Phorbol esters inhibit insulin-induced receptor down-regulation in cultured human lymphocytes: association with diminished insulin receptor autophosphorylation

Krikor TOROSSIAN, Peter NOWER, Thomas SCHWARTZ and I. George FANTUS*†

Protein and Polypeptide Hormone Laboratory, Department of Medicine, Royal Victoria Hospital and McGill University, Montreal, Quebec H3A 2B2, Canada

Exposure of cells to phorbol 12-myristate 13-acetate (PMA) has been reported to result in resistance to the acute biological effects of insulin and an associated reduction in insulin-receptor tyrosine kinase activity. To investigate the relationship of insulin receptor autophosphorylation with a longer-term action of insulin the effect of PMA on insulin-stimulated receptor down-regulation was examined in cultured human lymphocytes (IM-9). Lymphocytes bound [^{3}H]phorbol dibutyrate specifically with characteristics typical of binding to protein kinase C (PKC). Acute exposure (30 min) to PMA resulted in a transient decrease of insulin binding which is consistent with a decrease in receptor number. Chronic (18 h) exposure to PMA (5 nM) resulted in inhibition of insulin-induced down-regulation of its cognate receptor. Sphingosine, an inhibitor of PKC, or chronic pre-exposure to a high concentration of PMA (1 μM), which is known to inactivate PKC, blocked the effect of PMA. PMA inhibited insulin-stimulated receptor internalization by 26% and receptor degradation by 82%. Exposure of intact cells to PMA followed by insulin treatment inhibited insulin-receptor autophosphorylation subsequently assayed *in vitro*, as well as β-subunit tyrosine phosphorylation *in situ*. In summary, PMA inhibited insulin-stimulated receptor down-regulation via activation of PKC. This was associated with an inhibition of both receptor internalization and receptor degradation. There was a concomitant inhibition of receptor tyrosine autophosphorylation consistent with a requirement of receptor kinase activation for both short-term and long-term biological effects of insulin.

INTRODUCTION

A well-documented long-term action of insulin is to down-regulate its cell-surface receptors [1–5]. This process appears to involve two steps; receptor internalization followed by a slower phase of degradation [4,5]. The regulation mechanism of these events is not known. The appreciation that insulin-receptor autophosphorylation and its activation as a tyrosine-specific protein kinase is an essential step in mediating a number of insulin's biological effects has led to the hypothesis that these same biochemical events are necessary for insulin-mediated receptor down-regulation. Studies of transfected insulin receptors mutated at Lys-1018 (numbering according to Ullrich et al. [6]), a critical amino-acid residue involved in ATP binding, revealed that defects in autophosphorylation and tyrosine kinase activity were associated with an inability of insulin to stimulate receptor endocytosis and down-regulation [7–9]. In the study in which arginine [9] rather that alanine [7,8] was substituted for lysine, the mutant insulin receptors did undergo constitutive internalization, raising the possibility that this first step in down-regulation may not always be receptor-kinase-dependent. Similar conflicting results have been published for the epidermal growth factor (EGF)+ receptor. Thus, tyrosine kinase-defective EGF receptors did not undergo EGF-dependent internalization and down-regulation [10,11]. Also, micro-injected monoclonal anti-phosphotyrosine antibodies inhibited EGF-dependent internalization of native EGF receptors [11]. In contrast, other tyrosine kinase-deficient mutant EGF receptors did undergo ligand-dependent internalization, but not receptor degradation [12,13]. Overall, the evidence that receptor tyrosine kinase activity is required for receptor degradation is stronger than that for internalization.

Phorbol esters are known to bind to and activate protein kinase C (PKC), a Ca^{2+}- and phospholipid-dependent serine/threonine kinase [14,15]. Phorbol esters cause a rapid decrease in insulin binding in several cell types [16–18]. This has been associated with a decrease in receptor affinity in cultured human lymphocytes [16] and adipocytes [17], but enhanced receptor internalization in endothelial cells [18]. Similar effects of phorbol esters have been reported for insulin-like growth factor (IGF-1) [19], EGF [20], transferrin [21], and asialoglycoprotein [22] receptors. In Fao cells no acute effect on insulin binding was found [23]. Apart from these variable effects on binding, phorbol esters have been shown to induce insulin resistance to rat adipocytes [17,24] and Fao hepatoma cells [23,25]. Thus, phorbol 12-myristate 13-acetate (PMA) inhibited insulin-stimulated glucose transport in adipocytes, and glycogen synthase and tyrosine aminotransferase activities in hepatoma cells. Other studies demonstrated that phorbol esters stimulate insulin-receptor phosphorylation on serine residues in intact cells [18,25,26]. Such phosphorylation has been observed directly by PKC *in vitro* [27]. This phosphorylation was associated with an impairment of insulin-stimulated receptor-tyrosine-kinase activity [24,25]. Thus, it was postulated that the insulin resistance induced by phorbol esters is mediated by PKC-stimulated phosphorylation of the insulin receptor.

In this study we examined the relationship between insulin-receptor autophosphorylation and insulin-induced receptor

Abbreviations used: EGF, epidermal growth factor; IGF, insulin-like growth factor; FBS, fetal bovine serum; WGA, wheat germ agglutinin; PMA, phorbol 12-myristate 13-acetate; PDBU, phorbol dibutyrate; PDD, phorbol didecanoate; OAG, oleoyl-acetyl-rac-glycerol; PKC, protein kinase C.

* To whom correspondence should be addressed.

† Present address: Mount Sinai Hospital, Department of Medicine, Division of Endocrinology, Suite 780, 600 University Avenue, Toronto, Ontario M5G 1X5, Canada.

down-regulation in cultured human lymphocytes. We employed the phorbol ester, PMA, as a probe to induce insulin resistance. We demonstrate that PMA causes resistance to this long-term action of insulin and that this is associated with an inhibition of both steps: internalization and receptor degradation. PMA also inhibited insulin-stimulated receptor autophosphorylation in these cells, supporting the hypothesis that activation of the receptor kinase by insulin is a necessary event in this process.

