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Internalization of Endothelin by Cultured Human Vascular Smooth Muscle Cells: Characterization and Physiological Significance

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SUMMARY

The binding and internalization of ^{125}I-endothelin (^{125}I-ET-1) was studied in cultured human vascular smooth muscle cells (hVSMC). Discrimination between surface-bound and internalized radiolabeled ligand was achieved using either acetic acid or trypsin treatment of cell layers, with the two procedures yielding comparable results. Total cellular ^{125}I-ET-1 binding hVSMC at 37° was rapid and reached near equilibrium within 30 min. Such binding could be resolved into surface-bound (acid/trypsin-sensitive) and internalized (acid/trypsin-resistant) components. The accumulation of internalized ^{125}I-ET-1 was temperature dependent and occurred at 37° ($t_{1/2}$ ~15 min) but not at 4°. Internalization of ^{125}I-ET-1 by hVSMC was reversibly inhibited by the transglutaminase inhibitor dansylcadaverine (half-maximal inhibitory concentration, ~400 μM). Cytosolic acidification of hVSMC (from pH ~6.8 to ~6.3) by incubation with potassium acetate in a choline buffer also inhibited ^{125}I-ET-1 internalization. Our observation indicate that smooth muscle cells internalize ET-1 via the clathrin-mediated endocytotic pathway. Dansylcadaverine and other inhibitors of transglutaminase inhibited ET-1-stimulated inositol phospholipid hydrolysis in hVSMC and decreased ET-1-induced vasoconstriction in isolated endothelium-denuded blood vessels. Internalization of ET-1 may, therefore, be relevant to the characteristically protracted physiological effects of this peptide on the vasculature.

The 21-amino acid peptide ET-1 was originally isolated from the culture supernatant of porcine endothelial cells (1; 2). Stimulation of cultured endothelial cells by various peptide hormones (e.g., thrombin, angiotensin II, vasopressin, transforming growth factor-β) or by mechanical means (e.g., fluid shear stress) leads to enhanced expression of ET-1 mRNA and subsequent peptide secretion (1; 2). More recently, constitutive release of ET-1 from the intima of porcine aortic strips (3) has been demonstrated and, furthermore, secretion levels of this peptide can be increased following stimulation with thrombin or calcium ionophore (3). ET-1 was originally identified as a potent vasoconstrictor (1; 2). However, it has since been demonstrated to possess a wide spectrum of activities in tissues (e.g., intestine, lung, brain, heart, kidney, and adrenals) other than blood vessels (reviewed in Ref. 4); tissues in which specific high affinity ET-1-binding sites have also been demonstrated (5). The existence of three ET-1 isopeptides raises the possibility of multiple ET-1 receptor subtypes (4–6); and distinct receptor subpopulations have been demonstrated for chick cardiac membranes (7). Pharmacokinetic studies show that intravenously injected ^{125}I-ET-1 is rapidly eliminated from the bloodstream and that a major fraction of the radioactivity is located in the lungs, kidney, liver, and spleen (8, 9). It is noteworthy that these tissues are also functionally responsive to the peptide (reviewed in Ref. 4), but it remains to be elucidated whether such target/clearing tissues possess specific systems to metabolize ET-1.

Although there is some information on the postbinding events that mediate the cellular actions of ET-1 (e.g., activation of membrane phospholipases and Ca^{2+} channels, with subsequent generation of intracellular signals) (reviewed in Refs. 3 and 4); less is known about the fate of ET-1 and its surface receptors after binding. Once bound to surface receptors, many ligands are rapidly internalized via a process termed adsorptive endocytosis/receptor-mediated endocytosis (10–12). Following translocation from the cell surface to the cell interior, receptors may be degraded [e.g., epidermal growth factor receptor (13)], reinserted into the cell surface [e.g., low density lipoprotein receptor (12; 14) and angiotensin II receptor (15)], or extruded from cells [e.g., transferrin receptor (16)]. Whatever the fate of ligand-receptor complex, receptor internalization/cyclization constitutes an important mechanism for the control of ligand action in target tissues. This study investigates the process of

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ABBREVIATIONS: ET-1, endothelin; hVSMC, human vascular smooth muscle cells; HEPES, 4-(2-hydroxymethyl)-1-piperazine-*N*′-2-ethanesulfonate; TES, *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonate; DMO, 5,5-dimethyloxazolidine-2,4-dione; VSMC, vascular smooth muscle cells.

ET-1 receptor internalization in cultured hVSMC and its relevance to some biochemical and physiological effects of ET-1 on vascular tissue.

Experimental Procedures

Materials. With the exception of fetal serum (Fakola AG, Basel, Switzerland), all tissue culture material and chemicals were from GIBCO AG (Basel, Switzerland). ET-1 (porcine, human) was obtained from Nova Biochem (Läufelfingen, Switzerland) and ^{125}I-ET-1 (2000 Ci/mmol) from Anawa (Wangen, Switzerland). *myo*-[2-^{3}H]Inositol (16–20 Ci/mmol) and [2-^{14}C]DMO were purchased through Rahn and Co. (Amersham, Zurich, Switzerland). All other chemicals and reagents were purchased from Fluka AG (Buchs, Switzerland) or Sigma (St. Louis, MO).

Isolation and culture of VSMC. The isolation, characterization, and propagation of hVSMC was performed as described previously (17, 18); the tissue of origin was obtained from patients undergoing abdominal surgery and consisted of microarterioles associated with omental fat. Experiments described here used two isolates of hVSMC between passage 8 and 17. Before all experimentation, confluent hVSMC were rendered quiescent by serum deprivation and maintenance in serum-free medium, containing 0.1% (w/v) bovine serum albumin, for 48 h (with one medium change after 24 hr). Cell numbers were routinely determined by counting aliquots of cell suspension in Isoton with a Coulter counter (Coulter Electronics Inc., Hialeah, FL), after enzymatic disaggregation of cell layers (17, 18).

^{125}I-ET-1 binding and internalization. hVSMC were plated into six-well Costar multiwell plates (2×10^4 cells/cm^2). At confluence, cell layers were washed (2×2 ml) with minimal essential medium containing 0.1% (w/v) bovine serum albumin, 20 mM HEPES/20 mM TES (both pH 7.3), 2 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (buffer B), and then 2.0 ml of buffer B were added to each well. Dishes were maintained at either 37° or 4° before addition of ^{125}I-ET-1 (final concentration, 0.25 nM; 0.05 μCi/well), without or with inclusion of 200 nM unlabeled ET-1 for determination of nonspecific binding. After incubation (at 37° or 4°) for the appropriate time intervals, medium overlay was removed by aspiration and cell layers were washed (4×2 ml) with phosphate-buffered saline containing 0.2% (w/v) bovine serum albumin at 4°; all dishes were maintained at 4° thereafter. Surface-bound and internalized ^{125}I-ET-1 was determined using the acetic acid procedures as described by Haigler *et al.* (19); the appropriate methodology has been placed in the legend to Fig. 1 to facilitate symbol identification. In some experiments, binding incubations were performed as described above, but total, surface-bound, and internalized ^{125}I-ET-1 were determined using the trypsinization (0.05% trypsin/0.02% EDTA) procedures described by Aulinskas *et al.* (20) and Cheng *et al.* (21). Again, to facilitate symbol identification the appropriate methodology has been placed in the legend to Fig. 3. All results for ^{125}I-ET-1 binding (total, surface, and internalized) are expressed as specific binding after correction for nonspecific binding. Each experiment was performed on at least two separate occasions, and for any single experiment triplicate determinations were made. Experimental protocols for determination of the effects of dansylcadaverine and intracellular acidification on ^{125}I-ET-1 binding have been presented in the appropriate figure legends.

