Insulin Receptor Recycling in Vascular Endothelial Cells

REGULATION BY INSULIN AND PHORBOL ESTER*

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Endothelial cell insulin receptors mediate the transcytosis of insulin from luminal to abluminal cell surface. We have investigated the kinetics of insulin receptor translocation by immunoprecipitation of radiolabeled receptors at various times before and after trypsin treatment of intact endothelial cells. Insulin receptors were constitutively internalized with t1/2 = 18 ± 2 min and were recycled to the cell surface. Insulin stimulated receptor internalization and externalization rates 2.6- and 2.4-fold, respectively. Changes in cell-surface binding of 125I-insulin were consistent with the receptor translocation rates observed in surface-labeling experiments. Phorbol myristate acetate (PMA) treatment increased the rate of insulin-stimulated receptor externalization 1.7-fold. PMA treatment increased the constitutive externalization rate 3.5-fold without affecting the constitutive internalization rate, suggesting that recycling might occur via a mobilization of receptors from intracellular sites in a manner independent of internalization rate. Analysis of the intracellular distribution of receptors by 125I-insulin binding and immunogold electron microscopy revealed that less than one-third of the total insulin receptor pool resided on the cell surface. In summary, endothelial cell insulin receptors are constitutively recycled, and internalization and externalization rates are increased by receptor occupancy and PMA treatment.

Vascular endothelial cells possess insulin receptors that are structurally similar to those found in other tissues (1). An extracellular α-subunit containing the putative insulin binding domain, and a β-subunit containing transmembrane and tyrosine kinase domains, form a heterodimer linked by disulfide bonds (1, 2). Evidence indicates that two such heterodimers associate to form a tetramer capable of specifically binding insulin with high affinity (2). Microvascular endothelial cells in culture demonstrate biological responses to insulin, such as increased glucose and amino acid transport, and increased growth rate, as do many insulin target tissues (3, 4). Despite these similarities in receptor structure and function, endothelial cells process receptor-bound insulin differently than most other cell types. Endothelial cells cultured from aorta and several microvasculatures degrade less than 20% of the insulin they internalize and release the balance of internalized insulin ungraded (5–7). The receptor-mediated transcytosis of insulin by endothelial cells has been demonstrated in vitro (8, 9), and recent evidence strongly suggests that it also occurs in vivo (10).

Receptor-mediated transcytosis is also the most likely mechanism by which thyroglobulin crosses thyroid follicular cells (11), IgG (12), IgA (13, 14), and epidermal growth factor (15) cross-epithelial cells, and low density lipoprotein (16) cross-endothelial cells. As shown for low density lipoprotein, specific domains within receptor molecules may participate in the regulation of intracellular trafficking (18).

The binding of insulin to its receptor results in the autophosphorylation of specific tyrosine residues in the β-subunit and a less immediate increase in its level of serine phosphorylation (19, 20). The temporal coincidence of these events and receptor-mediated endocytosis suggest that they may signal or otherwise regulate receptor internalization and/or recycling. Recently published studies on the effects of phorbol ester on insulin-stimulated insulin receptor internalization in endothelial cells further implicate the phosphorylation state of the receptor in these processes (19). In the present study we demonstrate constitutive internalization and recycling of insulin receptors in endothelial cells, characterize the effects of receptor occupancy on these processes, and extend previous findings by characterizing the effects of phorbol ester on constitutive receptor traffic and insulin-stimulated receptor recycling.

EXPERIMENTAL PROCEDURES

Materials—The following materials were obtained from the indicated sources: [Na125I]iodide and Triton X-100 were from Du Pont–New England Nuclear; [125I]monodiodoinsulin (high performance liquid chromatography-purified) was from Amersham Corp.; HEPES1 and reagents used for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad; trypsin (t-1-tysoylamido-2-phenylethyl chloromethyl ketone-treated) was from Worthington; bovine serum albumin (insulin-free) from Arnel, NY; aprotinin, cycloheximide, β-phorbol 12-myristate 13-acetate (PMA), phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, lactoperoxidase, and glucose oxidase were from Sigma; human fibronectin was obtained from New York Blood Center, NY; porcine insulin (Lot 1JM95AN) was from Elianco; tissue culture medium, and plasma-derived equine serum were obtained from Gibco. All tissue culture supplies were from NUNC, Kamstrup, Denmark or Costar.

Cell Culture—All experiments were performed using microvascular endothelial cells derived from rat epididymal fat pad as described previously (5). Cells were subcultured for a minimum of 5 passages from aorta and several microvasculatures degrade less than 20% of the insulin they internalize and release the balance of internalized insulin ungraded (5–7). The receptor-mediated transcytosis of insulin by endothelial cells has been demonstrated in vitro (8, 9), and recent evidence strongly suggests that it also occurs in vivo (10).