EXPERIMENTAL

Materials

IM-9 (human lymphoblasts) cells were obtained from the American Type Culture Collection; RPMI-1640, fetal bovine serum (FBS) and Trypan Blue were from Gibco; Na^{125} I and Amplify were from Amersham Corp.; SDS was from Boehringer-Mannheim; wheat germ agglutinin (WGA)–agarose was from Pharmacia Biotechnology Inc.; Pansorbin was from Calbiochem; Hepes, PMA, phorbol dibutyrate (PDBU), phorbol didecanoate (PDD), oleoyl-acetyl-rac-glycerol (OAG), poly[Glu(sodium salt)/Tyr] (4:1, v/v), BSA, Triton X-100, *N*-acetyl-D-glucosamine, rabbit γ-globulin, glucose oxidase (Type V) and anti-phosphotyrosine antibodies were from Sigma; poly(ethylene glycol) (8000) was from Fisher; γ-[^{32}P]ATP (10–40 Ci/mmol), [^{3}H]PDBU (370–740 GBq/mmol), Econofluor and Protosol were from Dupont New England Nuclear; glass fibre filters (934-AH) were from Whatman; monocomponent pig insulin was a generous gift from Nordisk Laboratories (Gentofte, Denmark), human anti-(insulin receptor) antibodies and rabbit anti-960 antibodies (raised against the 960 region of the insulin-receptor β-subunit) were kind gifts from Dr. B. Posner.

Cell culture

Human lymphocytes (IM-9) were cultured in RPMI-1640 supplemented with 25 mM Hepes and 10% (v/v) FBS at 37 °C in a humidified atmosphere of 5% CO_2 and air as described in [28]. Cells in late logarithmic phase were harvested by centrifugation (10 min at 500 ***g***) and washed twice with PBS. They were resuspended in fresh medium containing pig insulin, PMA, or combinations at the concentrations indicated. After 18 h, or the times indicated at 37 °C, the cells were harvested and washed three times over a period of 60 min at 22 °C in PBS (pH 7.4) containing 1% (w/v) BSA to remove residual insulin [28]. Cell viability determined by Trypan Blue exclusion was always equal in control and treated cells and greater than 85%.

[^{3}H]PDBU binding to IM-9 lymphocytes

After washing, cells were resuspended in RPMI-1640 and [^{3}H]PDBU binding was performed as described in [29]. Cells (15×10^{6} cells/ml) were incubated with 5 nM [^{3}H]PDBU with shaking in the presence of increasing concentrations of unlabelled phorbol ester or its analogues for 120 min at 4 °C in a total volume of 500 μl. The assay was terminated by diluting the incubation mixture with 3 ml of ice-cold PBS. Cells were recovered on glass fibre filters by filtration and washed three times with 3 ml of ice-cold PBS. The filters were placed in scintillation vials and 0.5 ml of Protosol and 5 ml of Econofluor were added sequentially. Vial contents were mixed and radioactivity was determined in an LKB β-counter after 18 h in darkness. Non-specific binding in the presence of 5 μM PDBU was subtracted from total binding to yield specific binding. Results were corrected to 2×10^{7} cells/ml and expressed as mean ± S.E.M.

Cell solubilization

After washing and centrifugation, 3 ml of ice-cold solubilization buffer [1% (v/v) Triton X-100/50 mM Hepes (pH 7.6)/4 mM EDTA/1 trypsin-inhibitor-unit (TIU)/ml of aprotinin/20 mM NaF/1 mM sodium orthovanadate/1 mM phenylmethanesulphonyl fluoride] were added to the cell pellet which was then frozen to −70 °C. After at least 1 h, the cell suspension was thawed and homogenized at 4 °C in a Potter–Elvejhem-type homogenizer. The solubilized extract was centrifuged at 100000 ***g*** for 1 h at 4 °C. The supernatants were used for ^{125}I-insulin binding.

^{125}I-Insulin binding to IM-9 lymphocytes and solubilized cell extracts

After washing, cells were resuspended in binding buffer [118.5 mM NaCl/4.7 mM KCl/2.5 mM $CaCl_2$/1.2 mM KH_2PO_4/1.2 mM $MgSO_4$/30 mM Hepes (pH 7.6)/5 mM glucose/1% (w/v) BSA] and ^{125}I-insulin binding was performed as described previously [28]. Cells (8×10^{6} cells/ml) were incubated with ^{125}I-insulin (0.1 ng/ml), iodinated by the chloramine-T method to 200 Ci/g [28], and 0–100000 ng/ml of unlabelled insulin for 90 min at 15 °C in a total volume of 500 μl. The assay was terminated by layering duplicate 200 μl aliquots on 200 μl of ice-cold binding buffer followed by centrifugation for 5 min in Beckman Microfuge. The supernatants were aspirated and the cell pellets excised and counted in an LKB Quattro γ-counter. Non-specific binding, measured in the presence of 100 μg/ml insulin, was subtracted from total binding to yield specific binding. Results were corrected to 10^{7} cells/ml and expressed as mean ± S.E.M.

For ^{125}I-insulin binding to solubilized receptors, supernatants (50 μl) were incubated for 20 h at 4 °C with ^{125}I-insulin (0.1 ng/ml) in binding buffer (150 mM NaCl/50 mM Hepes/10 mM $MgSO_4$/0.15% BSA) pH 7.6, with and without 0.1–1000 ng/ml of unlabelled insulin in a total volume of 0.5 ml. The final Triton X-100 concentration in the assay was 0.016%. The assay was terminated by addition of 1 ml of 0.125% rabbit γ-globulin and 1.0 ml of 25% (w/v) poly(ethylene glycol), both dissolved in 50 mM Hepes (pH 7.6), followed by centrifugation at 2000 ***g*** for 30 min. The supernatants were aspirated and the radioactivity of the pellets was counted. Non-specific binding was designated as binding in the presence of 1 μg/ml insulin and subtracted from total binding to yield specific binding. Results were corrected for 100 μg of protein determined by the Bio-Rad assay [30] and expressed as mean ± S.E.M.

Iodination of cell-surface proteins

Labelling of cell-surface proteins was performed using the lactoperoxidase technique [31] modified by Taylor and Marcus-Samuels [32] as we described in [33]. Cells were washed three times in Dulbecco's PBS without Ca^{2+} and Mg^{2+} and resuspended (2×10^{7} cells/ml) in the same buffer containing 20 mM glucose at room temperature. Lactoperoxidase (25 μg/ml) was added to the cell suspension with constant stirring. The reaction was started by the addition of glucose oxidase (1100 munits/ml) and Na^{125}I (100 μCi/ml). The reaction was stopped by dilution into Dulbecco's PBS lacking Ca^{2+} and Mg^{2+} (10^{6} cells/ml). The suspension was centrifuged at 500 ***g*** for 10 min, the supernatant was discarded, and the cells were washed three times with the above-mentioned buffer. They were resuspended in culture medium and incubated at 37 °C for 60 min before additions were made. At the times indicated after additions of PMA and/or insulin, cells were harvested by centrifugation and solubilized in 3 ml of ice-cold

buffer as described above. An aliquot of the 100000 *g* supernatant was assayed for protein content using the Bio-Rad reagent and the remainder was used for immunoprecipitation.