Measurement of intracellular pH. Intracellular pH was measured using the weak acid [^{14}C]DMO following the procedures of Mendoza and Rozengurt (22), which we have previously detailed for hVSMC (17).

Measurement of phosphatidylinositol catabolism. All methodology for prelabeling of hVSMC (~2×10^6 cells/well with 5 μ Ci/ml *myo*-[^{3}H]inositol for 48 hr), stimulation with ET-1 (in the presence of 10 mM LiCl), and subsequent extraction and chromatographic resolution of inositol phosphates and phosphoinositol lipids (after deacylation) have been detailed previously (17, 23).

Organ chamber experiments. Pig hearts obtained from the local slaughterhouse were immersed in cold modified Krebs-Ringer bicarbonate solution (composition in mM: NaCl, 118.3; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_4$, 2.5; $NaHCO_3$, 25.0; CaEDTA, 0.016; glucose, 11.1; pH 7.4) (control solution). Left circumflex coronary arteries were excised, cleaned of loose connective tissue, and cut into rings of 4-mm length. The intimal layer of rings was removed deliberately by gentle rubbing of the luminal layer with a wetted cotton swab (24). The rings were mounted horizontally in organ chambers filled with control solution at 37° and gassed with 95% O_2/5% CO_2 (24). The preparations were attached to a strain gauge and then rings were progressively stretched until the contractile response evoked by 20 mM KCl was maximal (optimal tension) (24). Rings were equilibrated for 30 min before experimentation. The absence of endothelium was verified by the absence of relaxation to bradykinin (1 μM) during a contraction to prostaglandin $F_{2\alpha}$ (2 μM) (24). The rings were then washed with control solution and incubated for 45 min with either dansylcadaverine (100 μM), bacitracin (300 μM), or vehicle (0.6% dimethyl sulfoxide) before additions of ET-1. Results are given as means ± standard error where n refers to the number of pigs used. Contactile responses to ET-1 are expressed as the percentage of the maximal contraction induced by 100 mM KCl. For each concentration-response curve to ET-1, the area under the concentration-response curve (in arbitrary units from 0 to 1000) was calculated, and where possible the negative logarithm of the effective concentration of ET-1 causing 25% of that contraction induced by 100 mM KCl (ED_{25}) was calculated. Statistical evaluation of data was performed using Student's t test for paired observations. Differences were considered to be significant at $P < 0.05$.

Results

Acid release of surface-bound ^{125}I-ET-1. For many ligands, such as epidermal growth factor, insulin, angiotensin II, and α_2-macroglobulin, the pH of the medium in which binding occurs influences their receptor interactions (15, 19, 15–28). The dissociation of receptor-ligand complexes under acidic conditions has been successfully exploited experimentally in a variety of culture systems to differentiate between surface and internalized ligand (15, 19, 26, 28). We (23) and others (29, 30) have previously demonstrated that VSMC in culture possess specific high affinity receptors for ET-1, although in these studies only total cellular binding of radioactive ligand was determined.

To examine whether smooth muscle cells internalize ET-1, hVSMC were incubated with ^{125}I-ET-1 at both 37° and 4° [to inhibit internalization (10–12)] and subsequently exposed to 0.5 M NaCl/0.2 M acetic acid (pH 2.5) (19). The amount of cell-bound radioactivity (total, acid-sensitive, and acid-resistant) was determined as a function of time (Fig. 1). For incubations at 4°, most of the cell-associated radioactivity was released following acid treatment, and negligible amounts of ^{125}I-ET-1 remained associated with hVSMC layers regardless of the time of incubations (Fig. 1B). In contrast, for incubations performed at 37°, ^{125}I-ET-1 bound to hVSMC was both acid sensitive and acid resistant, with the sum of these components being quantitatively comparable to total cellular (cell layer lysis without prior acid treatment) ^{125}I-ET-1 bound (Fig. 1A). The proportional distribution of the two components varied with time. The acid-sensitive fraction reached plateau levels within 15 min of incubation time at 37°, whereas the acid-insensitive ^{125}I-ET-1 continuously increased ($t_{1/2}$ ~15 min), to reach maximum levels (~70% of total) within 30–60 min (Fig. 1A).

It has been well documented that at 4° receptor-ligand internalization is inhibited (10–12) and, because accumulation of acid-resistant ^{125}I-ET-1 occurred at 37° but not at 4°, this

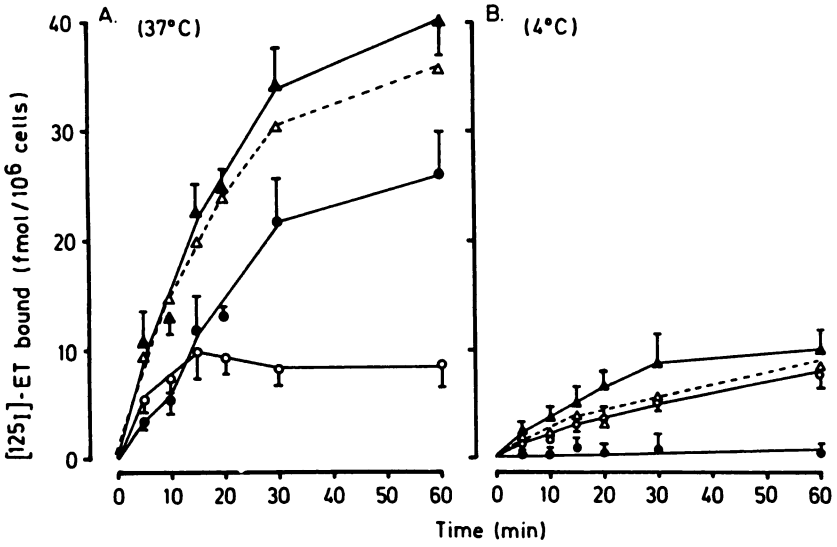

Fig. 1. Temperature-dependent kinetics of ^{125}I-ET-1 to hVSMC. Confluent quiescent hVSMC in buffer B (at 37° or precooled to 4°) were incubated with 0.25 nM ^{125}I-ET-1 at 37° (A) or 4° (B). At the indicated times, unbound hormone was removed by washing at 4° with phosphate-buffered saline/bovine serum albumin. ^{125}I-ET-1 bound to the cell surface was removed by incubation of cell monolayers with 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl (2 × 10 min at 4°) (acid-sensitive ^{125}I-ET-1) (○). The remaining cell-associated radioactivity was quantitated after solubilization of cell layers in 1 M NaOH containing 0.1% sodium dodecyl sulfate (acid-resistant ^{125}I-ET-1) (●). Total cell-bound radioactivity (△) was calculated by adding these two components. Cell layers in a parallel series of dishes were solubilized in 1 M NaOH/0.1% (w/v) sodium dodecyl sulfate without prior acid treatment, to experimentally determine total cell-bound ^{125}I-ET-1 (▲), and such values were not significantly different from those obtained by summation. Data (mean ± SD, n = 4) represent specific ^{125}I-ET-1 binding.

fraction was taken to represent internalized ligand. However, cellular binding of ^{125}I-ET-1 was always greater at 37° than at 4°, as previously observed (23, 29), and we considered that the temperature-dependent differential accumulation of acid-resistant ^{125}I-ET-1 might be due to the occurrence of nonspecific permeation/uptake during acid-washing procedures. To exclude such a possibility, cell layers were pretreated with 0.5 M NaCl/0.2 M acetic acid (pH 2.5) and thereafter repeatedly washed and maintained in binding buffer for 1 hr at either 37° or 4° before addition of ^{125}I-ET-1. Following such acid pretreatment, hVSMC were morphologically intact and binding of ^{125}I-ET-1 at 37° and 4° (Fig. 2, C and D) occurred with the same efficiencies as in control cultures (Fig. 2, A and B). Acid pretreatment did not influence the kinetics of total cellular ^{125}I-ET-1 binding at either 37° or 4°, although these cells exhibited an apparent accelerated initial rate of accumulation of acid-resistant radioactivity at 37° (compare Fig. 2, A and B). However, at least 95% of ligand bound at 4° remained acid sensitive (Fig. 2D), and the amounts of total and acid-sensitive ^{125}I-ET-1 were comparable to those in control cultures (compare Fig. 2, C and D). Such data indicate that the acid procedures employed do not render hVSMC permeable to hormone and that accumulation of acid-resistant ^{125}I-ET-1 is a specific temperature-dependent process in hVSMC.