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1The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; HBB, HEPES binding buffer; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline.
Insulin Receptor Recycling in Endothelial Cells

and a maximum of 20 passages. The homogeneity of endothelial cell cultures was confirmed by morphological and immunocytochemical criteria, including the presence of angiotensin converting enzyme activity and uptake of diacylated low density lipoprotein. All cultures were maintained at 37 °C in a humidified atmosphere composed of 95% air and 5% CO₂. Cells were grown in plastic tissue culture dishes (tissue culture treated) coated with 10% fetal bovine serum (Hoefer Scientific Instruments, San Francisco, CA).

Cell-surface Insulin Binding—[125I]-Insulin binding was measured as described previously (3, 22). Briefly, confluent monolayers of microvascular endothelial cells were grown in 6-well culture trays (NUNC). The cells were incubated for 16 h at 4 °C in 3 ml of HEPES binding buffer (HBB: 150 mM NaCl, 5 mM KC, 1.2 mM MgSO₄, 8 mM glucose, 0.5% bovine serum albumin, 100 mM HEPES, pH 7.6) containing [125I]monooiodoinsulin (10⁻⁸ M, 100,000 cpm), in the presence and absence of unlabeled insulin (1 μM). The unbound insulin was removed from the monolayers by three washes (2 ml) with phosphate-buffered saline (PBS; pH 7.4) at 4 °C. To assess surface-bound tracer, the cells received two additional washes for 7 min each using PBS (pH 4.0) at 4 °C, and the washes were saved and counted. Acid-sensitive radioactivity was used as an index of cell-surface [125I]-insulin, and the acid-resistant radioactivity was used as a measure of internalized [125I]-insulin (23). The cells were then solubilized with 1 ml of 0.1% SDS and the bound radioactivity was measured in a γ counter. Release of internalized [125I]-insulin and degradation of [125I]-insulin was assayed by trichloroacetic acid precipitation as described above (7).

Insulin Binding in Solubilized Cell Extracts—Total [125I]-insulin binding activity in microvascular endothelial cells was measured in Triton X-100 cell extracts as described previously (24). The portion of the total binding activity located inside the cells was estimated by trypsinization of intact cell monolayers at 4 °C as described below (cf. "Determination of Insulin Receptor Internalization and Externalization Rates") prior to performing binding assays on Triton X-100 extracts.

Internalization of Prebound Insulin—[125I]-Insulin was prebound to endothelial cells by incubation with 500,000 cpm of [125I]monooiodoinsulin (10⁻⁸ M) in HBB at 4 °C until the start of the internalization step. Unbound insulin was removed by extensive rinsing with cold HBB. The cells were then quickly warmed to 37 °C by addition of warm HBB and incubated at this temperature for specified time intervals. The dishes were subsequently placed on ice, and the HBB was collected. The cells were rinsed three times with 5 ml of cold PBS, and the rinses were combined with the HBB. An aliquot of this buffer was counted and the total amount of radioactivity released by the cells was calculated. The amount of radioactivity bound to the cell surface was ascertained by acid resistance as described above, and the radioactivity remaining inside the cells after acid washing was measured following solubilization with 0.5% SDS.

Surface 125I-Labelling of the Intact Endothelial Cells—Surface labeling of cultured cells was performed by lactoperoxidase-catalyzed iodination of extracellular proteins (25). Cell cultures in 10 cm dishes were washed three times in PBS (pH 7.4) to remove serum proteins, and then incubated at 4 °C with 3 ml of PBS containing 10 μM glucose, 2 units/ml lactoperoxidase, 1 unit/ml glucose oxidase, and 0.4 mM NaI. The reaction was carried out for 30 min, and the cells were subsequently washed several times with PBS to stop the reaction. The labeled cells were kept at 4 °C until the start of internalization or externalization time courses, at which time they were quickly warmed to 37 °C and incubated with or without insulin at variable concentrations (100 nM) for 30 min. Initially, the cells were washed three times with ice-cold PBS and trypsin-treated at 4 °C for 45 min. After three washes with cold PBS to remove the trypsin, the cells were rewarmed to 37 °C and incubated at this temperature for specified time intervals. Subsequently, the cells were rinsed in cold PBS, surface-labeled, solubilized, and immunoprecipitated as described above.