Before immunoprecipitation Triton X-100 present in the solubilization buffer was removed by shaking the 100000 *g* supernatant in the presence of Bio-Beads SM-2 for 6 h [34].

Immunoprecipitation of receptors

Protein A–Sepharose-affinity-column-purified anti-(insulin receptor) antibody (14 mg/ml) was added to solubilized receptors (0.9 ml) and incubated, with shaking, at 4 °C for 16 h. Pansorbin [200 μl of a 10 % (w/v) suspension] was added and the incubation was continued for 4 h at 4 °C. The immunoprecipitates were centrifuged (500 *g*, 5 min) and washed [2 × 900 μl of 50 mM Hepes (pH 7.6)/0.1 % Triton X-100; 1 × 900 μl of 50 mM Hepes (pH 7.6)/0.1 % SDS/0.1 % Triton X-100; 1 × 900 μl of 50 mM Hepes (pH 7.6)/0.1 % Triton X-100] and subjected to SDS/PAGE in a 7.5 % (w/v) gel. The gel was stained with Coomassie Brilliant Blue, destained, soaked in Amplify fluorography reagent, and dried. The driel del was exposed on Kodak X-Omat AR film for 1 week at −80 °C, and labelled proteins were detected by autoradiography and quantified by densitometry. Densitometric values with individual variances (expressed as percentages of radioactivity remaining in the insulin-receptor α-subunit) were fitted using an exponential function with the Multifit non-linear regression software (version 2.01) from Day Computing (Cambridge, U.K.).

Partial receptor purification

The 100000 *g* supernatant, after cell solubilization, was applied to a column (0.6 cm × 3.0 cm) of WGA–agarose and after washing with 100 ml of 50 mM Hepes buffer, pH 7.6, containing 150 mM NaCl and 0.1 % Triton X-100, the bound material was eluted with the above buffer supplemented with 0.3 M *N*-acetyl-D-glucosamine.

Insulin-receptor autophosphorylation

Aliquots (30–50 μl) containing equal amounts of receptor (350–450 fmoles) were added in 1.5 ml Eppendorf tubes to a reaction mixture containing final concentrations of 50 mM Hepes, 10 mM $MnCl_2$, 10 μM sodium orthovanadate and 250 μM NaF, pH 7.6, in a final volume of 90 μl. Phosphorylation was initiated by adding [γ-^{32}P]ATP (80 μCi) and unlabelled ATP to a final concentration of 25 μM. The phosphorylation reaction was allowed to proceed at 22 °C for 60 min and terminated by the addition of 50 μl of stopping solution (50 mM Hepes/50 mM EDTA/10 mM sodium pyrophosphate/1 mM benzamidine/20 mM sodium molybdate/40 mM NaF/1 mM PMSF/2 mM sodium orthovanadate/0.1 % Triton X-100, pH 7.6). The insulin receptors were immunoprecipitated and separated by SDS/PAGE as described. The phosphorylated receptor was visualized by autoradiography at −70 °C using enhancing screens and exposure for 1–8 h with Kodak X-Omat AR film. The intensities of the 95 kDa bands were quantified with a LKB ultrascan XL laser densitometer interfaced with an IBM 386 computer using the program GEL SCAN XL (Pharmacia) and are expressed in arbitrary units.

Western blotting with anti-phosphotyrosine antibody

After electrophoretic transfer of proteins from the gels to nitrocellulose membranes the latter were washed and blocked with 50 ml of PBS, pH 7.4, containing 10 % (v/v) FBS for 1 h at 22 °C. The solution was replaced with 50 ml of 1:300-diluted affinity-purified anti-phosphotyrosine antibody in PBS, pH 7.4, containing 2 % (w/v) BSA and incubated for 2 h at 22 °C. The membranes were washed and incubated with 50 ml of ^{125}I-labelled goat anti-(rabbit IgG) antibody (0.7 × 10^6 c.p.m./lane) in PBS containing 2 % (w/v) BSA for 1 h at 22 °C. The membranes were washed, dried and fixed. Labelled phosphotyrosine-containing proteins were visualized by radioautography, and intensities of the 95 kDa bands determined as above.

Receptor content was determined by immunoblotting with antibodies against the residue-960 region of the β-subunit. The above procedure, used for the anti-phosphotyrosine blots, was modified by using a solution of PBS, pH 7.4, with 3 % (v/v) Carnation milk for blocking followed by incubation with a 1:300 (v/v) dilution of anti-960 antibody in PBS with 3 % (v/v) Carnation milk for 2 h at 22 °C. The incubation with ^{125}I-labelled goat anti-(rabbit IgG) antibody was also carried out in PBS containing 3 % (v/v) milk. Radioactivity was quantified as above by densitometry of exposed films.

RESULTS

Phorbol ester binding

Phorbol esters are analogues of diacylglycerol and bind to and activate PKC [14,15]. The IM-9 lymphocytes specifically bound [^{3}H]PDBU (Figure 1a). Competition-binding assays with active analogues such as PMA, PDBU and OAG yielded EC_{50} values of