In another series of ^{125}I-ET-1 binding experiments, ligand compartmentalization (total, surface, and internalized) was investigated using either the 0.5 M NaCl/0.2 M acetic acid (pH 2.5) procedures, as in the foregoing experiments, or brief tryp-

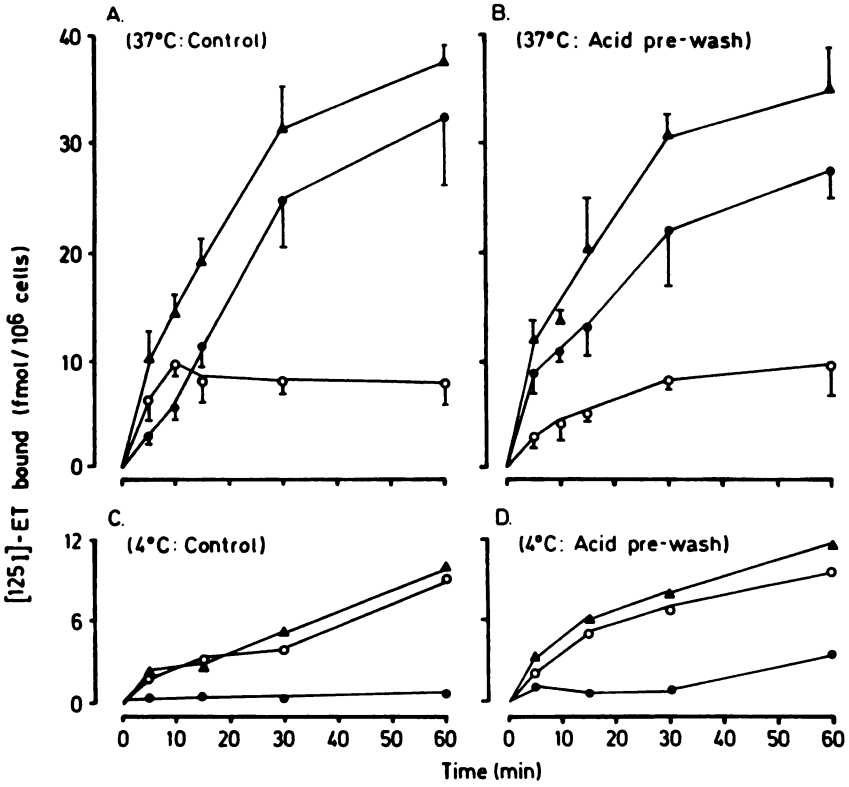

Fig. 2. ^{125}I-ET-1 binding to acid-treated hVSMC. hVSMC were incubated for 20 min at 4° with buffer B (A and C) or with 0.5 M NaCl/0.2 M acetic acid (pH 2.5) (B and D) and washed four times with and subsequently incubated in buffer B at either 37° (A and B) or 4° (C and D) for 1 hr. Thereafter, buffer overlay was replaced with fresh buffer B and binding was initiated by addition of ^{125}I-ET-1 (0.25 nM final concentration). After incubation for the indicated times at 37° (A and B) or 4° (C and D), total (▲), surface/acid-sensitive (○), and internalized/acid-resistant (●) ^{125}I-ET-1 binding was determined as described in Experimental Procedures and the legend to Fig. 1. Data for specific binding are shown and values in A and B represent mean ± standard deviation (n = 3), whereas in C and D values represent the mean of triplicate determination from two separate experiments.

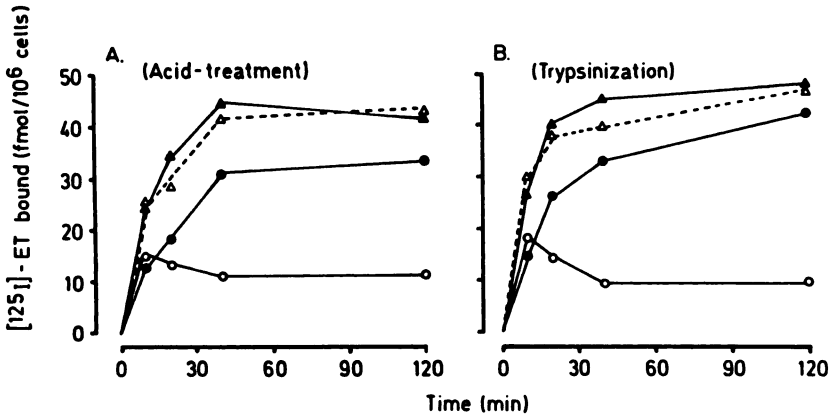

Fig. 3. Comparison of acid treatment and brief trypsinization procedures in determination of surface binding and internalization of ^{125}I-ET-1. hVSMC were incubated in buffer B at 37° in the presence of 0.25 nM ^{125}I-ET-1. At the indicated time points, cell layers were washed with phosphate-buffered saline/bovine serum albumin to remove unbound ligand and then processed using either the acid treatment described in Experimental Procedures and the legend to Fig. 1 (A) or brief trypsinization (B). For this trypsinization procedure, hVSMC were incubated with 0.05% trypsin/0.02% EDTA (w/v) for 20 min at 4°, the detached cells were transferred into an equal volume of phosphate-buffered saline/bovine serum albumin containing 2% (w/v) fetal calf serum (to inhibit trypsin), and the suspension was centrifuged at 1000 × *g* for 10 min, at 4°. The cell pellet was washed twice by recentrifugation in phosphate-buffered saline/bovine serum albumin, and all supernatant fractions were pooled for quantitation of trypsin-releasable (surface) ^{125}I-ET-1. Remaining cell-associated ^{125}I-ET-1 (internalized) was determined after solubilization in 1 M NaOH/0.1% (w/v) sodium dodecyl sulfate. Total binding for this series of dishes was determined after solubilization of the trypsin-containing cell suspension in 1 M NaOH/0.1% (w/v) sodium dodecyl sulfate. Data represent specific binding and values are the mean of quadruplicate determinations from two separate experiments. ○, surface-bound ^{125}I-ET-1 (acid- or trypsin-releasable); ●, internalized ^{125}I-ET-1 (acid- or trypsin-insensitive); ▲, experimentally determined total binding; △, calculated (○ plus ●) total binding.

sin/EDTA treatment of cell layers (see legend to Fig. 3). The latter proteolytic method (*vis à vis* the dissociative principle of acid treatment) has also been successfully employed to differentiate between surface binding and internalization of a number of ligands, such as low density lipoprotein (20), triiodothyronine (21), transferrin (28, 31), and heparin (32). Additionally, trypsin treatment of porcine aortic and rat lung membranes abolished subsequent specific receptor binding of ^{125}I-ET-1 (33), an observation we have also made for isolated plasma membranes of cultured smooth muscle cells.[1] As presented in Fig. 3, the acid and trypsin procedures yielded essentially identical results, both kinetically and quantitatively. Either procedure may, therefore, be employed to discriminate between cell surface-bound and internalized ^{125}I-ET-1. The use of an alternative acid method, namely washing with 50 mM glycine/100 mM NaCl at pH 4.0 (15, 24), was found to be inefficient and unsuitable for removal of surface ^{125}I-ET-1, with levels of "acid-resistant" radioactivity being high at 4° and correspondingly elevated at 37° (data not shown). In all the following experiments, we have utilized the acetic acid procedure in preference to trypsinization because of the obvious advantage of technical simplicity.