Immunoprecipitation of Insulin Receptor—Immediately following experimental incubations, cell monolayers were solubilized at 4 °C with 1 ml of a solution containing 1% Triton X-100, 50 mM HEPES (pH 7.4), 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 2 mM sodium vanadate, 1 mg/ml aprotinin, and 2 mM phenylmethylsulfon fluoride. All subsequent steps were performed at 4 °C unless otherwise noted. The cells were scraped from the dishes and cell material was sedimented by centrifugation in a Beckman Microfuge B for 5 min. The soluble cell extract was immunoprecipitated with anti-insulin receptor antibodies by incubation with 10 μg of anti-insulin receptor antibodies (B9) for 16 h at 4 °C, as described previously (21). The antibody complex was precipitated with 100 μl of Pansorbin (10% Pansorbin for 1 h) and separated on a resolving polyacrylamide gel (21). The amount of labeled insulin receptor in each lane of the autoradiogram was measured by scanning densitometry, expressed as a percentage of total surface-labeled receptor from untreated cells not subjected to trypsinization (included as a control in all experiments). Data used to characterize receptor internalization and externalization rates were obtained from three or more separate experiments. Initial rates (0-10 min) were computed by linear least squares analysis of data from three or more experiments using Graphwriter software (Lotus Development Corporation, Cambridge, MA) on an IBM PC XT. Statistically significant differences were determined by Student's t test, and are noted by p < 0.05 or lower.

The time course of insulin receptor externalization was constructed similarly, with the following modifications. Cells were incubated in the presence or absence of PMA for 30 min, at which time insulin was added to some dishes and the incubation was continued for another 30 min. The cells were then washed three times with ice-cold PBS and trypsin-treated at 4 °C for 45 min. After three washes with cold PBS to remove the trypsin, the cells were rewarmed to 37 °C and HBB and incubated at this temperature for specified time intervals. Subsequently, the cells were rinsed in cold PBS, surface-labeled, solubilized, and immunoprecipitated as described above.

A modification to the protocol was made to facilitate the measurement of receptor externalization that did not interfere with the measurement of receptor internalization. This modification to the protocol facilitated the measurement of receptor externalization essentially consisted of treating cells with trypsin before the 37 °C time course instead of after it. To examine the possibility that trypsin treatment might alter the subsequent recovery of receptor recycling activity, another protocol was devised that allowed the localization of receptor by protection from proteolysis, but also allowed receptor recycling to occur before trypsin treatment. In these experiments cell-surface protein was radiolabeled, and receptor internalization was stimulated with insulin for 30 min. The cells were then chilled, the medium was replaced, and externalization was facilitated by incubation with or without insulin (100 nM) for 30 min. At the end of the recycling period, the cells were trypsin-treated, solubilized, and the radiolabeled intracellular receptor was immunoprecipitated and quantitated as described above. Initial rates (0-10 min) were computed by linear least squares analysis of data from three or more separate experiments.

Transmission Electron Microscopy and Morphometric Analysis of Intracellular Insulin Receptor Distribution—Rat microvascular endothelial cells grown to confluence on fibronectin-coated dialysis membranes were fixed with 2.5% glutaraldehyde in 100 mM phosphate buffer, pH 7.4 for 1 h at room temperature. Osmication was omitted; otherwise, tissues were routinely dehydrated through graded ethanols and embedded in Araldite. Ultrathin sections were picked up on nickel grids, incubated with anti-insulin receptor antibodies (B-9, 100 μl of a solution containing 50 mM HEPES, 150 mM NaCl, 100 mM NaF, 1.6% Triton X-100, and 0.1% SDS (pH 7.4). The proteins were eluted from washes precipitated with 70 μl of Laemmli sample buffer, reduced with 100 μl dithiothreitol and separated by SDS-polyacrylamide gel electrophoresis on 7.5% resolving polyacrylamide gel as described previously (21). The labeled proteins were identified by autoradiography of the stained and dried gels using an X-ray film and autoradiography ( screens) (cf. "Determination of Insulin Receptor Internalization and Externalization Rates") prior to performing binding assays on Triton X-100 extracts.
no protein A-gold. After immunogold staining, grids were stained with uranyl acetate and lead citrate and viewed using a Phillips model 301 electron microscope. For each of two control and two anti-receptor antisera-stained samples, a montage of 16 micrographs taken at $x \times 25,000$ was constructed. The montage covered a distance of 11 $\mu m$, measured linearly along the substratum. Gold particles were counted over this distance and categorized as either intracellular or surface-associated. The mean negative control (non-immune serum) values in each category were subtracted from their respective counterparts among the anti-receptor antisera-stained samples to give corrected values shown in Table VI.

