

## Regulation of Insulin Receptor Internalization in Vascular Endothelial Cells by Insulin and Phorbol Ester\*

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Phorbol 12-myristate 13-acetate (PMA) was used to examine the role of insulin receptor phosphorylation in the regulation of insulin receptor internalization in vascular endothelial cells. Association of  $^{125}\text{I}$ -insulin in rat capillary and bovine aortic endothelial cells preincubated with PMA was increased by 80 and 64% over control, respectively. The increase was due to enhanced  $^{125}\text{I}$ -insulin internalization as opposed to an effect on surface-bound hormone. PMA had no significant effect on  $^{125}\text{I}$ -insulin degradation or on release of internalized insulin from the cells. Internalization of  $^{125}\text{I}$ -labeled insulin receptor was determined by the resistance of labeled receptor to trypsinization. At 10 °C, nearly all of the labeled receptor was sensitive to removal by trypsin, indicating that it was exposed on the cell surface. Exposure of labeled cells to insulin (100 nM) at 37 °C resulted in the rapid appearance of trypsin-resistant insulin receptor, indicating receptor internalization. Steady state for receptor internalization was attained at 10–15 min. When surfaced-labeled cells were preincubated with PMA at 37 °C, the rate of insulin receptor internalization was increased by  $3.6 \pm 0.2$ -fold and  $2.1 \pm 0.5$ -fold at 1 and 5 min of insulin exposure, respectively ( $\text{ED}_{50}$  at 16 nM PMA). This effect of PMA was associated with an increase in serine phosphorylation of the insulin receptor. Thus, PMA increased insulin internalization in the endothelial cells by modulating the insulin-induced internalization of the receptor. The additive effects of PMA and insulin on insulin receptor phosphorylation suggest that the phorbol ester and insulin act via independent signaling mechanisms.

The insulin receptor in vascular endothelial cells is structurally similar to that in other cell types and is composed of an  $\alpha$ - and  $\beta$ -subunit (1–3). In the basal state, the receptor contains phosphoserine and phosphothreonine, and upon insulin binding, the receptor is activated by tyrosine autophosphorylation of the  $\beta$ -subunit (4–6). In endothelial cells, the insulin receptor mediates the metabolic and growth actions of insulin (7, 8) and facilitates the transcytosis of insulin (9, 10). In muscle and adipose tissue, capillary endothelial cells form a barrier that prevents free diffusion of circulating insulin out of the vascular compartment (11, 12). The delivery

of insulin to subendothelial tissue may require specific transport of insulin by the receptor (9, 10). Thus, the concentration of insulin receptors in the plasma membrane of the endothelial cell may regulate the efflux of insulin from the vascular compartment and the transport of insulin to tissue sites of action. However, the mechanism that regulates the internalization of the insulin receptor in the endothelial cell is unknown.

The phorbol ester 4- $\beta$ -phorbol 12-myristate 13-acetate (PMA)<sup>1</sup> has been used to examine the role of serine/threonine phosphorylation in the internalization of the membrane receptor. Phorbol esters such as PMA produce a variety of effects on cellular metabolism, differentiation, and growth and in addition have recently been shown to induce the internalization of receptors for transferrin, epidermal growth factor (EGF), and  $\beta$ -adrenergic agonists (13–18). Protein kinase C is one of the major phorbol ester receptors in the cell (19, 20). It is a  $\text{Ca}^{2+}$ -dependent serine- and threonine-specific protein kinase that is activated by diacylglycerol produced during the hormone-mediated activation of phospholipase C. PMA substitutes for diacylglycerol and activates protein kinase C in the absence of other agonists (19, 20). The PMA-induced phosphorylation of the receptors for transferrin, EGF, and the  $\beta$ -adrenergic agonists is thought to occur through the activation of protein kinase C (17, 18, 21–25).

In this study, the role of receptor phosphorylation in the regulation of insulin receptor internalization was studied in the endothelial cell. PMA was used to evaluate the possible role of serine phosphorylation of the insulin receptor in receptor internalization.

### EXPERIMENTAL PROCEDURES

**Materials**—Materials were obtained from the following sources. [ $^{32}\text{P}$ ]orthophosphate and Triton X-100 were from New England Nuclear, and  $\text{Na}^{125}\text{I}$  and  $^{125}\text{I}$ [A<sup>14</sup>]monoiodoinsulin were from Amersham Corp. Phorbol 12-myristate 13-acetate, HEPES, aprotinin, phenylmethylsulfonyl fluoride, dithiothreitol, trichloroacetic acid, glucose oxidase, and lactoperoxidase were from Sigma. Porcine insulin was from Elanco. Staphylococcal protein A (Pansorbin) was from Behring Diagnostics. L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin was from Worthington, and the reagents for SDS-PAGE were from Bio-Rad.

**Cell Culture**—Endothelial cells from calf aorta were isolated and cultured as described previously (7). Capillary endothelial cells were isolated from the rat epididymal fat pad. Male rats, ~160 days old, were killed by anesthetizing in a  $\text{CO}_2$ -filled chamber. The epididymal fat pads were dissected from ~3–5 rats. The fat pads were minced, incubated in phosphate-buffered saline (PBS), pH 7.4, containing 0.2% collagenase and 0.5% bovine serum albumin for 45 min at 37 °C,

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<sup>1</sup> The abbreviations used are: PMA, 4- $\beta$ -phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

and then homogenized in a Dounce homogenizer. The homogenate was sequentially filtered through nylon screens with pore sizes of 210, 88, and 53  $\mu\text{m}$ . Endothelial cells and partially digested capillaries retained by the last nylon screen were collected by centrifugation and culture on fibronectin-coated tissue culture dishes. Capillary endothelial cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% plasma-derived horse serum (Irvine Scientific, Irvine, CA). Homogeneity of endothelial cell cultures was determined by morphology under phase-contrast microscopy and confirmed by the presence of angiotensin-converting enzyme activity and positive uptake of acetylated low density lipoprotein (26).

**Insulin Binding and Degradation**— $^{125}\text{I}$ -Insulin binding studies were performed on confluent cells cultured in 35-mm multi-well plates (Costar). Cells were incubated with and without PMA as indicated, and  $^{125}\text{I}$ -insulin binding was measured as previously described (3, 7). Surface-bound and internalized  $^{125}\text{I}$ -insulin was assessed by the resistance of cell-associated radioactivity to dissociate with acid wash (27). Acid-sensitive radioactivity was used as the index of cell surface  $^{125}\text{I}$ -insulin, and acid-resistant radioactivity was a measurement of internalized  $^{125}\text{I}$ -insulin. The release of internalized  $^{125}\text{I}$ -insulin and the degradation of  $^{125}\text{I}$ -insulin assessed by trichloroacetic acid precipitability were determined as previously described (2).