Effect of dansylcadaverine on ^{125}I-ET-1 binding and internalization. Internalization via adsorptive endocytosis requires clustering of receptor-ligand complexes in coated pits (10–12, 14). The enzyme transglutaminase is inhibitable by a variety of compounds including alkylamines, diamines, and lysylpeptides, and a quantitative correlation between inhibition of enzyme activity and inhibition of clustering has been established (35). Dansylcadaverine is a potent inhibitor of both the receptor clustering and internalization processes, as has been demonstrated for a variety of ligands such as α_2-macroglobulin, insulin, epidermal growth factor, and triiodothyronine (19, 21, 34–36). Therefore, in order to assess whether ET-1 internalization occurs via the receptor-mediated endocytotic pathway described for these ligands, we have analyzed the effect of dansylcadaverine on the binding and internalization of ^{125}I-ET-1 in hVSMC.

The kinetics of total ^{125}I-ET-1 binding to hVSMC was comparable in the absence (Fig. 4A) or presence (Fig. 4B) of dansylcadaverine (50 μM). However, in differential analysis of surface-bound and internalized ^{125}I-ET-1, dansylcadaverine exerted marked effects. As previously observed (Figs. 1–3) for control cultures, ^{125}I-ET-1 binding to the surface (acid sensitive) increased during the first 15 min of incubation and thereafter declined slightly to reach a plateau (~20% of total at 60 min), whereas intracellular radioactivity levels (acid resistant) steadily increased to reach ~70% of the total bound at 60 min (Fig. 4, A and C). However, in the presence of dansylcadaverine, surface-bound ^{125}I-ET-1 increased throughout the 60-min incubation, whereas the accumulation of internalized ^{125}I-ET-1 was significantly ($p < 0.001$ at >30 min versus control) reduced (Fig. 4B). At 60 min, ~60% of cellular radioactivity was acid sensitive and ~40% was acid resistant in dansylcadaverine-treated cultures (versus ~20% and ~70%, respectively, in control cultures) (Fig. 4C). The ability of dansylcadaverine to sustain surface accumulation and to inhibit internalization of ^{125}I-ET-1 was concentration dependent (Fig. 5A) and almost completely reversible (Fig. 5B).

Effect of cytosolic acidification on ^{125}I-ET-1 binding to hVSMC. In addition to a role for transglutaminase in receptor-mediated endocytosis, it has been proposed that the plasma membrane-bound Na^+/H^+ antiporter plays a role in ligand internalization through intracellular pH regulation (28, 37, 38). Cytosolic acidification has been demonstrated to efficiently block endocytosis of epidermal growth factor, transferrin, and diphtheria toxin (28, 37, 38).

To determine the effect of intracellular acidification on ET-

[1] T. J. Resink and T. Scott-Burden, unpublished observations.

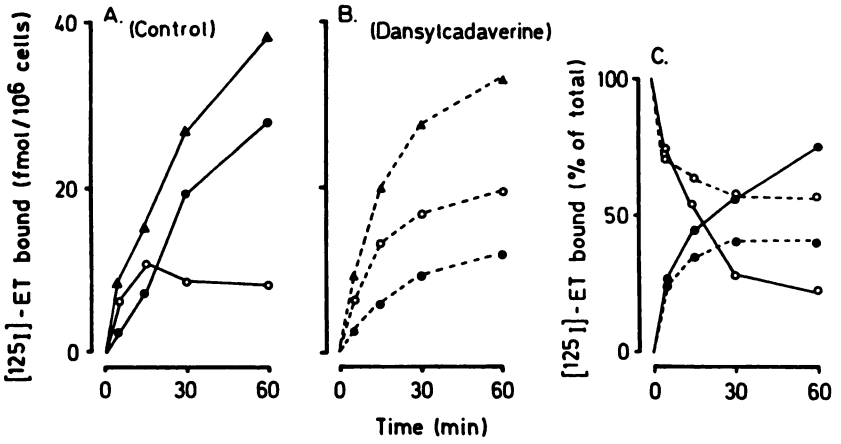

Fig. 4. Effect of dansylcadaverine on ^{125}I-ET-1 binding in hVSMC. Confluent quiescent hVSMC were preincubated in buffer B for 30 min at 37° in the absence (A, ——) or presence (B, – – –) of 500 μM dansylcadaverine and then further incubated at 37° for the indicated period in the presence of 0.25 nM ^{125}I-ET-1. After removal of unbound hormone, total (▲), surface (acid-sensitive) (○), and internalized (acid-resistant) (●) ^{125}I-ET-1 binding was determined as described in Experimental Procedures and the legend to Fig. 1. C, Effect of dansylcadaverine on surface (○) and internalized (●) ^{125}I-ET-1 binding, where data from Panels A and B were converted to express ^{125}I-ET-1 bound in each component as a percentage of total (100%) bound at each time interval. Values are the mean of quadruplicate determinations from two separate experiments and represent specific binding.

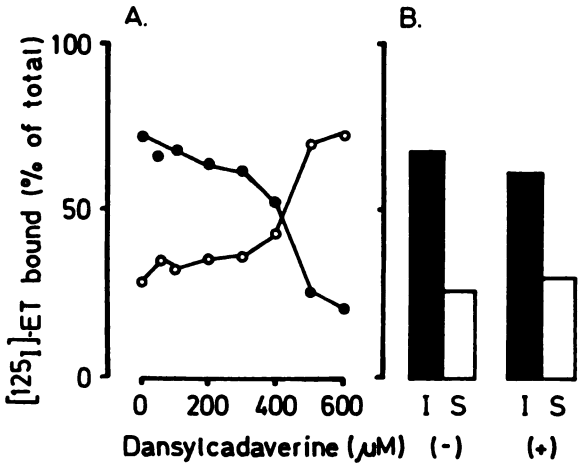

Fig. 5. Concentration dependence and reversibility of the effects of dansylcadaverine on ^{125}I-ET-1 binding. Confluent hVSMC were preincubated in buffer B without (vehicle, 0.1% dimethyl sulfoxide) or with the indicated concentrations of dansylcadaverine for 20 min at 37° before addition of 0.25 nM ^{125}I-ET-1 and further incubation at 37° for 60 min (A). In B, cultures were preincubated without (–) (vehicle, 0.1% dimethyl sulfoxide) or with (+) 500 μM dansylcadaverine in buffer B for 20 min at 37°, washed with phosphate-buffered saline/bovine serum albumin to remove the inhibitor, and then incubated for 15 min at 37° in buffer B before addition of ^{125}I-ET-1 (0.25 nM) and further incubation for 60 min. After termination of binding incubations and removal of unbound ligand, cell surface ^{125}I-ET-1 was removed with 0.5 M NaCl/0.2 M acetic acid (pH 2.5) (○ and *S*), and cell layers were subsequently solubilized in 1 M NaOH/0.1% (w/v) sodium dodecyl sulfate to determine internalized ^{125}I-ET-1 (● and *I*). Results were obtained from triplicate determinations in two separate experiments and data (specific binding) represent mean values expressed as the percentage of total cellular ^{125}I-ET-1 bound (100%) for each data point. Absolute values for total ligand bound were relatively constant (±13%) under all experimental protocols (~37–42 fmol of ^{125}I-ET-1 bound/10^6 cells at 37°).