**RESULTS**

**Insulin Receptor Internalization**—Microvascular endothelial cells constitutively internalized insulin receptors in the absence of insulin, as shown in Fig. 1. After radiolabeling the cell-surface protein by lactoperoxidase-catalyzed iodination, the cells were solubilized and the insulin receptor was immunoprecipitated with an anti-receptor antibody. *Lane 1* (Fig. 1A) shows that the $\alpha$-subunit of the receptor migrated at 140 kDa and the $\beta$-subunit at 95 kDa, consistent with their structural similarity with other insulin receptors (1–3). Bands seen at other molecular weights were also observed after immunoprecipitation with control antibodies, and thus can be considered non-specific. The $\beta$-subunit of the receptor has only a minor extracellular subunit which was only partially labeled by cell-surface iodination. For this reason, receptor translocation was quantitated by measuring changes in the appearance of the $\alpha$-subunit only. When the cells were treated with trypsin at 4 $^\circ C$ immediately after surface-labelling, more than 95% of the surface receptors were removed (Fig. 1A, lane 2). When radiolabeled cells were incubated at 37 $^\circ C$ before trypsin treatment, a portion of the receptors were internalized and thereby protected from proteolysis, as indicated in lanes 3–8. With increasing time at 37 $^\circ C$, these bands increase in intensity as more receptor is internalized. The data were quantitated by scanning densitometry (Fig. 1B), and internalization expressed as a percentage of the total radiolabeled surface receptor observed in the absence of trypsin treatment. Constitutive receptor internalization reached 50% of maximum after 25 min, maximum at 60 min, and decreased thereafter, probably as a result of receptor recycling. In the presence of 100 nM insulin, the rate of receptor internalization increased 2.2-fold (as quantitated from initial rates shown in Table I), and the time required to reach maximum decreased from 60 to 15 min, a 4-fold reduction ($p < 0.05$; Fig. 1B).

The initial rate of insulin-stimulated receptor internalization was confirmed by measuring changes in the surface binding of $^{125}$I-insulin (Table II). After exposure to 100 nM insulin for the time shown in the left-hand column, bound, unlabeled insulin was dissociated from cell surface receptors by rinsing with a low pH buffer at 4 $^\circ C$, and specific binding at the cell surface was measured. The values shown in the right-hand column show that the insulin binding activity at the cell surface decreased at a rate of 4.8 $\pm$ 0.4% of total surface receptor/min, comparable with the initial rate of receptor internalization (6.1 $\pm$ 1.1% total surface receptor/min; no significant difference) observed in surface labeling experiments.

**Table I**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial rate*</th>
<th>$t_{1/2}$</th>
<th>% Total surface IR/min</th>
<th>min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutive</td>
<td>2.8 $\pm$ 0.3</td>
<td>18</td>
<td>6.1 $\pm$ 1.1</td>
<td>7</td>
</tr>
<tr>
<td>Insulin (100 nM)</td>
<td>3.8 $\pm$ 0.3</td>
<td>12</td>
<td>12.4 $\pm$ 1.4</td>
<td>5</td>
</tr>
</tbody>
</table>

* Insulin rates were calculated as change in the amount of immunoprecipitable radiolabeled IR over 10 min at 37 $^\circ C$. Results are expressed as the percentage of total radiolabeled IR (obtained from control cells at $t = 0$, without subsequent trypsin treatment) internalized per min. Values shown are the mean $\pm$ S.E. from three replicate experiments.

$^b t_{1/2}$ = time required to internalize 50% of maximum.

**Table II**

<table>
<thead>
<tr>
<th>Initial rate of internalization of $^{125}$I-insulin binding activity</th>
</tr>
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<tbody>
<tr>
<td>Endothelial cells were exposed to 100 nM insulin for the time shown in the left-hand column. Bound, unlabeled insulin was then dissociated from surface receptors at low pH, and specific binding of $^{125}$I-insulin was measured as described under &quot;Experimental Procedures&quot; (&quot;Cell-surface Insulin Binding&quot;), corrected for total cell protein. Values shown are mean $\pm$ S.E. from three separate experiments. Initial rate (0–10 min) = 0.16% cpm/mg/min = 4.8% total surface binding activity/min.</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Time</th>
<th>Specific binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>% cpm/mg protein</td>
</tr>
<tr>
<td>0</td>
<td>3.39 $\pm$ 0.12</td>
</tr>
<tr>
<td>5</td>
<td>2.24 $\pm$ 0.18</td>
</tr>
<tr>
<td>10</td>
<td>1.79 $\pm$ 0.16</td>
</tr>
</tbody>
</table>
In the insulin-stimulated receptor internalization experiments described above, the time required for insulin to bind to its receptor was unavoidably included in the time required for internalization. To estimate the insulin-stimulated receptor internalization rate apart from the time required for binding, 125I-insulin was prebound to cell-surface receptors, and, after various time periods at 37 °C, the fate of the prebound insulin within extracellular, surface, and intracellular compartments was measured. As shown in Fig. 2, the internalization of a cohort of surface receptors reached a maximum within 3 min and coincided with an 11.5-fold decrease in the amount of surface-bound tracer. After 7.5 min, the amount of surface-bound insulin increased 79%, suggesting the return of internalized insulin to this compartment; the amount of internalized insulin showed a 52% decrease over this period. Two factors may contribute to the rapid rise in insulin released over the 30-min time course: first, as the cells were warmed to 37 °C, much of the insulin bound to its receptor at 4 °C may dissociate before internalization, and second, internalized insulin may be released intact through an exocytic process, as demonstrated previously in endothelial cells (5, 6).