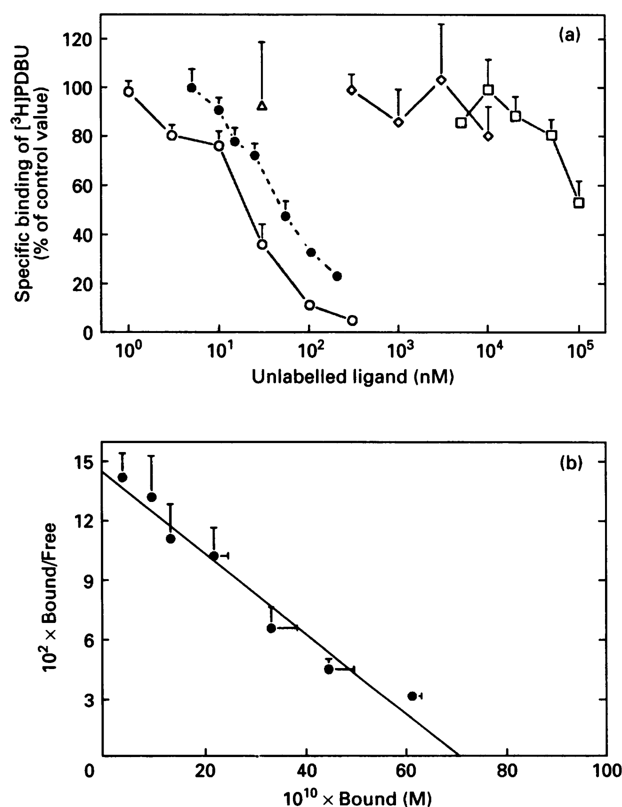

Figure 1 [^{3}H]PDBU binding to cultured human lymphocytes

(**a**) [^{3}H]PDBU binding was assayed in the presence of the indicated concentrations of PDBU (●), PMA (○), PDD (◇), OAG (□) and insulin (△) as described in the Experimental section. Results are expressed as the mean ± S.E.M. of three experiments. Mean specific binding of [^{3}H]PDBU was 13 ± 1 %/2 × 10^7 cells per ml. (**b**) Scatchard analysis of binding competition by unlabelled PDBU ($n = 3$).

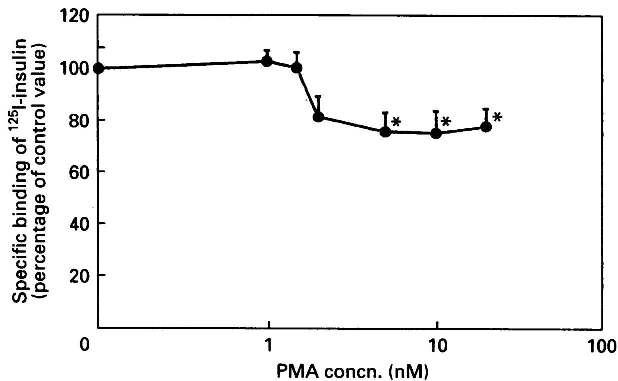

Figure 2 Acute effect of PMA on decrease of insulin binding

Cells were incubated for 15 min at 37 °C with the indicated concentrations of PMA, cooled to 15 °C and then ^{125}I-insulin binding to intact cells was assayed as described in the Experimental section. Results are expresseed as the mean ± S.E.M. of three to ten experiments. Control ^{125}I-insulin binding was 18.1 ± 1.4%/10^7 cells per ml; * $P < 0.05$.

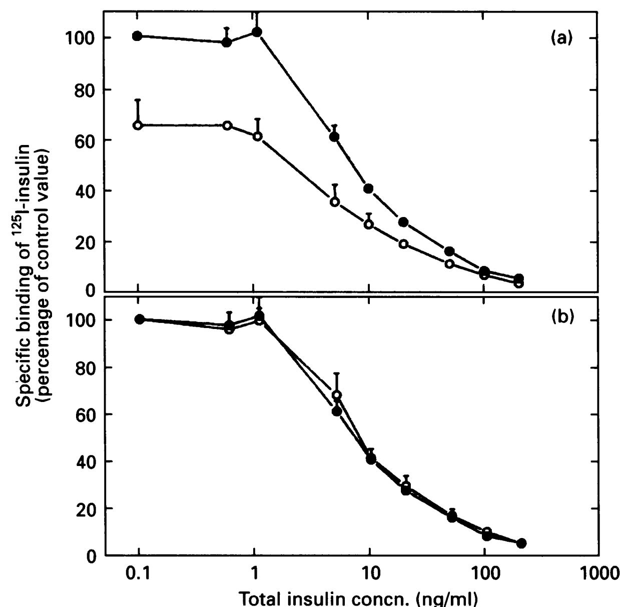

Figure 3 Acute effect of PMA on insulin binding

(**a**) illustrates a competition curve. Cells were incubated as described in the legend to Figure 2 with vehicle (●) or 50 nM PMA (○). Results (mean ± S.E.M., $n = 3$) were normalized to control tracer binding. (**b**) Results of each individual binding curve were normalized to a tracer binding of 100%. IC$_{50}$, apparent receptor affinity, was 9 ng/ml for control and PMA-treated cells.

20 nM, 52 nM and 70 μM respectively. Competition with an inactive phorbol ester, α-PDD, and with insulin showed no displacement of bound [^{3}H]PDBU (Figure 1a). Scatchard analysis of the PDBU competition data (Figure 1b) revealed that binding capacity was 7 nM/10^7 cells or 2×10^5 sites/cell. This value agrees closely with the number of binding sites for PKC reported previously in freshly isolated human lymphocytes [35].

Acute effects of PMA on insulin binding

Incubation for 15 min with PMA at 37 °C led to a significant dose-dependent decrease in ^{125}I-insulin binding to a nadir of 75 % of control levels at 5 nm (Figure 2). Competition-binding curves revealed that the decrease caused by exposure to PMA

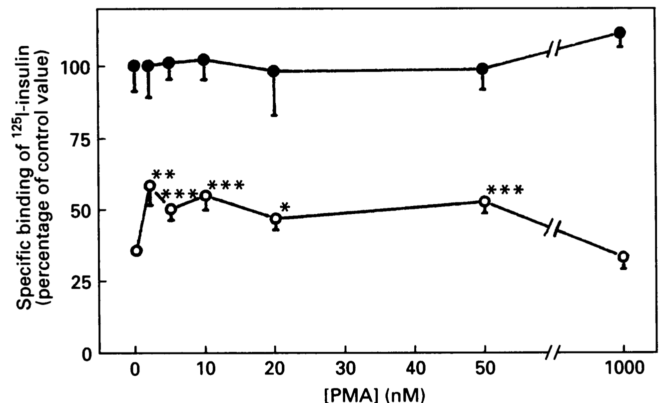

Figure 4 Long-term effect of PMA on inhibition of insulin-stimulated down-regulation in cultured human lymphocytes

Cells were exposed to the indicated concentrations of PMA (●) or PMA plus 10^{-7} M insulin (○) for 18 h at 37 °C. Cells were washed thoroughly and ^{125}I-insulin binding was assayed. Results are expressed as mean ± S.E.M. of two to seven experiments. Statistical significance: * $P < 0.05$; ** $P < 0.025$; *** $P < 0.01$.

was apparent at all insulin concentrations (Figure 3a). When the two curves were normalized to their respective tracer binding values, they were superimposable (Figure 3b). These results indicated that apparent receptor affinity (IC$_{50}$ = 9.0 ng/ml insulin) remained unchanged and that short-term PMA treatment resulted in a decrease in the number of cell-surface insulin receptors.