1-binding, quiescent hVSMC were incubated in a choline buffer (130 mM choline chloride substituted for NaCl) with addition of potassium acetate (20 mM) to cause cytosolic acidification (39). Using the fluorescent pH indicator 2′,7′bis(carboxylethyl)-5(6)carboxyfluorescein, such incubation conditions have been shown to decrease intracellular pH by ~0.4 units (39). In this study, intracellular pH was determined by measurement of the distribution of the weak acid [^{14}C]DMO, and in control and choline chloride/potassium acetate-treated cultures the values for cytosolic pH were calculated to be 6.82 ± 0.16 and 6.38 ± 0.09 (mean ± SD, $n = 4$), respectively.

Such cytosolic acidification resulted in an accelerated initial rate of total cellular ^{125}I-ET-1 binding (Fig. 6, A and B). This observation becomes more evident when the data are transformed into an activity ratio (Fig. 6C), whereby total binding in acidified hVSMC was ~50% greater than in control cells during the first 15 min of incubation. Thereafter, this difference in binding declined, and by 60 min both acidified and control cells bound equivalent total amounts of ^{125}I-ET-1 (Fig. 6). Differential analysis (i.e., surface and internalized) revealed a reduced capacity for ligand internalization in acidified hVSMC (Fig. 6, A and B), such that after 60 min these cells internalized only ~34% of total ^{125}I-ET-1 bound, compared with 70% for the corresponding control hVSMC (Fig. 6C). The reversibility of these effects in choline chloride/potassium acetate-pretreated cells (i.e., by restoration to control NaCl-containing medium) was not investigated. However, from data presented in Fig. 2, in which cultures were pretreated with acetic acid [which also produces intracellular acidification (27)] and then returned to conditions permitting normalization of pH before binding, it was evident that such cells retained their full capacity for ligand internalization. Therefore, the inhibitor effects of intracellular acidification on endocytosis/internalization of ^{125}I-ET-1 appear to be reversible.

Effects of dansylcadaverine on the responses of hVSMC and isolated blood vessels to ET-1. In order to assess whether internalization of ET-1 may be relevant to the biochemical response of cultured smooth muscle cells to this peptide, *myo*-[^{3}H]inositol-prelabeled hVSMC were exposed to 500 μM dansylcadaverine before stimulation with ET-1. Dansylcadaverine exerted a potent inhibitory effect on the ability of ET-1 to induce the hydrolysis of phosphatidylinositol bisphosphate (Fig. 7A) and accumulation of inositol trisphosphate (Fig. 7B). Dansylcadaverine (500 μM, 30 min) by itself did not influence the inositol trisphosphate [see absolute values (dpm/well) in Fig. 7). The inhibitory effect of dansylcadaverine on ET-1-stimulated phospholipase C activity was dose dependent (IC_{50}, 177 ± 40 μM; Fig. 8). Two other transglutaminase inhibitors, bacitracin and ethylamine (35), were also found to effectively inhibit the ability of ET-1 to promote accumulation of inositol trisphosphate (IC_{50}, 288 ± 70 μM and 4.2 ± 1.5 mM, respectively), whereas methylamine was ineffective in this regard (Fig. 8). The relative potency of these compounds in inhibiting the biochemical response of hVSMC to ET-1 is similar to that previously reported (35) for inhibition of transglutaminase activity and α_2-macroglobulin receptor clustering in fibroblasts.

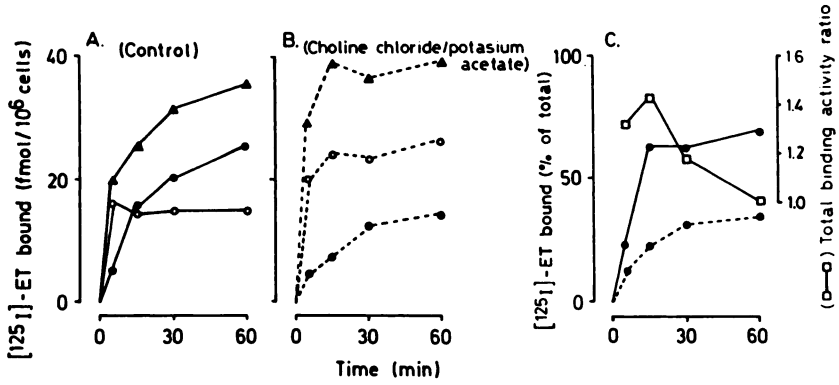

Fig. 6. Cytosolic acidification inhibits ^{125}I-ET-1 internalization by hVSMC. Confluent hVSMC were washed twice with either buffer N [130 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, 25 mM glucose, 20 mM HEPES-NaOH (pH 7.3), 0.2% (w/v) bovine serum albumin] or buffer A (same composition as buffer N except that 130 mM choline chloride was substituted for NaCl). Each series of cultures was then incubated at 37° for 30 min in the same buffer used for washing; for cultures in buffer A, potassium acetate was included (final concentration, 20 mM) during the final 10 min of this incubation. Thereafter, ^{125}I-ET-1 (0.25 nM) was added (without or with 200 nM unlabeled ET-1 for determination of nonspecific binding) and incubations were continued at 37°. At appropriate time periods, cultures were processed for determination of total (▲), surface (○), and internalized (●) ^{125}I-ET-1 binding, as described in Experimental Procedures and the legend to Fig. 1, except that buffer N and buffer B were used to remove unbound ligand instead of the phosphate-buffered saline/bovine serum albumin. A, ——, cultures washed and incubated with buffer N; B, – – –, cultures washed and incubated with buffer A. C (—— and – – – as above), ^{125}I-ET-1 internalized as a percentage of total (100%) binding at each time interval. Data are given as mean values of triplicate determinations from two separate experiments and represent specific binding to hVSMC. The binding activity ratio (C, □) was obtained by dividing total specific cellular binding in the choline chloride/potassium acetate-pretreated cultures (B, ▲) by that in control cultures (A, ▲) for each time point.

In porcine coronary arteries without endothelium, KCl (100 mM) induced a contraction that was not significantly different between control and transglutaminase inhibitor-treated groups (control, 12.4 ± 1.4 g; dansylcadaverine, 10.7 ± 1.9 g; bacitracin, 12.0 ± 0.9 g; n = 5). However, the contraction (expressed as percentage of that induced by 100 mM KCl) induced by 100 nM ET-1 (104 ± 9.7%) was significantly decreased by 100 μM dansylcadaverine (45.8 ± 16.8%) and 3×10^{-4} M bacitracin (77.2 ± 12.6%) (Fig. 9). Dansylcadaverine significantly decreased the area under the concentration-response curve to ET-1 (control, 185.8 ± 41.4; dansylcadaverine, 72.8 ± 32.6 arbitrary units), whereas bacitracin was not significantly effective (163 ± 32.5) in this regard (Fig. 9). Bacitracin did not modify the ED_{25} (−log M) of the response to ET-1 (control, 8.26 ± 0.23; bacitracin, 8.26 ± 0.24). An ED_{25} value for experiments in the presence of dansylcadaverine could not be reliably determined.