Phorbol esters, such as PMA, are diacylglycerol analogs that exert a wide variety of effects on cell metabolism, growth, and differentiation and induce the internalization of receptors for epidermal growth factor, transferrin, and β-adrenergic agonists (27-31). We have examined the effects of PMA on constitutive and insulin-stimulated insulin receptor internalization in microvascular endothelial cells (Fig. 3). No significant change in the rate or extent of constitutive receptor internalization was observed in response to phorbol treatment (not significant; Fig. 3A). However, in the presence of 100 nM insulin added at the start of the time course, significant increases in the amount of receptor internalized at 2.5 and 5 min were observed (Fig. 3B). The initial rate (0-10 min) of insulin-stimulated receptor internalization, as determined by linear least squares analysis, was increased 1.7-fold by PMA treatment (p < 0.05). The effects of insulin, PMA, and insulin plus PMA on the initial rate and time required to reach 50% of maximum insulin receptor internalization are summarized in Table I. The biologically inactive 4α-isomer of PMA had no significant effects on constitutive or insulin-stimulated receptor internalization (data not shown).

**Insulin Receptor Externalization—**Endothelial cells were treated with 160 nM PMA for 30 min prior to surface labeling; otherwise the experiments were performed as outlined in Fig. 1 and under “Experimental Procedures.” Values are the mean ± S.E. from three replicate experiments. Inset, linear regression computed from three 0-10 min time course experiments used to calculate initial rates shown in Table II. B, the effects of PMA on the time course of constitutive insulin receptor internalization. Cells were pretreated with 100 nM PMA for 30 min before surface labeling, and 100 nM insulin was added to the cells at the start of the 37 °C incubation. Values are the mean ± S.E. from three or more replicate experiments. Inset, linear regression computed from three 0-10 min time course experiments used to calculate initial rates shown in Table II.

Fig. 2. Representative time course of the redistribution of prebound 125I-insulin between intracellular, cell-surface, and intracellular compartments. 125I-Insulin was prebound to the surface of endothelial cells at 4 °C, unbound insulin was removed, and the cells were rapidly warmed to 37 °C for the time intervals shown. The fate of labeled insulin among the three compartments was determined as described under “Experimental Procedures.” Similar results were observed in three different experiments.

Fig. 3. A, the effects of PMA on the time course of constitutive insulin receptor internalization. Cells were pretreated with 160 nM PMA for 30 min prior to surface labeling; otherwise the experiments were performed as outlined in Fig. 1 and under “Experimental Procedures.” Values are the mean ± S.E. from three replicate experiments. Inset, linear regression computed from three 0-10 min time course experiments used to calculate initial rates shown in Table II. B, the effects of PMA on the time course of insulin-stimulated insulin receptor internalization. Cells were pretreated with 160 nM PMA for 30 min before surface labeling, and 100 nM insulin was added to the cells at the start of the 37 °C incubation. Values are the mean ± S.E. from three or more replicate experiments. Inset, linear regression computed from three 0-10 min time course experiments used to calculate initial rates shown in Table II.

Insulin Receptor Recycling in Endothelial Cells

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**Insulin Receptor Externalization—**Endothelial cells were incubated in the presence and absence of 100 nM insulin for 30 min at 37 °C. During this period insulin-stimulated receptor internalization reached a maximum, as determined in the experiments described above. The cells were then treated with trypsin, and externalization of the receptors that had internalized during the first 37 °C incubation could be observed by cell-surface labeling after a second 37 °C incubation of variable duration. The surface-labeled insulin receptor was immunoprecipitated and quantitated as described above. The data indicate that insulin receptors in microvascular endothelial cells were constitutively recycled, requiring 60 min to replenish 89% of the steady-state complement of surface receptors (Fig. 4). Pretreatment with 100 nM insulin significantly accelerated receptor externalization from 0 to 15 min by 1.8-fold (p < 0.05); thereafter the rate quickly decreased to a level similar to that observed in untreated cells.