Chronic effects of PMA on insulin binding

To determine the long-term effects of PMA, cells in culture were exposed to various concentrations of PMA for 18 h at 37 °C after which cells were washed and ^{125}I-insulin binding was assayed. There was no effect of PMA on insulin binding after 18 h exposure, indicating that the acute effect was transient (Figure 4).

To determine whether chronic exposure to PMA affected insulin-stimulated down-regulation of its own receptor, cells were co-incubated with insulin (10^{-7} M) and various concentrations of PMA. Insulin alone decreased numbers of cell-surface receptors by 64.8 % after 18 h at 37 °C. Low concentrations of PMA (2–50 nM) inhibited the insulin-induced down-regulation significantly (Figure 4). In the presence of 5 nM PMA, insulin binding was decreased in the presence of 10^{-7} M insulin by only 49.7 ± 4 %. ($P < 0.05$). This represented a 23.3 % inhibition of insulin-induced down-regulation. When a high concentration of PMA (1 μM) was used, there was no effect on insulin-induced down-regulation (Figure 4). An inactive phorbol ester analogue, α-PDD, also did not inhibit insulin-induced down-regulation (results not shown).

PMA inhibits the insulin-induced decrease in total cellular receptors

The total complement of cellular insulin receptors was assessed to determine the effect of PMA on insulin-receptor metabolism. After incubation of cells in the presence and absence of insulin and PMA for 18 h as described above, lymphocytes were homogenized and solubilized in Triton X-100 and ^{125}I-insulin binding determined as described in the Experimental section. Insulin binding to total cellular solubilized receptors showed that 10^{-7} M insulin decreased specific binding to 30.2 ± 7.5 % of control (mean ± S.E.M., $n = 3$). PMA (5 nM) alone had no effect

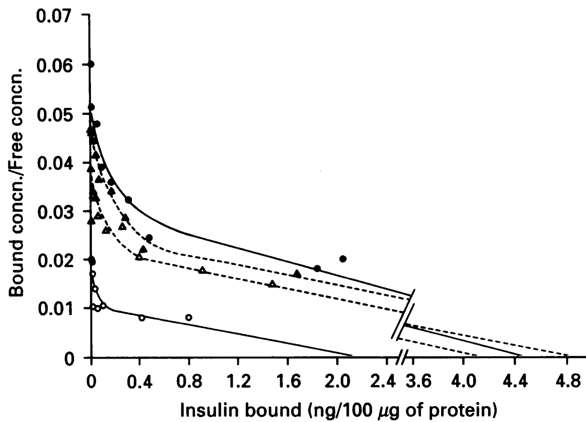

Figure 5 Effect of PMA on insulin-stimulated down-regulation in human lymphocytes

Cells were cultured in the absence (●) or presence (○) of insulin (10^{-8} M), PMA (2.5 nM) (▲) or PMA plus insulin (△) for 20 h at 37 °C. Cells were washed, homogenized and solubilized as described in the Experimental section. ^{125}I-Insulin binding to solubilized receptors was performed. Mean Scatchard analyses are shown of three binding-competition experiments, each performed in triplicate. Mean ± S.E.M. (%) specific tracer (0.1 ng/ml) insulin binding values corrected for 100 μg of protein were: control, 5.68 ± 0.20; insulin, 1.93 ± 0.46 ($P < 0.01$ compared with control); PMA, 4.51 ± 0.95; and PMA plus insulin, 3.75 ± 1.00 (P not significant compared with PMA alone or with control levels).

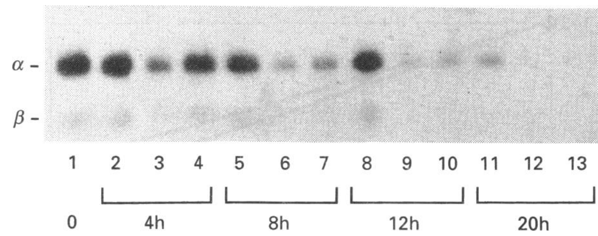

Figure 6 Effect of PMA on insulin-stimulated insulin-receptor degradation in cultured human lymphocytes

Cells were labelled with Na^{125}I using the lactoperoxidase technique and incubated for 0–20 h in RPMI-1640 medium with 10% (v/v) FBS in the presence and absence of 5 nM PMA, 10^{-7} M insulin or 5 nM PMA plus 10^{-7} M insulin. After cell solubilization, the insulin receptor was immunoprecipitated and subjected to SDS/PAGE under reducing conditions. The gel was dried and exposed to film for autoradiography. Lanes 1, 2, 5, 8 and 11: α- and β-subunits of insulin receptors from control cells at 0, 4, 8, 12 and 20 h; lanes 3, 6, 9, and 12: α- and β-subunits of insulin receptors from insulin-treated cells at 4, 8, 12, and 20 h; lanes 4, 7, 10, and 13: α- and β-subunits of insulin receptors from PMA plus insulin-treated cells at 4, 8, 12, and 20 h.

on binding (94.9 ± 4.1 % of control value). In the presence of 10^{-7} M insulin, 5 nM PMA consistently inhibited the insulin-induced decrease in a similar manner to that observed in intact cells (54.4 ± 9.6 % of control value).

To determine whether this effect on insulin binding may have been mediated by an alteration in receptor affinity, complete competition-binding curves were generated after incubation for 20 h in the presence and absence of 2.5 nM PMA and/or 10^{-8} M insulin. The competition-binding curves and Scatchard plots [36] indicate that PMA inhibited the decrease in total cellular insulin-receptor number induced by insulin (Figure 5).