**Determination of Insulin Receptor Internalization**—Cells were surface-labeled with  $\text{Na}^{125}\text{I}$ . Confluent cells cultured on 100 mm plates were washed three times with PBS and incubated with 3 ml of PBS containing 10 mM glucose, 2 units/ml of lactoperoxidase, 0.5 nM of  $\text{NaI}$ , 1 unit/ml of glucose oxidase, and 0.15–0.30 mCi/ml of  $\text{Na}^{125}\text{I}$  at 10 °C for 30 min, with intermittent stirring. After iodination, the cells were washed four to five times at 4 °C with PBS. Labeled cells were incubated with and without PMA (160 nM) in HEPES binding buffer (0.1 M HEPES, pH 7.6, 0.1 M NaCl, 5 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 8 mM glucose, and 1% bovine serum albumin) for 30 min at 37 °C. Cells then were incubated with or without insulin (100 nM) at 37 °C for the indicated time intervals. Internalized insulin receptor was determined by its resistance to proteolysis by trypsin treatment of the labeled cells. Cells were exposed to 0.05% TPCK/trypsin for 30 min at 10 °C. The low temperature was used to inhibit surface protein internalization. These conditions effectively removed nearly all surface-labeled receptors (described in Fig. 5). Trypsinization was stopped by addition of 0.1% soybean trypsin inhibitor, and the cell suspension was placed on ice. Cells were centrifuged at 5000  $\times g$  at 4 °C and washed twice at 4 °C with PBS to remove trypsinized surface-labeled peptides from the cell suspension. Labeled insulin receptor was immunoprecipitated with anti-receptor IgG (purified from human B-9 serum) and protein A (Pansorbin), reduced with dithiothreitol (100 nM), and analyzed by SDS-PAGE (38). The  $^{125}\text{I}$ -labeled proteins were identified by autoradiography of stained and dried gels using Kodak X-Omat film, and the bands were quantitated by scanning densitometry.

**Insulin Receptor Phosphorylation in Intact Cells**—The culture medium of confluent cells was changed to Dulbecco's modified Eagle's medium, 0.5% serum, and 0.25% bovine serum albumin prior to the experiment. Intact cells were then incubated with phosphate-free and serum-free RPMI 1640 medium containing carrier-free  $^{32}\text{P}$ orthophosphate (0.5 mCi/ml) for 90 min at 37 °C. The cells were treated with and without PMA (160 nM) for 30 min and afterwards with and without insulin (100 nM) for 5 min. The reaction was stopped by rapidly removing the incubation medium by aspiration and freezing the cells by the addition of liquid nitrogen to the plates (5, 6). The cells were thawed immediately in 1 ml of a solution containing HEPES (50 mM), pH 7.4, sodium pyrophosphate (10 mM), sodium fluoride (100 mM), EDTA (4 mM), sodium vanadate (2 mM), aprotinin (1 mg/ml), and phenylmethylsulfonyl fluoride (2 mM) (5). Cells were scraped from the plates and centrifuged at 50,000 rpm in a Beckman Ti-70.1 rotor at 4 °C for 60 min. Thereafter, all steps were carried out at 4 °C. The pellet was then solubilized in 1% Triton X-100 in the same solution described above. Insoluble material was sedimented by a second centrifugation, and the supernatant was used for immunoprecipitation.

Two procedures were used to immunoprecipitate the insulin receptor. The first immunoprecipitation used anti-insulin receptor IgG ( $\alpha\text{IR}$ ) and protein A (Pansorbin). To characterize further the population of phosphorylated insulin receptors, the receptor was sequentially immunoprecipitated by anti-phosphotyrosine IgG ( $\alpha\text{PY}$ ), followed by  $\alpha\text{IR}$ . The  $\alpha\text{PY}$  has previously been shown to immunoprecipitate phosphopeptides specific for tyrosine residues (5, 6). Thus, the first immunoprecipitation identified the phosphotyrosine-containing subset of insulin receptors, and the second immunoprecipitation identi-

fied the phosphotyrosine-free subset of receptors, that is, those phosphorylated on serine and threonine residues (5, 6). Phosphoproteins were separated by SDS-PAGE under reducing conditions with dithiothreitol and identified by autoradiography. The phosphoproteins were quantitated by Cerenkov counting, and the intensity of the autoradiographic bands was measured by scanning densitometry.

Phosphoamino acid analysis of the insulin receptor was also performed, as previously described (28, 29). Briefly, the 95-kDa  $\beta$ -subunit of the receptor identified by autoradiography was excised from the dried gel and digested with TPCK/trypsin. Tryptic peptides were then dried and partially hydrolyzed in HCl (6 M) for 90 min at 110 °C. Samples were lyophilized, and the phosphoamino acids were separated by high voltage electrophoresis on cellulose thin-layer plates using pyridine/acetic acid/water (1:10:89). The migration of labeled amino acids was identified by autoradiography and compared to that of authentic phosphoserine, phosphothreonine, and phosphotyrosine.

## RESULTS

**The Effect of PMA on  $^{125}\text{I}$ -Insulin Binding and Internalization**—The binding and internalization of  $^{125}\text{I}$ -insulin in endothelial cells was measured after incubation without or with PMA (160 nM) for 30 min. At 37 °C, PMA increased cell-associated  $^{125}\text{I}$ -insulin in endothelial cells in a time-dependent manner. After 60 min, an 80% increase (from  $2.4 \pm 0.3$  to  $4.3 \pm 0.4\%$  bound) was measured in rat capillary endothelial cells, and a 64% increase was observed in bovine aortic endothelial cells (Fig. 1). The half-maximal PMA concentration that increased cell-associated  $^{125}\text{I}$ -insulin was 10 ng/ml (16 nM), and the maximum effect was observed at concentrations greater than 100 ng/ml (160 nM) (Fig. 2). These effects were specific for PMA, as the biologically inactive 4- $\alpha$ -phorbol (160 nM) had no effect on cell-associated  $^{125}\text{I}$ -insulin. All subsequent experiments were carried out with 100 ng/ml (160 nM) PMA.

At 4 °C, PMA had no effect on cell-associated  $^{125}\text{I}$ -insulin in endothelial cells, and at 15 °C the increase was only 18% (data not shown). The temperature dependence suggests involvement of energy-dependent processes, such as hormone-receptor internalization. To examine this possibility, the surface-bound and internalized components of cell-associated  $^{125}\text{I}$ -insulin were determined. Internalized  $^{125}\text{I}$ -insulin was determined by its resistance to dissociation from the cells with an acid wash (27). As shown in Fig. 3A, PMA increased internalized  $^{125}\text{I}$ -insulin in endothelial cells by 85%, from  $1.7 \pm 0.2$  to  $3.2 \pm 0.3\%$  at 60 min. In contrast, surface-bound or acid-sensitive  $^{125}\text{I}$ -insulin was not affected by PMA (Fig. 3B). Thus, PMA increased cell-associated  $^{125}\text{I}$ -insulin in the endothelial cell by increasing the amount of internalized insulin.