To examine the possibility that trypsin treatment might alter the subsequent receptor recycling activity, an alternative protocol was devised that allowed the localization of receptor by protection from proteolysis but also allowed receptor externalization to occur before trypsin treatment. Cell surface protein was radiolabeled, and receptor internalization was
Fig. 4. Time course of constitutive and insulin-stimulated insulin receptor recycling. Cells were incubated in the presence and absence of insulin for 30 min at 37 °C, then chilled, trypsin-treated, and rewarmed to 37 °C for the time intervals indicated. At the end of this time cell-surface protein was radiolabeled at 4 °C, and the cells were solubilized and immunoprecipitated as described under "Experimental Procedures." The intact, radiolabeled receptor was purified by SDS-PAGE, and autoradiograms were analyzed by scanning densitometry. Values are the mean ± S.E. from three replicate experiments. Inset, linear regression computed from three 0–10-min time course experiments used to calculate initial rates shown in Table IV.

stimulated with insulin for 30 min. The cells were then chilled, the medium was replaced, and externalization was facilitated by a second 37 °C incubation of variable duration. The cells were subsequently trypsin-treated, solubilized, and the radiolabeled intracellular receptor was quantitatively immunoprecipitated as described above. With increasing time at 37 °C in the second incubation, the amount of intact receptor decreased as more of it recycled to the cell surface and became susceptible to trypsin digestion. The initial rate of insulin-stimulated insulin receptor depletion was 6.8 ± 0.9% of total surface receptor/min. Thus, the rate and total extent of receptor externalization measured as receptor depletion was not significantly different from the rate and extent of insulin-stimulated receptor externalization as assessed using the original protocol (8.2 ± 1.1% of total surface receptor/min, Table IV, no significant difference). These results suggested that trypsin treatment had no adverse effects on insulin receptor recycling, and so the original protocol was used in subsequent studies.

The insulin-stimulated increase in receptor externalization rate was further confirmed by binding studies using 125I-insulin. In these experiments cells were treated identically to those used in immunoprecipitation experiments, except that instead of surface labeling at the end of the second 37 °C incubation, the surface binding activity of the intact cells was measured (Table III). Within 10 min at 37 °C, the rate of return of insulin binding activity to the cell surface increased 1.8-fold, consistent with rates of receptor externalization measured by immunoprecipitation. To show that the reappearance of receptors and insulin binding activity was due to receptor recycling and not the delivery of newly synthesized receptors to the plasma membrane, receptor externalization (as assessed by immunoprecipitation of radiolabeled receptors) was measured in the presence of cycloheximide to block protein synthesis. For 1 h before the start of the experiment, and during the experimental time course, endothelial cells were incubated in 10 μg/ml cycloheximide in the presence and absence of insulin. At the end of 1 h, cycloheximide-treated cells replenish 96% of the amount of receptor constitutively recycled by control cells, and 89% of control levels in the presence of 100 nM insulin (no significant difference). These data strongly suggest that the delivery of newly synthesized receptors to the cell surface did not contribute significantly to the reappearance of receptors measured in externalization experiments.

The effects of PMA on constitutive and insulin-stimulated receptor externalization were assessed by immunoprecipitation of surface-labeled receptor as described above. PMA treatment significantly increased the amount of receptor externalized in the absence of insulin between 5 and 20 min and reduced the time required to reach 50% of maximum from 9 to 3.5 min (p < 0.05; Fig. 5A). These results are in contrast to the effects of PMA on constitutive receptor internalization, where no significant increase was observed. PMA treatment significantly increased the amount of insulin-stimulated receptor externalization between 5 and 40 min by a maximum of 2.3-fold and prevented the drop to the constitutive externalization rate after 20 min observed for insulin treatment alone (p < 0.05; Fig. 5B). The initial rates and times required to reach 50% of maximum for constitutive and insulin-stimulated receptor externalization in the presence and absence of PMA, as determined by linear least squares analysis of data from surface-labeling experiments, are summarized in Table IV. The initial rate of receptor externalization was increased 2-fold when endothelial cells were stimulated with 100 nM insulin (p < 0.05). PMA treatment alone increased the initial rate of insulin receptor externalization by 2.3-fold, and the combination of insulin and PMA further increased this rate by 75% (p < 0.05).

Intracellular Insulin Receptor Distribution—The data in Figs. 3 and 5 and Tables II and IV indicate that PMA significantly increased constitutive receptor externalization without significantly affecting constitutive insulin receptor internalization. Indeed, microvascular endothelial cells pretreated with 160 nM PMA for 30 min displayed a 35% increase in 125I-insulin binding at the cell surface (p < 0.05). One possible scheme by which PMA could increase the apparent initial rate of receptor externalization in the absence of a coordinate increase in receptor internalization consists of a mobilization of receptors from an intracellular pool. We have begun to investigate this hypothesis by estimating the distribution of insulin receptors in endothelial cells between surface and intracellular pools in two ways: 125I-insulin binding and immunogold electron microscopy.

Table V shows the distribution of insulin receptors in endothelial cells measured by 125I-insulin binding. Cells were
plexes were precipitated with polyethylene glycol, and specific trypsin-treated under conditions identical to those used in surface-labeling experiments and then solubilized in Triton X-100 and incubated with $^{125}$I-insulin in the presence and absence of excess unlabeled insulin. Insulin-receptor complexes were precipitated with polyethylene glycol, and specific binding was determined. Trypsin treatment removed 35 ± 2.7% of the total cellular binding activity, indicating that 65% of the binding activity was located inside the cells under steady-state conditions.