Discussion

This study has demonstrated that VSMC bind and internalize endothelin rapidly and that the process of internalization may be integral to some biochemical/physiological responses elicited by this peptide. Our conclusions are based on the application of a number of established methods for the discrimination between surface-bound and internalized ligand. Using either acid treatment or brief trypsinization following incubation of intact hVSMC with ^{125}I-ET-1, two binding components could be differentiated, namely, acid and/or trypsin sensitive and acid and/or trypsin resistant. Furthermore, the acid- and/or trypsin-resistant ^{125}I-ET-1 component was demonstrable only for incubations performed at 37° and not at 4°. Internalization/endocytosis is effectively arrested at 4° and ligand binding at this temperature is held to represent surface binding sites for many physiologically important macromolecules that enter cells by receptor-mediated endocytosis (see Refs. 10–12 and 14 for reviews). Therefore, by analogy with previous similar studies on ligand-receptor binding for diverse ligands such as epidermal growth factor, low density lipoprotein, heparin, triiodothyronine, transferrin, insulin, α_2-macroglobulin, and angiotensin II (15, 19–21, 26, 28, 31, 32), the acid/trypsin-sensitive and acid/trypsin-resistant ^{125}I-ET-1 binding components may be taken to represent surface-bound and internalized ligand, respectively.

Using rat aortic VSMC we have made observations[1] similar to those presented for hVSMC in Figs. 1–3. At present, there is no available equivalent information for other cell types, although ET-1 receptor internalization may be relevant to rapid clearance of the peptide from the circulation by tissues such as lung, kidney, liver, and spleen (8, 9). This study has employed only isotopic methods to discriminate between surface and internalized ^{125}I-ET-1. However, other methods such as video intensification microscopy (using fluorescent-labeled ligands) and electron microscopy (using colloidal gold, ferritin, or peroxidase ligand conjugates) have, for ligands such as insulin, epidermal growth factor, low density lipoprotein, α_2-macroglobulin, and triiodothyronine, yielded essentially identical findings in terms of their time- and temperature-dependent surface and internal localization (10–12, 21, 28, 34, 35, 40, 41).

Diverse molecules enter cultured cells via receptor-mediated endocytosis (10–15, 21, 25, 31). Although intracellular transport and processing vary markedly between different receptor-ligand systems and different cell types, initial steps in ligand internalization are similar. Following ligand binding to specific and diffusely distributed cell surface receptors, ligand-receptor complexes cluster in specialized structures termed clathrin-coated pits (10–12, 14). Clustering is followed by "pinching off" of the coated region to form endocytotic vesicles, which transfer ligands to other intracellular organelles (10–12, 14). The clustering process involves transglutaminase activity (34, 35) for the

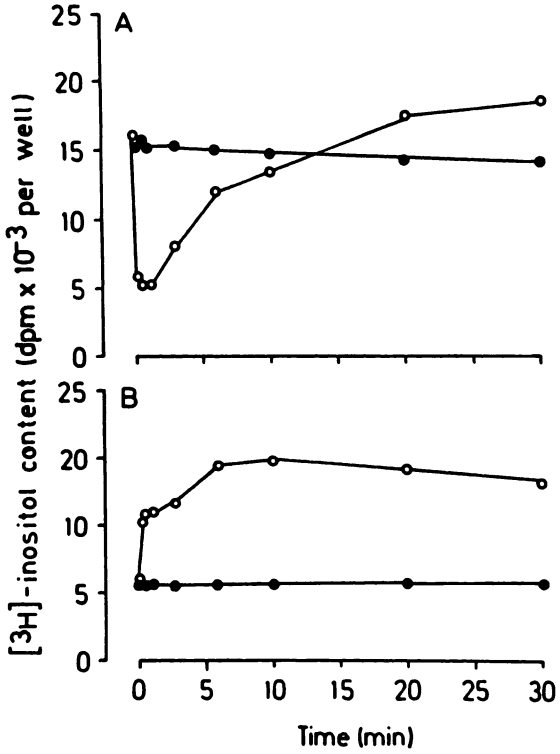

Fig. 7. Inhibition of ET-1-stimulated phospholipase C activity by dansylcadaverine. *myo*-[^{3}H]Inositol-prelabeled hVSMC were preincubated with 500 μM dansylcadaverine (●) or vehicle (0.1% dimethyl sulfoxide) (○) for 30 min before stimulation with ET-1 (50 nM) for the indicated times. After extraction and chromatographic resolution, [^{3}H]inositol contents of phosphatidylinositol bisphosphate [after deacylation (17, 23)] (A) and inositol trisphosphate (B) were determined. Data (2 dpm/well) are mean values of quadruplicate determinations (SD < 10%) from a single representative experiment, and comparable findings were made in two additional experiments.

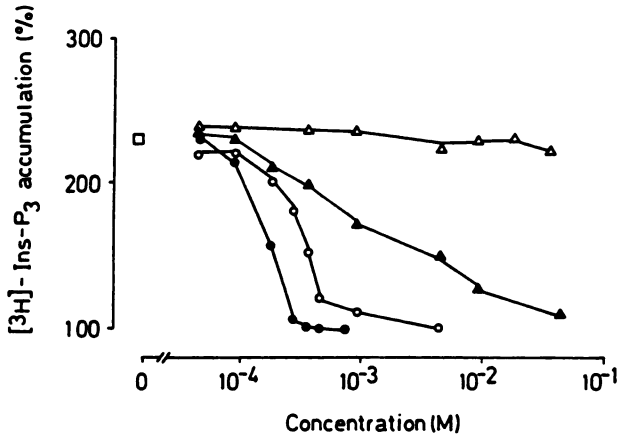

Fig. 8. Dose-dependent inhibition of ET-1-promoted inositol trisphosphate accumulation by inhibitors of transglutaminase. *myo*-[^{3}H]Inositol-prelabeled hVSMC were preincubated for 30 min with vehicle (0.1% dimethyl sulfoxide) (□) or the indicated concentrations of dansylcadaverine (●), bacitracin (○), ethylamine (▲), or methylamine (△) before stimulation with ET-1 (50 nM, 3 min). After extraction and resolution, ^{3}H content of inositol trisphosphate (*Ins-P$_3$*) was determined. Data express the changes in levels of [^{3}H]inositol trisphosphate after exposure to ET-1 as the percentage of that present (100%) in control samples (vehicle only and without ET-1 exposure). Absolute values (dpm/well) for control and ET-1-stimulated (without inhibitors) (□) samples were 5,524 ± 323 and 13,240 ± 871 (mean ± SD, n = 3). Data points represent mean values obtained from three experiments performed in duplicate; half-maximal inhibitory concentrations (IC_{50}) were separately determined in each experiment and are given in the text (mean ± standard deviation).

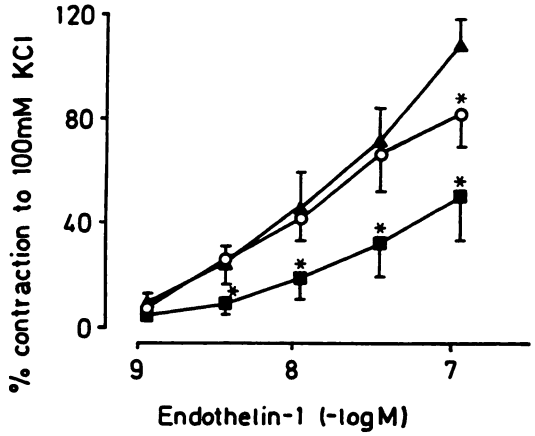

Fig. 9. Effect of transglutaminase inhibitors on ET-1-induced concentration of isolated arterial vessels. Isolated, endothelium-denuded, porcine coronary artery rings were contracted with the indicated concentrations of ET-1 after preincubation without (control (▲) or with 100 μM dansylcadaverine (■) or 300 μM bacitracin (○). All methods are detailed in Experimental Procedures. Data (mean ± standard error, n = 5) express the contractile response to ET-1 as a percentage of that (100%) to 100 mM KCl (see Experimental Procedures). * Contractile responses in the presence of inhibitors differ significantly from those in control rings (p at least < 0.05).

formation of ε-(γ-glutamyl)lysine cross-linkage between proteins (42), and inhibition of this enzyme blocks both ligand clustering and internalization (34, 36). We observed a marked inhibitory effect of the transglutaminase inhibitor and dansylcadaverine on ^{125}I-ET-1 internalization by hVSMC, and such effects were consistent with those made for epidermal growth factor binding in fibroblasts (19). Likewise, (19), dansylcadaverine did not influence total cellular ^{125}I-ET-1 binding, and the inhibitory effects on ^{125}I-ET-1 internalization were reversible.