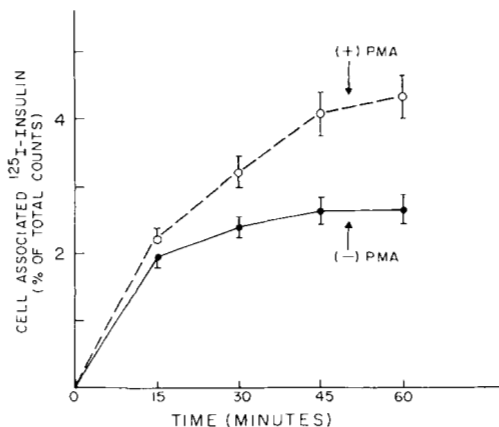
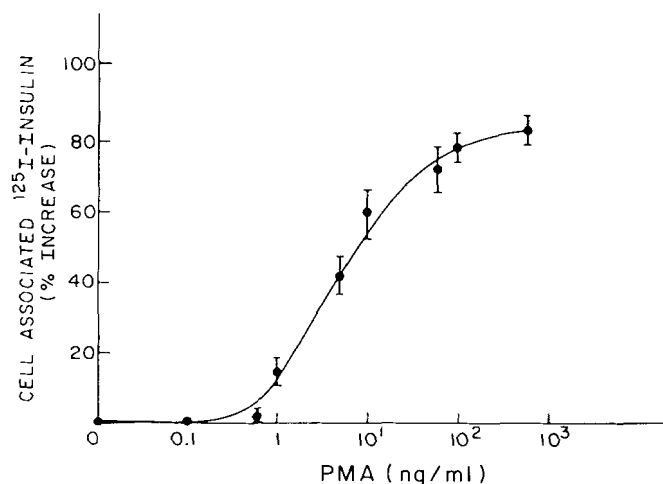


FIG. 1. Effect of PMA on  $^{125}\text{I}$ -insulin binding to rat capillary endothelial cells. PMA ( $\circ$ ) or buffer ( $\bullet$ ) was added to confluent endothelial cells 30 min prior to the addition of  $^{125}\text{I}$ -insulin at 37 °C.  $^{125}\text{I}$ -insulin binding was determined under "Experimental Procedures." Values are the mean  $\pm$  S.E. of four experiments.

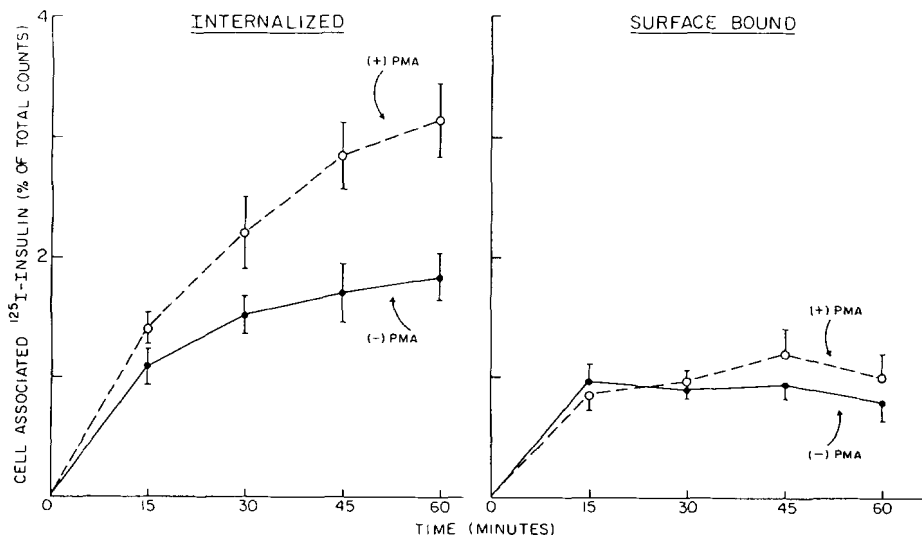
**The Effect of PMA on the Release and Degradation of Internalized Insulin**—PMA may increase internalized  $^{125}\text{I}$ -insulin in the endothelial cells by affecting the degradation or the release of internalized insulin from the cells. Insulin degradation in endothelial cells was measured by trichloroacetic acid precipitation. At 60 min, 25% of cell-associated  $^{125}\text{I}$ -insulin was degraded by the cells (trichloroacetic acid soluble). PMA had no significant effect on  $^{125}\text{I}$ -insulin degradation (Fig. 4A). Release of internalized insulin from the cells was measured after treatment with or without PMA. Cells were loaded with  $^{125}\text{I}$ -insulin by incubation at  $37^\circ\text{C}$  for 1 h. The surface-bound component was removed by an acid wash. Thereafter, the cells were incubated in fresh medium for various time intervals at  $37^\circ\text{C}$ , and the release of  $^{125}\text{I}$ -insulin from the cells was monitored. PMA had little or no effect on the release of internalized insulin from the cells (Fig. 4B). Thus, PMA had no effect on surface binding, degradation, or release of insulin from the cell. This suggests that the increase in cell-associated insulin by PMA may be due to increased internalization of the receptor-insulin complex.

**Effect of PMA on Insulin Receptor Internalization**—Insulin receptors were labeled by surface iodination of endothelial



**FIG. 2. Dose response of PMA effect on  $^{125}\text{I}$ -insulin binding.** Capillary endothelial cells were incubated with PMA at the indicated concentrations for 30 min at  $37^\circ\text{C}$  prior to the addition of  $^{125}\text{I}$ -insulin.  $^{125}\text{I}$ -Insulin binding was determined as described under "Experimental Procedures." Results are the mean  $\pm$  S.E. of three experiments.