Cultured endothelial cells were also prepared for electron microscopy using conventional techniques, and thin sections were incubated with anti-receptor antiserum or non-immune serum and then stained with protein A-gold. Electron micrographs of endothelial cells stained with anti-receptor antiserum revealed that gold particles were associated with the cell surface and a variety of subcellular organelles. The quantitation of total and surface-associated gold particles is summarized in Table VI. The total and surface-associated counts from the non-immune samples were subtracted from their respective counterparts in the anti-receptor antiserum samples to give the corrected values shown in the middle column. The right-hand column shows that the amount of surface-associated receptor was 20%.

### Table V

**Distribution of insulin receptors in endothelial cells as assessed by $^{125}$I-insulin binding**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific binding</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.4 ± 0.28</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin (0.05%, 60 min, 4°C)</td>
<td>4.15 ± 0.17</td>
<td>65</td>
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</tbody>
</table>

Insulin receptor traffic in insulin-sensitive cells includes the pathways involved in receptor synthesis and delivery to the plasma membrane, and those involved in the internalization of hormone-receptor complexes, in the degradation of hormone and receptor, and in the recycling of internalized receptor to the plasma membrane. As an insulin-sensitive cell type, microvascular endothelial cells possess these pathways and also possess any additional pathways needed for the receptor-mediated transcytosis of intact insulin across the vascular wall. Unique biochemical signals may be required to target internalized receptors into either transport or degradative pathways. We have attempted to identify discrete biochemical changes in endothelial cell insulin receptors associated with their recycling, as a first step toward understanding the mechanism of receptor-mediated transcytosis and the regulation of intracellular insulin receptor traffic in general.

### Discussion

The results show that insulin receptors are constitutively internalized in microvascular endothelial cells. Upon insulin binding, the receptor undergoes autophosphorylation within 20 s and becomes an active tyrosine kinase (2). Insulin-binding is also associated with a 2.3-fold increase in the initial rate of receptor internalization and a 4-fold decrease in the time required to reach 50% of maximum internalization. The rate at which cell-surface binding activity decreases in response to insulin treatment parallels the rate of receptor internalization. The results of experiments in which insulin was prebound to its receptor suggest that insulin-stimulated receptor internalization, apart from insulin binding, can occur in less than 3 min. These time intervals suggest that receptor occupancy, phosphorylation of the insulin receptor β-subunit at tyrosine and serine residues, and tyrosine kinase activation may be involved in the signaling and regulation of insulin-stimulated receptor internalization. Furthermore, insulin receptors purified from unstimulated endothelial cells contain...
Distribution of insulin receptors in endothelial cells as assessed by immunogold electron microscopy

Each sample was a montage of 16 micrographs at x25,000, representing 11 μm of cells in cross-section (see text). Preparation as described by Roth et al. (26).

<table>
<thead>
<tr>
<th>Ab Sample</th>
<th>Total particles</th>
<th>Surface-associated</th>
<th>Corrected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-9 (1:100)</td>
<td>1</td>
<td>397</td>
<td>83</td>
</tr>
<tr>
<td>2</td>
<td>617</td>
<td>114</td>
<td>5</td>
</tr>
<tr>
<td>Control (1:100)</td>
<td>3</td>
<td>19</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>28</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

*Mean of control subtracted from each group.

phosphoserine (19), as observed in other insulin target cell types (2, 20, 39), suggesting that serine phosphorylation may play a role in the constitutive insulin receptor internalization displayed by endothelial cells. The rates of insulin-stimulated receptor internalization reported here are comparable to rates observed in other cell types, such as rat adipocytes and 3T3 L1 cells, published previously (32–34).

We have used the diacylglycerol analog PMA to help evaluate the role of serine phosphorylation in insulin receptor internalization by endothelial cells. Diacylglycerol activates a family of Ca2+-dependent, serine- and threonine-specific kinases known as kinases C (35), which most likely also mediate the effects of PMA on the insulin receptor and other receptors (35–38). PMA treatment of intact endothelial cells results in increased serine phosphorylation of the insulin receptor β-subunit (19), similar to its effects on insulin receptors from FA0 rat hepatoma cells (33). Endothelial cells may be unique in that treatment with insulin and PMA together results in an additive increase in insulin receptor serine and tyrosine phosphorilation (19); in other cell types, PMA plus insulin partially inhibits autophosphorylation at tyrosine residues and decreases receptor kinase activity (33, 39). PMA did not significantly affect constitutive insulin receptor internalization in endothelial cells but did increase the initial rate of insulin-stimulated receptor internalization 2-fold. The PMA-associated increase in insulin-stimulated receptor internalization rate is consistent with that reported previously for this cell type (19) and for insulin receptors in a promyelocyte cell line (37). Thus, although a PMA-induced increase in serine phosphorylation on the endothelial cell insulin receptor has been documented, significantly increased internalization rate was observed only under conditions in which both tyrosine and serine phosphorylations are reportedly enhanced. It is tempting to speculate that increased phosphoserine content alone may be insufficient for increased receptor internalization; specific serine residues phosphorylated only after receptor activation, or a particular combination of serine and tyrosine phosphorylations, may be required.