Inhibition of the PMA effect by sphingosine

Sphingosine, a phospholipid commonly found in membranes, has been shown to be a potent inhibitor of PKC and a phorbol ester antagonist [37]. To confirm that the PMA effect was mediated by PKC, cells were treated for 18 h with sphingosine (10 μM) alone, sphingosine in the presence of insulin (10^{-7} M), and sphingosine with insulin (10^{-7} M) plus PMA (5 nM). In the presence of sphingosine insulin was able to down-regulate total cellular insulin receptors to the same extent in the absence and presence of PMA (ratio of bound/free ^{125}I-insulin; sphingosine alone 0.094, sphingosine and insulin 0.050, sphingosine and insulin plus PMA 0.049). In these experiments the effect of PMA on inhibition of insulin-induced receptor down-regulation in the absence of sphingosine was similar to that indicated above.

Effect of PMA on insulin-induced receptor internalization

Experiments in 3T3C2 fibroblasts [4], rat adipocytes [5] and our previous results in IM-9 lymphocytes [33] showed that insulin-stimulated down-regulation involves two steps, receptor internalization followed by a slower phase of degradation. The ability of insulin to induce internalization was assessed by exposing cells to insulin at 37 °C for 1 h. This was followed by a cold acid wash to remove residual insulin. We demonstrated previously that under these conditions the loss of insulin binding to intact cells is completely recoverable upon cell solubilization [33]. After 18 h of exposure of lymphocytes to 5 nM PMA, the ability of insulin to induce internalization was decreased by 26.4 ± 5.4 % (percentage of cell-surface receptors internalized; control, 41.7 ± 3.0; 5 nM PMA-treated cells, 30.7 ± 2.4; $P < 0.05$, $n = 3$). Thus, the inhibition of down-regulation by PMA is associated with a decrease of receptor internalization.

PMA inhibits insulin-induced receptor degradation

To assess the phase of receptor degradation cells were surface-labelled with ^{125}I and treated with PMA, insulin, PMA plus insulin, or vehicle alone for various times, solubilized, and the insulin receptors immunoprecipitated and separated by SDS/PAGE as described in the Experimental section. Insulin (Figure 6, lanes 3, 6, 9 and 12) accelerated the degradation of the surface-labelled receptors compared with control levels (Figure 6, lanes 1, 2, 5, 8 and 11). This effect was clearly inhibited by PMA (Figure 6, lanes 4, 7, 10 and 13). The half-life of the α-subunit of the insulin receptor was determined from the densitometric analysis of autoradiographs of gels at times 4–20 h. In the presence of 10^{-7} M insulin the amount of ^{125}I-labelled insulin receptor was significantly decreased at each time point (Figure 7b). Receptor half-life ($t_{\frac{1}{2}}$) was shortened by 51 % from 8.1 h to 4.0 h, consistent with previous studies [3,32,33]. PMA alone (5 nM) decreased insulin receptor $t_{\frac{1}{2}}$ from 8.1 to 6.9 h, reflecting a small increase in the rate of degradation (Figure 7a). However, in the presence of PMA, the effect of insulin on receptor degradation was severely inhibited (6.9 h to 6.3 h) (Figure 7c).

Effect of PMA on insulin-receptor autophosphorylation

Since the inhibitory effects of PMA on insulin action have been associated previously with a defect in insulin-receptor autophosphorylation [24,25], we determined the effect of PMA on autophosphorylation in these cultured IM-9 lymphocytes. Cells were incubated at 37 °C in the absence and presence of PMA (5 nM or 100 nM) for 30 min, followed by the addition of insulin (10^{-7} M) or vehicle for 1 min. The reactions were stopped and the insulin receptors were partially purified and autophosphorylation assayed in the presence of 25 μM [γ-^{32}P]ATP as

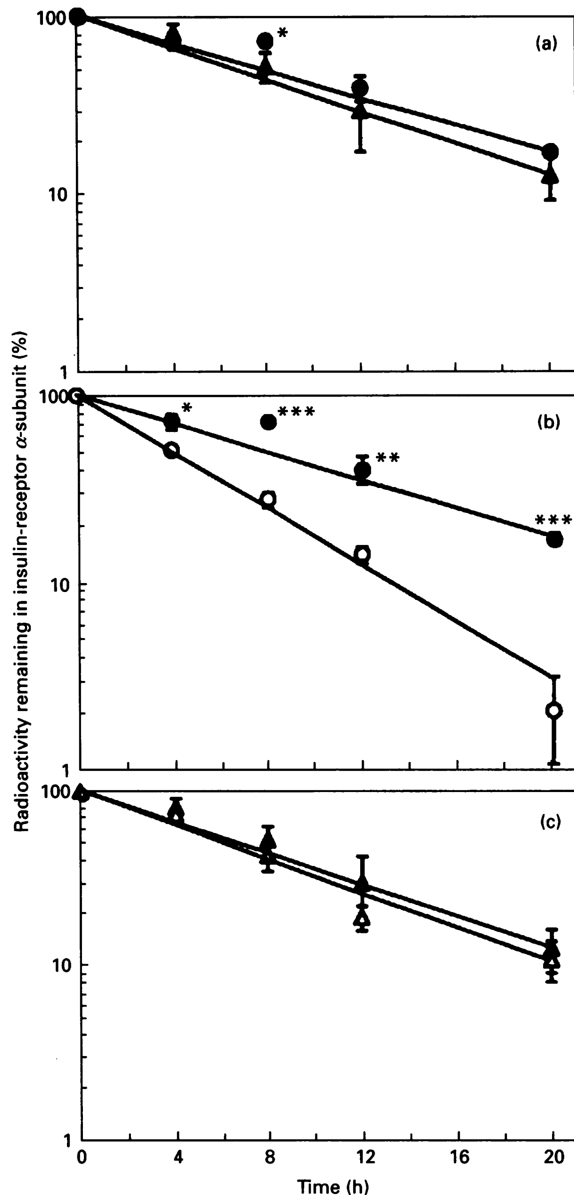

Figure 7 Effect of PMA and insulin on the half-life ($t_{\frac{1}{2}}$) of the insulin receptor

Autoradiographs from three to seven separate lactoperoxidase-labelling experiments as described in Figure 6 were scanned and densitometric readings were plotted as mean ± S.E.M. (percentage of control value). Comparison of control versus PMA-treated (**a**), control versus insulin-treated (**b**) and PMA-treated versus PMA plus insulin-treated (**c**). Control, $t_{\frac{1}{2}} = 8.1$ h (●); 10^{-7} M insulin, $t_{\frac{1}{2}} = 4.0$ h (○); 5 nM PMA, $t_{\frac{1}{2}} = 6.9$ h (▲); 10^{-7} M insulin plus 5 nM PMA, $t_{\frac{1}{2}} = 6.3$ h (△). Statistical significance: * $P < 0.05$; ** $P < 0.005$; *** $P < 0.001$.