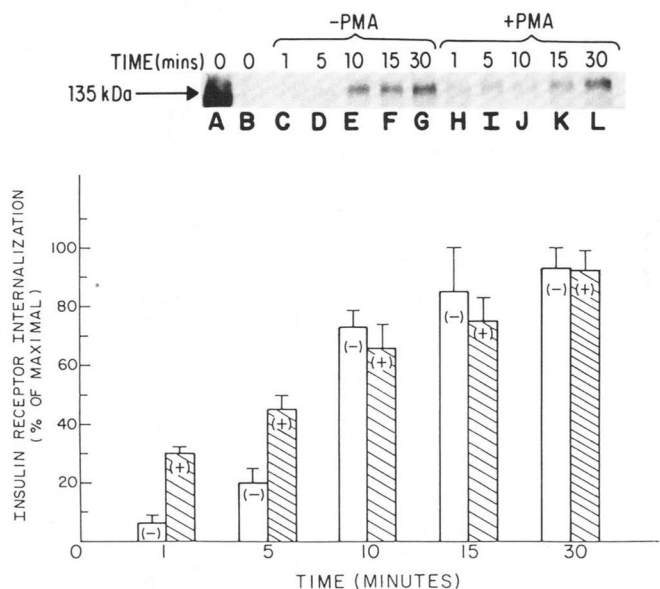
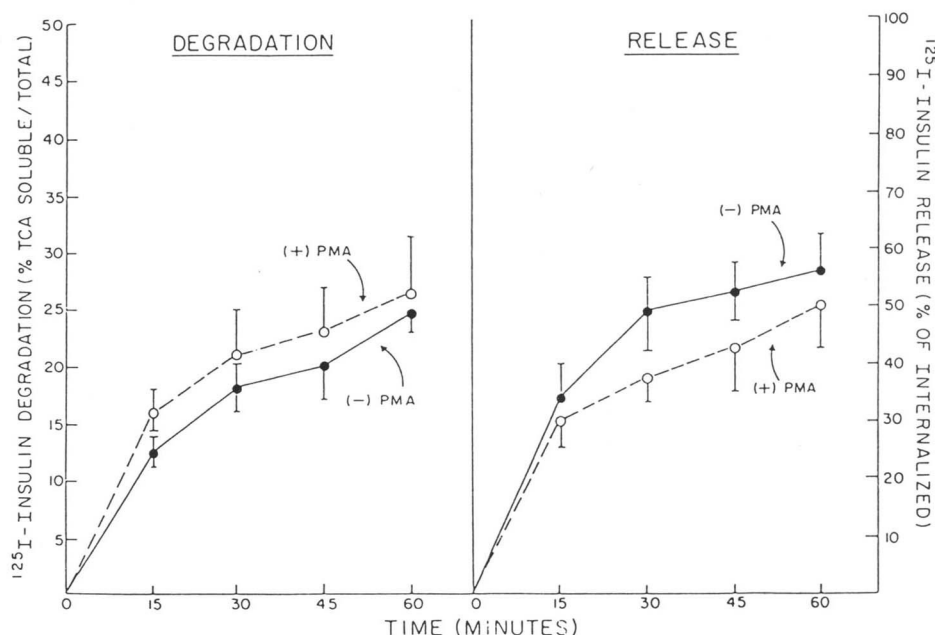
**FIG. 3. Effect of PMA on  $^{125}\text{I}$ -insulin internalization and surface binding.** Surface-bound and internalized components of  $^{125}\text{I}$ -insulin binding were assessed by the resistance of cell-associated radioactivity to dissociate from the cells with an acid wash. Acid-sensitive radioactivity was used as the index of surface-bound  $^{125}\text{I}$ -insulin, and acid-resistant radioactivity was a measurement of internalized  $^{125}\text{I}$ -insulin. Values are the mean  $\pm$  S.E. of four experiments.



cells at  $10^\circ\text{C}$ . The low temperature was used to inhibit the internalization of surface proteins during the iodination. The labeled cells were treated with and without PMA (160 nM) and then exposed to insulin (100 nM) for 1, 5, 10, 15, and 30 min at  $37^\circ\text{C}$  to induce receptor internalization. Internalized  $^{125}\text{I}$ -labeled receptor was determined by the resistance of the  $\alpha$ -subunit to trypsin digestion (28, 30). After trypsinization, the cells were solubilized, the insulin receptor was immunoprecipitated with anti-insulin receptor IgG, and the iodinated protein was reduced with dithiothreitol and separated by SDS-PAGE. In the absence of PMA and insulin, and before trypsinization, the total surface iodinated insulin receptor was immunoprecipitated (Fig. 5, lane A). Trypsin treatment of the cells for 30 min at  $10^\circ\text{C}$  removed almost all of the labeled receptor, indicating that it is located entirely at the cell surface (Fig. 5, lane B). After treatment of the cells with PMA and insulin, the resistance of the  $\alpha$ -subunit to trypsin digestion was measured (Fig. 5, lanes C-L). In the absence of PMA, insulin induced rapid receptor internalization as determined by the appearance of the trypsin-resistant  $\alpha$ -subunit (Fig. 5, lanes C-G). Quantitation of these lanes by scanning densitometry from four similar studies is shown in Fig. 5B (open bars). Receptor internalization over time is expressed as the percent of the maximum receptor internalized. In the absence of PMA (Fig. 5, open bars), steady-state insulin receptor internalization was reached at 10–15 min. After PMA treatment, insulin-induced receptor internalization was significantly increased over control at 1 and 5 min, by  $3.6 \pm 0.2$ -fold and  $2.1 \pm 0.5$ -fold, respectively (Fig. 5, lanes H-L and hatched bars). PMA had no effect on the internalization of the unoccupied insulin receptor as determined by the absence of trypsin-resistant receptor immediately after incubation with PMA (data not shown). At 10–30 min, the percent of receptors internalized in the absence and presence of PMA reached a plateau and was similar. This may reflect the reappearance of trypsin sensitivity of the labeled receptor due to receptor recycling to the cell surface; PMA does not affect the steady-state distribution of receptors.

**Effect of PMA on Insulin Receptor Phosphorylation**—The effect of PMA and insulin on the phosphorylation state of the insulin receptor in endothelial cells was examined. The autoradiogram from a representative study is shown in Fig. 6, and the analysis by scanning densitometry of the  $\beta$ -subunit from four similar studies is shown in Fig. 6B, right panel. Insulin stimulated the phosphorylation of the receptor 2.8-fold (Fig. 6, lanes and bars 1 and 2), whereas PMA treatment

**FIG. 4. Effect of PMA on  $^{125}\text{I}$ -insulin degradation and the release of internalized  $^{125}\text{I}$ -insulin from endothelial cells.** Cells were treated with and without PMA and incubated with  $^{125}\text{I}$ -insulin as described in Fig. 1. Surface-bound  $^{125}\text{I}$ -insulin was removed with an acid wash; thereafter, the internalized  $^{125}\text{I}$ -insulin was assayed for degradation by trichloroacetic acid (left), or the  $^{125}\text{I}$ -insulin released from the cells into the medium was monitored over time (right). Results are the mean  $\pm$  S.E. of four experiments.