Evidence in several internalization experiments suggested that the insulin receptor was recycled in endothelial cells. For example, during the latter half of the time course of insulin receptor internalization, the amount of receptor internalized decreased in both the absence and presence of insulin. The rise in the amount of surface-associated 125I-insulin after an initial decrease, and the coincidental decrease in the amount of internalized 125I-insulin, also suggested that the insulin receptors were recycled. The data in Fig. 4 and Table III confirm that insulin receptors and insulin binding activity were recycled in endothelial cells and that this process was enhanced by pretreatment with insulin. Experiments performed in the presence of cycloheximide demonstrated that the delivery of newly synthesized receptors did not contribute significantly to the externalization measured in binding and surface labeling experiments. A comparison of the data in Tables II and IV reveals that while there was little difference between the rates of constitutive internalization and externalization, both processes were enhanced to a similar degree upon addition of insulin. This suggests that receptor occupancy (and/or receptor kinase activation) affect both processes coordinately.

Further comparison of Tables II and IV indicates that cells pretreated with PMA and then treated with insulin internalized and externalized insulin receptors at similarly enhanced rates. PMA treatment also counteracted the drop to the constitutive externalization rate after 20 min, characteristic of insulin treatment alone. Thus, it appears that PMA not only enhanced the initial insulin-stimulated receptor externalization rate but interfered with the “switching off” of the increase rate observed in insulin-treated cells, and temporarily maintained the signal that triggered an accelerated rate of receptor externalization. As shown previously, under these conditions the insulin receptor undergoes enhanced phosphorylation at both serine and tyrosine residues in the β-subunit (19). The effect of PMA on occupied receptors suggests that enhanced serine phosphorylation alone may be insufficient to signal receptor internalization, but that a specific combination of tyrosine and serine phosphorylations may be required to produce the increases observed in internalization and externalization rates. Because the insulin receptors in endothelial cells contain phosphoserine in the unstimulated state, the effect of PMA on occupied receptors may simply exaggerate a pattern of phosphorylation involved in insulin-stimulated receptor processing.

The PMA-associated increase in unoccupied insulin receptor externalization was the single example of an effect on receptor externalization independent of internalization; the effects of receptor occupancy and PMA treatment in the presence of insulin suggest that the opposite is true, that internalization and externalization pathways are coordinately regulated in endothelial cells. This suggests that PMA may have exerted this effect through the translocation of insulin receptors from an intracellular pool. We have estimated the distribution of insulin receptors between cell-surface and intracellular pools to be 35% surface-associated by insulin binding, and 20% by morphometric analysis of immunogold-stained insulin receptors. Although not in exact agreement with the distribution as measured by insulin-binding, the quantitation by electron microscopy tends to underestimate the amount of surface-associated receptor, because invaginated cell-surface would appear intracellular in thin sections. The data from both estimates suggest that the majority of receptors are located inside the cell, while approximately one-third of the total reside at the cell surface. It is therefore possible that a PMA-induced mobilization of insulin receptors from this intracellular pool might contribute to a PMA-induced increase in receptor externalization independent of
receptor internalization. The intracellular distribution reported here may represent a unique feature of endothelial cells; in adipocytes approximately 90% of the insulin receptors reside on the cell surface, and the 10% located within the cell are thought to be newly synthesized receptors en route to the plasma membrane (40).

In summary, we have shown that in endothelial cells the insulin receptor is constitutively internalized and recycled. The binding of insulin to its receptor is associated with significant increase in phosphorylation at serine and tyrosine residues in the β-subunit of the receptor; these events are likely to involve the kinase C system. Occupied receptors internalized in the presence of PMA are also externalized at a comparably increased rate. The increase in initial rate of externalization induced by PMA suggests that the regulation of receptor externalization may be independent of internalization. We further speculate that the PMA-induced increase in receptor serine phosphorylation reported previously may somehow help trigger receptor externalization. PMA may exert this effect through the mobilization of receptors from an intracellular pool to the cell surface. These data, together with our previous study (19), provide evidence that serine and tyrosine phosphorlations on the β-subunit of the receptor may play an important role in the regulation of insulin receptor traffic in endothelial cells. Since these insulin receptors have been shown to mediate the transcytosis of insulin, these specific biochemical changes may also regulate insulin transport across the vascular wall.

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REFERENCES