described in the Experimental section. PMA alone had no effect on total insulin-receptor phosphorylation. However, there was a dose-dependent inhibition of *in situ* insulin activation of autophosphorylation by pre-exposure of cells to PMA. (Figure 8a). This was confirmed, in separate experiments, to be tyrosine phosphorylation by washing the gels in KOH to remove phosphoserine and phosphothreonine (results not shown). Although the autophosphorylation experiments demonstrated the inhibitory effect of PMA on *in situ* insulin activation, the phosphorylation reaction itself was carried out *in vitro* with

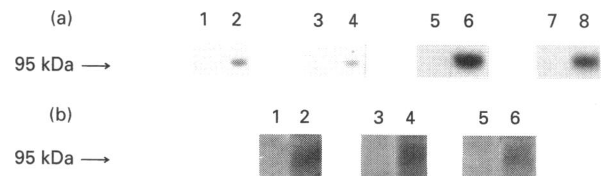

Figure 8 Effect of PMA on (a) insulin-receptor autophosphorylation and (b) insulin-stimulated tyrosine phosphorylation of the insulin receptor in intact cultured human lymphocytes

(**a**) Cells were incubated in the absence (lanes 1, 2, 5, and 6) or presence of 5 nM (lanes 3 and 4) or 100 nM (lanes 7 and 8) PMA for 30 min at 37 °C followed by the addition of 10^{-7} M insulin (lanes 2, 4, 6, and 8) or vehicle (lanes 1, 3, 5 and 7) for 1 min. Preliminary experiments revealed that peak insulin activation of autophosphorylation occurred after 1 min of exposure. The reaction was terminated by the addition of ice-cold stopping solution containing protease- and phosphatase-inhibitors and immediate freezing to −70 °C. Following solubilization and partial receptor purification by WGA-chromatography insulin-receptor autophosphorylation was assayed as described in the Experimental section. Autoradiographs of the 95 kDa β-subunit are shown. Results of densitometric analyses of the bands yielded the following values expressed in arbitary units: lane 1, 352; lane 2, 2757; lane 3, 176; lane 4, 1959; lane 5, 352; lane 6, 2757; lane 7, 181; lane 8, 1734. Results are from one of two separate experiments each performed in triplicate yielding similar results. An additional experiment was performed with a KOH wash of the gel to remove [^{32}P]phosphoserine and [^{32}P]phosphothreonine which demonstrated the same degree of inhibition of insulin-stimulated phosphorylation by PMA. (**b**) The experiments were performed as described in (**a**): Control (lane 1), 5 nM PMA (lane 3), 100 nM PMA (lane 5), insulin (10^{-7} M) (lane 2), 5 nM PMA plus insulin (lane 4) and 100 nM PMA plus insulin (lane 6). Following solubilization the insulin receptors were immunoprecipitated and separated by SDS/PAGE, transferred to nitrocellulose membranes and blotted with anti-phosphotyrosine antibodies. Anti-phosphotyrosine antibody was detected with ^{125}I-labelled second antibody. Autoradiographs of the 95 kDa β-subunit are shown. Densitometric analyses of the bands were corrected for β-subunit content quantified by anti-(β-subunit) (anti-960) antibody immunoblot (not shown) and yielded the following values expressed in arbitrary units: lane 1, 67; lane 2, 641; lane 3, 56; lane 4, 350; lane 5, 26; lane 6, 290. Results are from one of two experiments each performed in duplicate yielding similar results.

partially purified insulin receptor. To confirm that this inhibitory effect of PMA was associated with diminished insulin stimulation of receptor tyrosine autophosphorylation in the intact cells, the isolated receptors were separated by SDS/PAGE, transferred to nitrocellulose membranes and blotted with both anti-(phosphotyrosine) and anti-(β-subunit) antibodies. Pre-exposure to PMA for 30 min inhibited the response to insulin of *in situ* phosphotyrosine incorporation in parallel to the inhibition of autophosphorylation observed above (Figure 8b).

DISCUSSION

Short-term exposure of cultured human lymphocytes [16], U-937 monocytes [16] and adipocytes [17] to phorbol esters has been reported to cause a decrease in insulin-receptor affinity. In contrast, our results from cultured human lymphocytes suggest that similar to effects on a number of other receptors, e.g. EGF [20,38], transferrin [21,22], tumour necrosis factor-α [39] and low-density lipoprotein [40] phorbol esters induce a rapid, dose-dependent decrease in cell-surface insulin receptor number. In the case of the insulin receptor it has been reported that in some cells internalization of only the hormone-occupied receptor but not that of the unoccupied receptor is increased by PMA [41]. The effect of PMA may depend on the localization of receptors in coated pits. Thus, under our cell-culture conditions, a fraction (about one third) of the insulin receptors may be located in coated pits while the remainder are on the microvilli. Occupancy of insulin appears to stimulate micro-aggregation and movement of these receptors to coated pits [42] which would explain the discrepant obervations in different cell types.

PMA is known to activate the phospholipid- and Ca^{2+}-dependent serine/threonine kinase, PKC. PMA stimulates

insulin-receptor serine phosphorylation in these [26,43] and other cell types [18,25]. Whether the PMA-stimulated internalization is mediated via insulin-receptor phosphorylation is not clear. Two lines of evidence raise questions about this possible mechanism. First, studies with a mutated transferrin receptor indicated that PMA could exert its effect on receptor movement despite substitution of the serine residue (Ser-24), which is phosphorylated, for alanine [44]. Secondly, in Fao hepatoma cells, PMA-stimulated insulin-receptor phosphorylation was not associated with any change in insulin binding [23,25].

In adipocytes and Fao hepatoma cells, exposure to PMA has been associated with resistance to acute biological effects of insulin [17,23]. In this study, we found that PMA induced resistance to a long-term effect of insulin, i.e. down-regulation of its own receptor. In the presence of PMA, insulin-stimulated down-regulation of cell-surface receptors was inhibited by 23 %.