**FIG. 5. Effect of PMA on the internalization of surface-labeled insulin receptor.** Insulin receptors on the surface of the cells were labeled with  $^{125}\text{I}$  and lactoperoxidase. Labeled receptors were internalized by incubation with insulin (100 nM) for the indicated times at 37 °C. Surface-labeled receptor was removed by trypsin. Cells were solubilized, and the trypsin-resistant or internalized receptors were quantitated by immunoprecipitation and autoradiography as described under "Experimental Procedures." Upper panel, insulin receptor 135-kDa  $\alpha$ -subunit over time. Lanes (from left to right): total surface-labeled receptor (A), labeled receptor after trypsin treatment of cells without insulin exposure (B), trypsin-resistant labeled receptor after insulin exposure for the indicated times, in the absence (C-G) and presence (H-L) of PMA. Lower panel, quantitation of 135-kDa  $\alpha$ -subunit by scanning densitometry from four similar studies. Insulin receptor internalization over time is shown as the percent of maximum internalized at 30 min in the absence (open bars) or presence (hatched bars) of PMA.

of the cells increased the phosphorylation 2.1-fold (Fig. 6, bars 1 and 3). Incubation of the cells with PMA followed by insulin increased 3.6-fold the  $\beta$ -subunit phosphorylation, suggesting that the effect of PMA and insulin is additive (Fig. 6, bars 1 and 4).

Phosphoamino acid analysis of the 95-kDa  $\beta$ -subunit of the insulin receptor was performed to further characterize the PMA- and insulin-stimulated phosphorylation of the receptor in the endothelial cell (Fig. 7). In the basal state the insulin receptor was phosphorylated on serine residues only (Fig. 7, lane 1). Incubation of the endothelial cells with insulin for 5 min stimulated phosphorylation of the receptor solely on tyrosine residues (Fig. 7, lane 2). In contrast, PMA treatment of the cells stimulated serine phosphorylation of the receptor (Fig. 7, lane 3), whereas PMA treatment followed by insulin stimulated both serine and tyrosine phosphorylation of the insulin receptor (Fig. 7, lane 4).

The insulin- and PMA-stimulated phosphorylation of the insulin receptor was further characterized by sequential immunoprecipitation of the receptor by anti-phosphotyrosine IgG ( $\alpha\text{pTy}$ ) and anti-insulin receptor IgG ( $\alpha\text{IR}$ ) (6). The  $\alpha\text{pTy}$  was used to separate phosphotyrosine-containing receptor, and the remaining receptors in the supernatant were precipitated with  $\alpha\text{IR}$ . The first immunoprecipitation with  $\alpha\text{pTy}$  identified the tyrosine-phosphorylated subset of insulin receptors (Fig. 8, upper panel). The second immunoprecipitation identified the phosphotyrosine-free subset of insulin receptors, presumably phosphorylated on serine and threonine residues (Fig. 8, lower panel). Densitometric analysis of the 95-kDa  $\beta$ -subunit of the receptor from these studies is shown in Fig. 9. The first immunoprecipitation with  $\alpha\text{pTy}$  revealed that basal tyrosine phosphorylation of the receptor is very low and not detectable above the background (Fig. 9, upper panel, bar 1). Insulin stimulated the tyrosine phosphorylation of the receptor by more than 20-fold (Fig. 9, upper panel, bars 1 and 2). In contrast, PMA had no effect on tyrosine phosphorylation of the receptor (Fig. 9, upper panel, bars 1 and 3). Tyrosine phosphorylation of the receptor by insulin and PMA was similar to the increase by insulin stimulation alone (Fig. 9, upper panel, bars 1 and 4).

The second immunoprecipitation with  $\alpha\text{IR}$  identified the phosphotyrosine-free subset of insulin receptors (Fig. 9, lower panel). These studies revealed that the insulin receptor in the intact endothelial cell is constitutively phosphorylated on serine residues (Fig. 9, lower panel, bar 1). Insulin stimulation had minimal effect on this subset of receptors (Fig. 9, lower

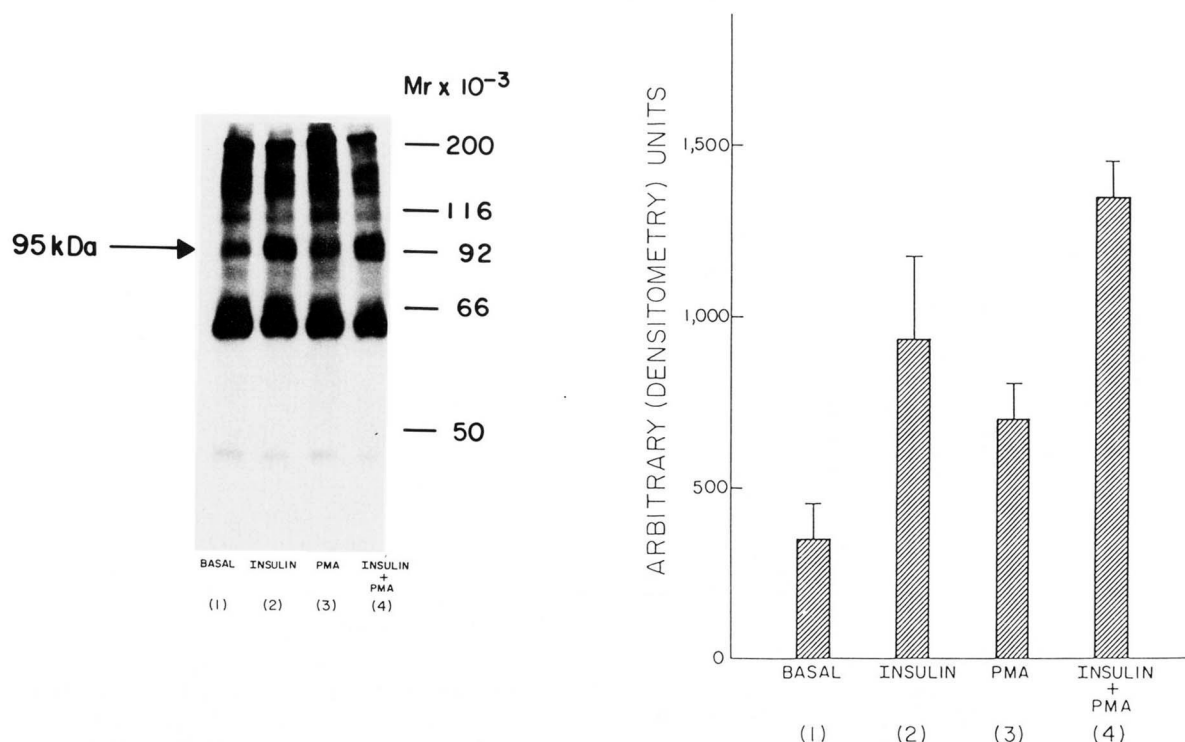


FIG. 6. **Effect of PMA on insulin receptor phosphorylation.** Endothelial cells were labeled with [<sup>32</sup>P] orthophosphate and then incubated in the absence (1), (2) or presence (3), (4) of PMA (160 nM) for 30 min at 37 °C. Cells were then stimulated with (2), (4) or without (1), (3) insulin (100 nM) for 5 min, as indicated. Insulin receptor was immunoprecipitated with anti-receptor immunoglobulin from human B-9 serum, as detailed under "Experimental Procedures." *Left*, a representative autoradiogram with the  $\beta$ -subunit of the insulin receptor indicated by the *arrow*. Molecular weights are shown  $\times 10^{-3}$ . *Right*, quantitation of 95-kDa  $\beta$ -subunit of the insulin receptor by scanning densitometry from four similar experiments.