Changes in intravesicular pH are important for cellular responses to ligand-receptor complexes, and the dissociation of these complexes following transfer from coated pits to endosomes requires a low pH (10–12, 25, 27). However, it is becoming increasingly evident that cytosolic pH is also important in receptor-mediated endocytotic processes (28, 37, 38). Cytosolic acidification has been shown to inhibit endocytotic uptake of a number of ligands, including epidermal growth factor, transferrin, and diphtheria toxin (28, 38). In concurrence with these studies, we observed that intracellular acidification of hVSMC efficiently blocked ^{125}I-ET-1 internalization. Furthermore, the initial rate of total cellular ligand binding was increased in acidified hVSMC, and this was not due to increase in available binding sites. As shown for epidermal growth factor (27), the inhibition of internalization by cytosolic acidification has been selectively attributed to inhibition of pinching off of coated pits (27). Therefore, our observations concerning the effects of cytosolic acidification, together with those related to the effects of dansylcadaverine, provide strong evidence that ET-1 binding and uptake are mediated via the clathrin-coated pit pathway for receptor-mediated endocytosis.

The ability of smooth muscle cells to internalize ET-1 may be physiologically important for the markedly protracted effects of this peptide on the vasculature (1, 2, 4). In such a context, receptor sequestration has been demonstrated to correlate with and to be integral to the second sustained phase of diacylglyc-

erol formation elicited by angiotensin II in cultured VSMC (26). A biphasic diacylglycerol accumulation response in VSMC exposed to ET-1 has also been demonstrated (43, 44), although its relationship to ET-1 internalization is currently unknown. Nevertheless, our data demonstrate that various inhibitors of transglutaminase [and hence of receptor clustering and internalization (35)] can exert potent inhibitory effects on the ability of ET-1 to promote receptor-coupled phospholipase C-mediated phosphoinositide catabolism. Furthermore, dansylcadaverine, and to a lesser extent bacitracin, decreased vasoconstriction induced by ET-1 but not that induced by KCl, which further supports their specific effect on receptor-mediated processes.

Hormone signalling from intracellular compartments has been suggested to occur (26, 45), and many cells have intracellular pools of ligand-specific binding sites (46, 47). Autoradiographic studies have indicated the existence of ET-1 receptors in both the plasma membrane and intracellular compartments of rat aortic smooth muscle cells (48). Additionally, in the myocardium, ischemia was shown to increase plasma membrane receptors for ET-1, with a concomitant decrease of intracellular membrane ET-1-binding (49). Therefore, it is indeed possible that ET-1 has surface (via plasma membrane receptor-coupled transduction pathways) and intracellular (requiring internalization of hormone-receptor complex) sites of action, which may be of importance to its diverse physiological actions (*vis à vis* circulatory peptide clearance). The inhibition of internalization of ET-1 by dansylcadaverine and/or other amines could prove useful in understanding the various cellular functions of this peptide.

