol/kg) of ET into the circulation of a rat results in a sustained increase in blood pressure for >2 hr (15). Furthermore, in cultured cells expressing $ET_A$, addition of ET-1 results in an elevation of cytosolic $Ca^{2+}$ that lasts at least 30 min, while addition of agonists of adrenergic and other receptors results in an elevation of cytosolic $Ca^{2+}$ that persists for only 1–2 min (M.C., U.K.L., E. Lobo, H. Ives, and H.F.L., unpublished data). One possible explanation for the long-lasting signal generated by the $ET_A$ receptor relates to its localization in caveolae, since these organelles also contain proteins such as $G_{\alpha q}$ that are required for signal transduction by $ET_A$. Frequently, plasma membrane caveolae close off and form

endosome-like structures (30). It is likely that the $ET_A$ receptor with its bound ET-1 ligand is incorporated in these vesicles. Importantly, over half of the ET-1 remains intact 1 hr after internalization and remains bound to $ET_A$ receptors (31). Thus, signal transduction by the ET receptor might occur in internalized caveolae as well as in caveolae that are continuous with the plasma membrane, accounting for the long-lasting elevation of cytosolic $Ca^{2+}$ and contraction of smooth muscle cells following a single addition of ET.

Caveolin, a protein component of the cytosolic coat of caveolar membranes, was first identified as a major substrate for v-*Src* in cells transformed by Rous sarcoma virus (RSV) (32). A possible role for caveolae in signal transduction stems from the correlation between RSV transformation and phosphorylation of caveolin by $pp60^{vsrc}$. This suggests that abnormal cell–cell interactions may be the consequence of tyrosine phosphorylation of caveolin (33). Thus caveolin could be a critical substrate for transmembrane signaling and cellular transformation and could also be involved in transduction of some intracellular signals by the $ET_A$ receptors.

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