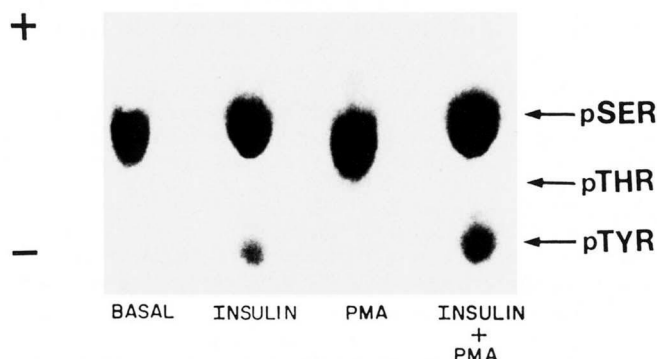


FIG. 7. **Phosphoamino acid analysis of the  $\beta$ -subunit of the insulin receptor.** Insulin receptors were phosphorylated in intact cells treated with or without PMA and insulin as described in Fig. 6, immunoprecipitated by anti-insulin receptor antibody, and separated by SDS-PAGE. The 95-kDa band was excised from the dried gel, trypsinized, and subjected to acid hydrolysis. Phosphoamino peptides were separated by high voltage electrophoresis and identified by the migration of known standards as described under "Experimental Procedures."

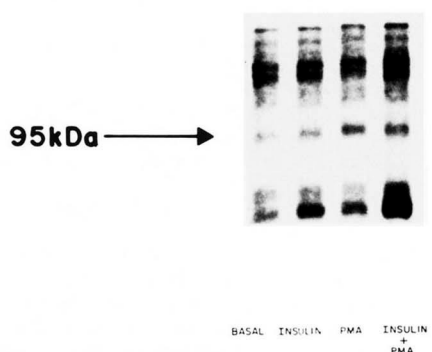
panel, bars 1 and 2). In contrast, PMA increased serine phosphorylation of the insulin receptor by 64% over basal (Fig. 9, lower panel, bars 1 and 3). A similar increase in serine phosphorylation of the receptor was seen with insulin plus PMA and was 73% increased over basal (Fig. 9, lower panel, bars 1 and 4). Thus, PMA increased serine phosphorylation of the insulin receptor. In conjunction with the studies which examined the effect of insulin and PMA on total insulin receptor phosphorylation, these data suggest that the effect

of PMA on serine phosphorylation are additive with the insulin-stimulated tyrosine phosphorylation of the receptor.

#### DISCUSSION

The insulin receptor mediates the metabolic and growth actions of insulin in capillary endothelial cells (7, 8) and facilitates the transport of insulin across the cells (9, 10). Regulation of the insulin receptor at the cell surface may therefore modulate insulin delivery and action at subendothelial tissue sites. The mechanisms that regulate the number of receptors at the cell surface are not clear. Multiple factors are known which can influence the receptors, including hormone-receptor affinity changes and receptor desensitization by hormone, drug, or general metabolic status (30–32). The role of phosphorylation of membrane proteins in the internalization of the membrane receptor has been examined in recent studies using the phorbol esters (13–18). In these studies, the phorbol esters have been shown to induce the internalization of membrane receptors for EGF, transferrin, and  $\beta$ -adrenergic agonists (13–18). Related studies have shown that the receptors undergo stimulated phosphorylation by the phorbol esters (17, 18, 21–25).

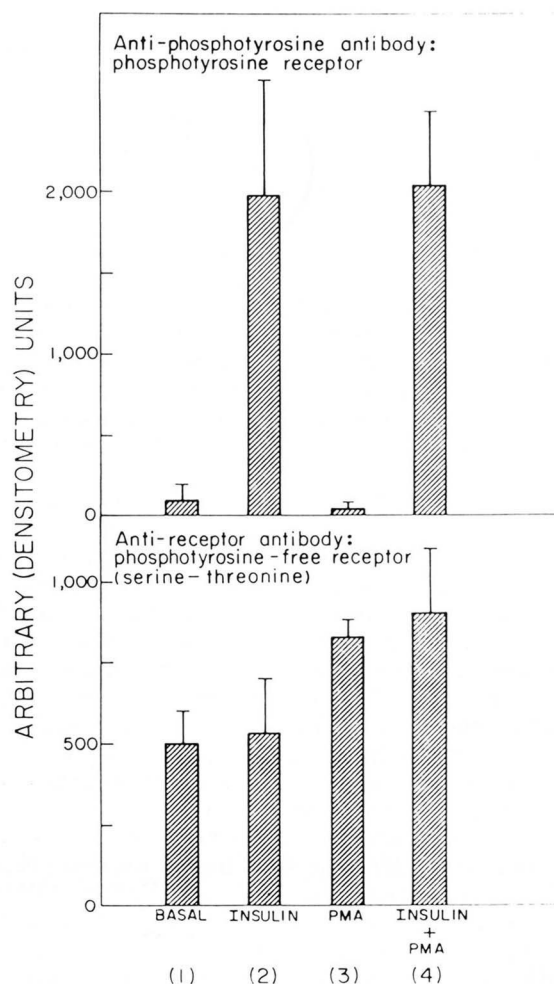
In the present study, the effect of PMA on the internalization of insulin and the insulin receptor in endothelial cells was examined. PMA increased insulin internalization in the endothelial cell. This effect was independent of effects on surface-bound insulin or on the percent of internalized insulin that was degraded or subsequently released from the cell. In previous studies, we have shown that the degradation and release of internalized insulin in the endothelial cell occurs via two functionally distinct pathways, a lysosome-targeted

**Anti-phosphotyrosine antibody:****Anti-receptor antibody:**

**FIG. 8. Effect of PMA on tyrosine and serine phosphorylation of the insulin receptor by sequential immunoprecipitation with anti-phosphotyrosine antibody (upper panel) and anti-insulin receptor antibody (lower panel).** Cells were labeled with [ $^{32}$ P]orthophosphate and stimulated with and without PMA and insulin as described in Fig. 6. *Upper panel*, insulin receptor immunoprecipitated with anti-phosphotyrosine antibody to identify phosphotyrosine-containing receptors. *Lower panel*, supernatant from the first precipitation after immunoprecipitation with antireceptor antibody, to identify the phosphotyrosine-free or serine-, threonine-phosphorylated subset of receptors. The arrow identifies the  $\beta$ -subunit of the insulin receptor. Molecular weights are shown  $\times 10^{-3}$ .