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References

1. Yanagisawa, M., H. Kurihara, S. Kumura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature (Lond.) 332:* 411–415 (1988).
2. Yanagisawa, M., and T. Masaki. Endothelin, a novel endothelium-derived peptide: pharmacological activities, regulation and possible roles in cardiovascular control. *Biochem. Pharmacol.* **28:** 1877–1883 (1989).
3. Boulanger, C., and T. F. Lüscher. Release of endothelin from the porcine aorta: inhibition by endothelium-derived nitric oxide. *J. Clin. Invest.*, **85:** 587–590 (1990).
4. Yanagisawa, M., and T. Masaki. Molecular biology and biochemistry of the endothelins. *Trends Pharmacol. Sci.* **10:** 374–378 (1989).
5. Koseki, C., M. Imai, Y. Hirata, M. Yanagisawa, and T. Masaki. Autoradiographic distribution of binding sites for [^{125}I]-endothelin in rat tissues. *Am. J. Physiol.* **256:** R858–R866 (1989).
6. Inoue, A., M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, K. Goto, and T. Masaki. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. *Proc. Natl. Acad. Sci. USA* **86:** 2863–2867 (1989).
7. Watanabe, H., H. Miyazuki, M. Kondoh, Y. Masuda, S. Kimura, M. Yanagisawa, T. Masaki, and K. Murakami. Two distinct types of endothelin receptors are present on chick cardiac membranes. *Biochem. Biophys. Res. Commun.* **161:** 1252–1259 (1989).
8. Shiba, R., M. Yanagisawa, T. Miyauchi, Y. Ishii, S. Kimura, Y. Uchiyama, T. Masaki, and K. Goto. Elimination of intravenously injected endothelin from the circulation of the rat. *J. Cardiovasc. Pharmacol.* **13** (Suppl. 5): S98–S101 (1989).
9. Angarrd, E., S. Galton, G. Rae, R. Thomas, L. McLoughlin, G. de Nucci, and J. R. Vane. The fate of radioiodinated endothelin-1 and endothelin-3 in the rat. *J. Cardiovasc. Pharmacol.* **13** (Suppl. 5): S46–S49 (1989).
10. Pastan, I. H., and M. C. Wilingham. Receptor-mediated endocytosis of hormones in cultured cells. *Annu. Rev. Physiol.* **43:** 239–250 (1981).
11. Wileman, T., C. Harding, and P. Stahl. Receptor-mediated endocytosis. *Biochem. J.* **232:** 1–14 (1985).
12. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russel, and W. J. Schneider. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell. Biol.* **1:** 1–39 (1985).
13. Carpenter, G., and S. Cohen. [^{125}I]-labeled human epidermal growth factor: binding, internalization and degradation in human fibroblasts. *J. Cell. Biol.* **71:** 159–171 (1976).
14. Brown, M. S., R. G. W. Anderson, and J. L. Goldstein. Recycling receptors: the round tip itinerary of migrant membrane proteins. *Cell* **32:** 663–667 (1983).
15. Ullian, M. E., and S. L. Linas. Role of receptor cycling in the regulation of angiotensin II surface receptor number and angiotensin II uptake in rat vascular smooth muscle cells. *J. Clin. Invest.* **84:** 840–846 (1989).
16. Pan, B. J., and R. M. Johnstone. Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. *Cell* **33:** 967–977 (1983).
17. Scott-Burden, T., T. J. Resink, A. W. A. Hahn, U. Baur, R. G. Box, and F. R. Bühler. Induction of growth-related metabolism in human vascular smooth muscle cells by low density lipoprotein. *J. Biol. Chem.* **264:** 12582–12589 (1989).
18. Jones, P. A., T. Scott-Burden, and W. Gevers. Glycoprotein, elastin and collagen by rat smooth muscle cells. *Proc. Natl. Acad. Sci. USA* **76:** 353–357 (1979).
19. Haigler, H. T., F. R. Maxfield, M. C. Willingham, and I. Pastan. Dansylcadaverine inhibits internalization of [^{125}I]-epidermal growth factor in BALB 3T3 cells. *J. Biol. Chem.* **255:** 1239–1241 (1980).
20. Aulinskas, T. H., A Van der Westhuyzen, and G. A. Coetzee. Ascorbate increases the number of low density lipoprotein receptors in cultured arterial smooth muscle cells. *Atherosclerosis* **47:** 159–171 (1983).
21. Cheng, S. Y., F. R. Maxfield, J. Robbins, M. C. Willingham, and I. H. Pastan. Receptor-mediated uptake of 3,3,5-triiodo-*c*-thyronine by cultured fibroblasts. *Proc. Natl. Acad. Sci. USA* **77:** 3425–3429 (1980).
22. Mendoza, F. S. A., and E. Rozengurt. Measurement of ion flux and concentration in fibroblastic cells. *Methods Enzymol.* **146:** 384–399 (1987).
23. Resink, T. J., T. Scott-Burden, and F. R. Bühler. Activation of multiple signal transduction pathways by endothelin in cultured human vascular smooth muscle cells. *Eur. J. Biochem.*, **189:** 415–421 (1990).
24. Shimokawa, H., L. L. Aarhus, and P. M. Vanhoutte. Porcine coronary arteries with regenerated endothelium have a reduced endothelium-dependent responsiveness to aggregating platelets and serotonin. *Circ. Res.* **61:** 256–270 (1987).
25. Posner, B. I., Z. Josefsberg, and J. J. M. Bergeron. Intracellular polypeptide hormone receptors: characterization of insulin binding sites in Golgi fractions from the liver of female rats. *J. Biol. Chem.* **253:** 4067–4073 (1977).
26. Griendling, K. K., P. Delafontaine, S. E. Rittenhouse, M. A. Gimbrone, Jr., and R. W. Alexander. Correlation of receptor sequestration with sustained diacylglycerol accumulation in angiotensin II-stimulated cultured vascular smooth muscle cells. *J. Biol. Chem.* **262:** 14555–14562 (1987).
27. Maxfield, F. R. Weak bases and ionophore rapidly and reversibly raise the pH of endocytotic vesicles in cultured mouse fibroblasts. *J. Cell. Biol.* **95:** 676–681 (1982).
28. Sandvig, K., S. Olsnes, O. W. Petersen, and B. van Deurs. Acidification of the cytosol inhibits endocytosis from coated pits. *J. Cell. Biol.* **105:** 679–689 (1987).
29. Hirata, Y., H. Yoshimi, S. Takata, T. X. Watanabe, S. Kumagai, K. Nakajima, and S. Sakakibara. Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **154:** 868–875 (1988).
30. Clozel, M., W. Fischli, and C. Guilly. Specific binding of endothelin on human vascular smooth muscle cells in culture. *J. Clin. Invest. 83:* 1758–1761 (1989).
31. Ciechanover, A., A. L. Schwartz, A. Dautry, and H. F. Lodish. Kinetics of internalization and recycling of transferrin and the transferrin receptor in a human hepatoma cell line. *J. Biol. Chem.* **258:** 9681–9689 (1983).
32. Castellot, J. J., K. Wong, B. Hermann, R. L. Hovver, D. F. Albertini, T. C. Wright, B. L. Caleb, and M. J. Karnovsky. Binding and internalization of heparin by vascular smooth muscle cells. *J. Cell. Physiol.* **124:** 13–20 (1985).
33. Kanse, S. M., M. A. Ghatei, and S. R. Bloom. Endothelin binding sites in porcine aortic and rat lung membranes. *Eur. J. Biochem.* **182:** 175–179 (1989).
34. Maxfield, F. R., M. C. Willingham, P. J. A. Davies, and I. Pastan. Amines inhibit the clustering of α_2-macroglobulin and EGF on the fibroblast cell surface. *Nature (Lond.)* **277:** 661–663 (1979).
35. Davies, P. J. A., D. R. Davies, A. Levitzky, F. R. Maxfield, P. Milhaud, M. C. Willingham, and I. R. Pastan. Transglutaminase is essential in receptor-mediated endocytosis of α_2-macroglobulin and polypeptide hormones. *Nature (Lond.)* **283:** 162–167 (1980).
36. Maxfield, F. R., J. Schlessinger, Y. Schechter, I. Pastan, and M. C. Willingham. Collection of insulin and α_2-macroglobulin in the same patches on the surface of cultured fibroblasts and common internalization. *Cell* **14:** 805–810 (1978).
37. Huang, J., J. Pouyssegur, M. C. Willingham, and I. Pastan. The role of intracellular pH in ligand internalization. *J. Cell Physiol.* **128:** 18–22 (1986).
38. Sandvig, K., T. I. Tonnessen, O. Sand, and S. Olsnes. Requirement of a transmembrane pH gradient for the entry of diphtheria toxin into cells at low pH. *J. Biol. Chem.* **261:** 11639–11644 (1986).
39. Griendling, K. K., B. C. Berk, and R. W. Alexander. Evidence that Na^+/H^+ exchange regulates angiotensin II-stimulated diacylglycerol accumulation in vascular smooth muscle cells. *J. Biol. Chem.* **263:** 10620–10624 (1988).
40. Haigler, H. T., J. A. Mckanna, and S. Cohen. Direct visualization of the

binding and internalization of a ferritin conjugate of epidermal growth factor in human carcinoma cells. *J. Cell. Biol.* **81:** 382–395 (1979).
41. Willingham, M. C., and I. Pastan. The visualization of fluorescent proteins in living cells by video intensification microscopy. *Cell* **13:** 501–507 (1978).
42. Folk, J. E., and J. S. Finlayson. The *epsilon*-[*gamma*-glutamyl]lysine crosslink and the catalytic role of transglutaminase. *Adv. Protein Chem.* **31:** 1–133 (1977).
43. Griendling, K. K., T. Tsuda, and R. W. Alexander. Endothelin stimulates diacylglycerol accumulation and activates proteins kinase C in cultured vascular smooth cells. *J. Biol. Chem.* **264:** 8237–8240 (1989).
44. Sunako, M., Y. Kawahara, K. Kariya, S. Araki, H. Fukuzaki, and Y. Takai. Endothelin-induced biphasic formation of 1,2-diacylglycerol in cultured rabbit vascular smooth muscle cells: mass analysis with a radioenzymatic assay. *Biochem. Biophys. Res. Commun.* **160:** 744–750 (1989).
45. Richards, D. E., R. F. Irvine, and R. M. C. Dawson. Hydrolysis of membrane phospholipids by phospholipase of rat liver lysosomes. *Biochem. J.* **182:** 599–606 (1979).
46. Oppenheimer, J. H. Thyroid hormone action at the cellular level. *Science (Washington, D.C.)* **203:** 971–979 (1979).
47. Goldfine, I. D., G. J. Smith, K. Y. Wong, and A. L. Jones. Cellular uptake and nuclear binding of insulin in human cultured lymphocytes: evidence for potential intracellular sites of insulin action. *Proc. Natl. Acad. Sci. USA* **74:** 1368–1372 (1977).
48. Hirata, Y., H. Yoshimi, S. Takaichi, M. Yanagisawa, and T. Masaki. Binding and receptor down-regulation of a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells. *FEBS Lett.* **239:** 13–17 (1988).
49. Liu, J., D. G. Casley, and W. G. Nayler. Ischaemia causes externalization of endothelin-1 binding sites in rat cardiac membranes. *Biochem. Biophys. Res. Commun.* **164:** 1220–1225 (1989).

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