degradation pathway and a nondegradative release pathway that mediates the transport of insulin across the cell (2, 33). The present results suggest that PMA acts at a site proximal to the divergence of the two pathways in the cell and, more specifically, at the level of insulin-receptor internalization. The ability of PMA to increase insulin internalization in the endothelial cell differs from the effects of the phorbol ester with insulin studied previously in other cell lines (34–38). In these studies, labeled insulin binding to the cells was decreased or unaffected by PMA (34–38). These differences may be ascribed to cell type-specific effects of the phorbol esters. In addition, the majority of these studies, as well as studies with the EFG and transferrin receptor, examined the effects of PMA on the unoccupied receptor. Indeed, occupancy of the insulin receptor was an important factor in the PMA effect on insulin and insulin receptor internalization in the endothelial cell. The effect of PMA in these cells was dependent on the presence of both insulin and PMA.

The effect of PMA on the insulin receptor in the endothelial cell was directly assessed by surface labeling the receptor and measuring the appearance of trypsin-resistant receptor. Insulin induced the rapid internalization of the receptor in the endothelial cell. At 10–30 min, the level of trypsin-resistant receptor induced by insulin reached a plateau, suggesting that



**FIG. 9. Effect of PMA on tyrosine and serine phosphorylation of the insulin receptor.** Quantitation by scanning densitometry of the 95-kDa  $\beta$ -subunit of the insulin receptor under conditions described in Fig. 7. The *upper panel* shows quantitation of the phosphotyrosine subset of insulin receptors (immunoprecipitated with anti-phosphotyrosine antibody), and the *lower panel* shows the phosphotyrosine-free or serine-, threonine-phosphorylated subset of receptors (from the second immunoprecipitation with antireceptor antibody). 1, basal; 2, insulin stimulation; 3, PMA treatment; 4, insulin + PMA treatment. Results are the mean  $\pm$  S.E. from three similar studies.

a steady state between receptor internalization and receptor recycling to the cell surface was attained at this time. The initial rate of insulin-induced receptor internalization in the endothelial cell was increased 2- to 4-fold by PMA, but the steady-state distribution of receptors was unaffected by PMA. Because the extent of receptor loss due to degradation is minimal, as expected, over the 30-min period examined in the present study (39, 40), this result suggests that PMA may affect the rate of both insulin-induced receptor internalization and recycling in the cell. Similarly rapid insulin receptor internalization and recycling have been observed in the rat adipocyte and 3T3 fibroblasts studied by photoaffinity labeled receptor (41–43) and direct  $^{125}$ I-insulin binding (44). In contrast, insulin receptor recycling in the rat hepatocyte, measured by photoaffinity labeled receptor, was prolonged and required 3–4 h (45). In the endothelial cell, insulin is rapidly internalized and transported across the cell via the receptor (3). The rapid internalization and recycling of the insulin receptor in the endothelial cell is consistent with a transport function for the receptor in the cells.

The internalization of insulin and the insulin receptor was increased in the endothelial cell, although the time course for these effects differed. The effect of PMA on the internalization of labeled insulin receptor was evident at 1 and 5 min of insulin exposure. In contrast, the effect of PMA on the insulin receptor measured by labeled insulin binding was first apparent at 15 min and continued thereafter. This may reflect differences between the measurements of receptor internalization by surface iodinated receptor and labeled hormone binding. Measurements by surface iodinated receptor are direct. In contrast, measurement of receptor internalization by insulin binding studies are indirect and in addition assess multiple cycles of hormone-receptor internalization. The latter factor may result in the accumulation of  $^{125}\text{I}$ -insulin within the cell and therefore contribute to the continued increase in cell-associated  $^{125}\text{I}$ -insulin observed with PMA in the labeled insulin binding studies.

The effect of PMA on the internalization of the insulin receptor in these cells was dependent on the presence of both insulin and PMA; PMA alone had no effect on the level of trypsin-resistant receptor. The effect of PMA on the internalization of the occupied insulin receptor, has also been shown by Iacopetta *et al.* (46) in a promyelocyte cell line using  $^{125}\text{I}$ -insulin and quantitative electron microscopic autoradiography. The effect of PMA on the occupied receptor suggests that PMA may act to modulate an insulin-induced signal for receptor internalization.

Interestingly, the ability of PMA to increase insulin and insulin receptor internalization was associated with alterations in the phosphorylation state of the receptor. Treatment of the cells with PMA stimulated insulin receptor phosphorylation exclusively on serine residues. The effect of PMA on serine phosphorylation was additive to the insulin-stimulated tyrosine phosphorylation of the receptor. The additive effects of PMA and insulin on receptor phosphorylation suggest that PMA and insulin act via independent signaling mechanisms; multiple independent signals may interact to regulate insulin receptor internalization in the endothelial cell. Whether PMA modulates the internalization of the insulin receptor in endothelial cells by protein kinase C-mediated serine phosphorylation remains to be determined.

Phorbol esters have previously been shown to induce the internalization of cell surface receptors for EGF, transferrin, and  $\beta$ -adrenergic agonists (13–18). The effect of PMA on the internalization of the receptors was associated with alterations in the phosphorylation state of the receptors (17, 18, 21–25). Understanding the functional significance of the phosphorylation of receptors by PMA is difficult, owing to the multiple effects demonstrated by PMA in receptor regulation. For example, PMA causes the loss of cell surface EGF receptors due to receptor internalization (14–16) and the loss of high affinity EGF binding sites (13, 45, 46). Functional distinction also exists between the effects of the phorbol esters on receptor internalization compared to the induction of receptor internalization by hormone. For example, although PMA has been shown to induce internalization of the EGF and transferrin receptor, in both cases the receptor was not targeted for degradation. In contrast, the internalized receptor induced by transferrin and EGF was degraded (12, 16). In addition, transfection of cells with an EGF receptor having an alanine for threonine 654 substitution (a PMA phosphorylation site) suggests that the phorbol ester and EGF act via independent mechanisms in internalization of the EGF receptor (49).

In summary, insulin induces the rapid internalization of its receptor in endothelial cells. This process is modulated by

PMA and is associated with the stimulation of serine phosphorylation of the receptor. The additive effects of PMA and insulin on insulin receptor phosphorylation, however, suggest that the phorbol ester and insulin act via independent mechanisms. However, in conjunction with the reported effects of PMA on the internalization of a number of other receptor systems (13–18), this suggests that activation of protein kinase C may represent a general signaling mechanism that modulates the internalization of the membrane receptors.

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#### REFERENCES

- Jialal, I., Crettaz, M., Hachiya, H. L., Kahn, C. R., Moses, A. C., Buzney, S. M., and King, G. L. (1985) *Endocrinology* **117**, 1222–1229
- Jialal, I., King, G. L., Buchwald, S., Kahn, C. R., and Crettaz, M. (1984) *Diabetes* **33**, 794–800
- Bar, R. S., and Boes, M. (1984) *Biochem. Biophys. Res. Commun.* **124**, 203–209
- Kasuga, M., Zick, Y., Blithä, D. L., Karlsson, F. A., Haring, H. U., and Kahn, C. R. (1982) *J. Biol. Chem.* **257**, 9891–9894
- White, M. F., Takayama, S., and Kahn, C. R. (1985) *J. Biol. Chem.* **260**, 9470–9478
- Pang, D. T., Sharma, B. R., Shafer, J. A., White, M. F., and Kahn, C. R. (1985) *J. Biol. Chem.* **260**, 7131–7136
- King, G. L., Buzney, S. M., Kahn, C. R., Hetu, N., Buchwald, S., McDonald, S., and Rand, J. (1983) *J. Clin. Invest.* **71**, 974–979
- King, G. L., Goodman, A. D., Buzney, S. M., Moses, A., and Kahn, C. R. (1985) *J. Clin. Invest.* **75**, 1028–1036
- King, G. L., and Johnson, S. (1985) *Science* **227**, 1583–1586
- Dernovsek, K. D., and Bar, B. S. (1985) *Am. J. Physiol.* **248**, E244–E251
- Rosio, E. A., Harpers, C. L., Soeldner, J. S., and Cahill, G. F., Jr. (1967) *J. Clin. Invest.* **46**, 905–910
- Sonksen, P. H., McCormick, J. R., Egdall, R. H., and Soeldner, J. S. (1971) *Am. J. Physiol.* **221**, 1672–1680
- Klausner, R. D., Harford, J., and Van Renswoude, J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 3005–3009
- Lee, L. S., and Weinstein, I. B. (1978) *Science* **202**, 313–315
- Magum, B. E., Matrisian, L. M., and Bowden, G. T. (1980) *J. Biol. Chem.* **255**, 6373–6381
- Beguino, L., Hanover, J. A., Ito, S., Richert, N. D., Willingham, M. C., and Pastan, I. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 2774–2778
- Kelleher, D. J., Pessin, J. E., Rusho, A. E., and Johnson, G. L. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 4316–4320
- Nambi, P., Peters, J. R., Sibley, D. R., and Lefkowitz, R. J. (1985) *J. Biol. Chem.* **260**, 2165–2171
- Castagna, M., Takai, Y., Karibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) *J. Biol. Chem.* **257**, 7847–7851
- Neidel, J. E., Kuhn, L. J., and Vandenberg, G. R. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 36–40
- Davis, R. J., and Czech, M. P. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 1974–1978
- Hunter, T., Ling, N., and Cooper, J. A. (1984) *Nature* **311**, 480–483
- May, W. S., Sahyoun, N., Jacobs, S., Wolf, M., and Cuatrecasas, P. (1985) *J. Biol. Chem.* **260**, 9419–9426
- May, W. S., Jacobs, S., and Cuatrecasas, P. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 2016–2020
- Davis, R. J., Johnson, G. L., Kelleher, D. J., Anderson, J. K., Mole, J. E., and Czech, M. P. (1986) *J. Biol. Chem.* **261**, 9034–9041
- Zetter, B. R. (1981) *Diabetes* **30**, 24–28
- Shimizu, N., Shimizu, Y., and Fuller, B. B. (1981) *J. Cell Biol.* **88**, 241–244
- Hunter, T., and Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 1311–1315
- Kasuga, M., White, M. F., and Kahn, C. R. (1984) *Methods Enzymol.* **109**, 609–621
- Salhanick, A. I., West, H., and Amatruda, J. M. (1985) *J. Biol. Chem.* **260**, 16232–16236

31. Arsenis, G., and Livingston, J. N. (1986) *J. Biol. Chem.* **261**, 147-153
32. Freidenberg, G. R., Klein, H. H., Kladdé, M. P., Cordera, R., and Olefsky, J. M. (1986) *J. Biol. Chem.* **261**, 752-757
33. Hachiya, H. L., Carpentier, J. L., and King, G. L. (1986) *Diabetes* **35**, 1065-1072
34. Thomopoulos, P., Testa, U., Gourdin, M. F., Hervy, C., Titeux, M., and Vainchenker, W. (1982) *Eur. J. Biochem.* **29**, 389-393
35. Grunberger, G., and Gorden, P. (1982) *Am. J. Physiol.* **243**, E319-E324
36. Shoyab, M., Delarco, J. E., and Todaro, G. J. (1979) *Nature* **279**, 387-391
37. Lee, L. S., and Weinstein, I. B. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 5168-5172
38. Takayama, S., White, M. F., Lauris, V., and Kahn, C. R. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81**, 7797-7801
39. Kosmakos, F. C., and Roth, J. (1980) *J. Biol. Chem.* **255**, 9860-9869
40. Heidenreich, K. A., Brandenburg, D., Berhanu, P., and Olefsky, J. M. (1984) *J. Biol. Chem.* **259**, 6511-6515
41. Reed, B. C., Glasted, K., and Miller, B. (1984) *J. Biol. Chem.* **259**, 8134-8143
42. Huecksteadt, T., Olefsky, J., Brandenburg, D., and Heidenreich, K. A. (1986) *J. Biol. Chem.* **261**, 8655-8659
43. Reed, B. C., Glusted, K., and Miller, B. (1984) *J. Biol. Chem.* **259**, 8134-8143
44. Marshall, S. (1985) *J. Biol. Chem.* **260**, 4136-4144
45. Fehlmann, M., Carpentier, J. L., Van Obberghen, E., Freychet, P., Thannan, P., Saunders, D., Brandenburg, D., and Orci, L. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79**, 5921-5925
46. Iacopetta, B., Carpentier, J. L., Pozzasn, T., Leu, D. P., Gorden, P., and Orci, L. (1986) *J. Cell. Biol.* **103**, 851-856
47. Downward, J., Waterfield, M. D., and Parker, P. J. (1985) *J. Biol. Chem.* **260**, 14538-14546
48. Shoyab, M., Delarao, J. E., and Todaro, G. T. (1979) *Nature* **279**, 387-391
49. Lin, C. R., Chen, W. S., Lazar, C. S., Carpenter, C. D., Gill, G. N., Evans, R. M., and Rosenfeld, M. G. (1986) *Cell* **44**, 839-848