At a concentration of 1 μm, PMA did not inhibit receptor down-regulation. It is known that exposure to high concentrations of PMA for a prolonged time results in down-regulation and degradation of PKC in a variety of cell types [45]. After such treatment cells become resistant to the actions of phorbol esters which are mediated via PKC activation [46,47]. Thus, the absence of an inhibitory effect on insulin-receptor down-regulation at this high PMA concentration suggests that the PMA effect was mediated by activation of PKC. The blockade of the PMA effect in the presence of 10 μM sphingosine further supports the interpretation that PKC is the putative mediator of the inhibitory effect of PMA on insulin-receptor down-regulation.

PMA inhibited the early phase (1 h) of disappearance of surface receptor by 26 %, similar to the extent of inhibition of down-regulation assessed by insulin binding to intact cells. The extent of internalization at 1 h reflects a steady state between the rates of endocytocis and recycling. We have not determined whether either rate is altered. However, the lack of internalization of tyrosine kinase-defective mutant receptors [7,8] suggests the possibility that neither rate may be affected, but that a population of receptors which are inhibited by PMA is generated which do not internalize at all.

The results observed with solubilized receptors suggested that the effect of PMA was not confined to receptor internalization. Indeed, PMA inhibited the effect of insulin on stimulation of insulin-receptor degradation. The transient nature of the decrease in cell-surface insulin receptors induced by PMA indicates that the short-term decrease in receptor availability could not account for these long-term effects of PMA. A possible mechanism of the inhibitory effect of PMA was suggested by studies in which mutated tyrosine kinase-defective insulin receptors could not be stimulated by insulin to undergo down-regulation [7,8]. Thus, the inhibitory effect of the phorbol ester could be mediated via an inhibition of insulin-receptor autophosphorylation. Pre-exposure of intact cells to 5 nM PMA inhibited insulin activation of receptor autophosphorylation assayed *in vitro*. Furthermore, PMA inhibited insulin stimulation of insulin-receptor tyrosine phosphorylation *in situ*. These results are in agreement with previous results obtained in these and other cell types [25,27]. However, it should be noted that inhibition of insulin-stimulated insulin-receptor autophosphorylation by PMA has not been consistently observed by all investigators [43].

Recently, Lewis et al. demonstrated that Thr-1336 in the C-terminal region of the β-subunit is the major amino-acid residue phosphorylated in response to PMA *in vitro* [48]. *In situ* stimulation by PMA led to similar phosphorylation, as well as the appearance of additional serine-phosphorylated peptides [48]. The relationship of the receptor kinase inhibitory effect of PMA to the Ser/Thr phosphorylation has also been questioned. Thus, Anderson and Olefsky demonstrated that PMA inhibited insulin-stimulated receptor autophosphorylation, tyrosine kinase activity and glucose incorporation into glycogen in Rat-1 fibroblasts expressing a 43 amino-acid C-terminal-truncated receptor [49]. The mechanism of the PMA effect remains to be determined.

Thies et al. have recently defined a submembrane region of the insulin receptor encoded by exon 16 which is necessary but not sufficient for insulin-induced internalization [50]. The activation of the receptor tyrosine kinase has also been demonstrated to be required [51]. However, Backer et al. reported that depletion of cellular ATP resulted in a decrease of insulin-stimulated receptor autophosphorylation but did not affect internalization [52]. There is strong support for the hypothesis that the second phase of the down-regulation process, i.e. receptor degradation, is tyrosine kinase dependent. Thus, mutagenesis studies uniformly indicate defective insulin-induced down-regulation of tyrosine kinase-defective receptors, even with a Lys to Arg-1018 mutant, which undergoes constitutive internalization [9]. Furthermore, Marshall and Monzon have demonstrated [53], and we have confirmed (C. Walker and I. G. Fantus, unpublished work), that vanadate, a protein tyrosyl phosphatase inhibitor which mimics insulin in adipocytes, can induce ligand-independent insulin-receptor degradation. The results in this study are consistent with the requirement of insulin-stimulated receptor autophosphorylation for both phases of down-regulation.

The observation that insulin-induced down-regulation of its own receptor was normal after exposure to high concentrations of PMA indicates that PKC is not involved in this action of insulin. Similarly, EGF-mediated down-regulation of its receptor does not require PKC-mediated receptor phosphorylation [38]. There is still controversy regarding the potential role of PKC in other actions of insulin [47,54–56] and whether high concentrations of PMA can completely remove PKC [56,57]. The normal extent of insulin-receptor down-regulation during chronic exposure to high PMA concentrations, as well as in the presence of sphingosine, lends support to the view that PKC is not involved.

In summary, this study demonstrates that PMA inhibits insulin-stimulated down-regulation of its own receptor. The disappearance of the effect at high PMA concentrations and inhibition by sphingosine are consistent with activation of PKC. The inhibition involves both receptor internalization and degradation. At the same concentrations PMA also inhibited insulin-stimulated insulin-receptor tyrosine autophosphorylation. Taken together, these results strongly support the results from mutagenesis studies that insulin-receptor autophosphorylation is a requirement for insulin-induced receptor internalization and degradation.

We thank Drs. B. Posner and J.-L. Carpentier for helpful discussions, N. De Luca and B. Baubinas for excellent secretarial assistance. This work was supported by the Canadian Diabetes Association. I. G. F. is a Recipient of a Chercheur Bousier award from the Fonds de la Recherche en Sante du Quebec.

REFERENCES

1 Gavin, J. R., Roth, J., Neville, D. M., Jr., De Meyts, P. and Buell, D. N. (1974) Proc. Natl. Acad. Sci. U.S.A. **71**, 84–88

2 Kosmakos, F. C. and Roth, J. (1980) J. Biol. Chem. **255**, 9860–9869

3 Kasuga, M., Kahn, C. R., Hedo, J. A., Van Obberghen, E. and Yamada, K. M. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 6917–6921

4 Knutson, V. P., Ronnett, G. V. and Lane, M. D. (1983) J. Biol. Chem. **258**, 12139–12142

5 Green, A. and Olefsky, J. M. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 427–431

6 Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. and Ramachandran, J. (1985) Nature (London) **313**, 756–761

7 Russell, D. S., Gherzi, R., Johnson, E. L., Chou, C. K. and Rosen, O. M. (1987) J. Biol. Chem. **262**, 11833–11840
8 McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A. and Olefsky, J. M. (1987) J. Biol. Chem. **262**, 14663–14671
9 Hari, J. and Roth, R. A. (1987) J. Biol. Chem. **262**, 15341–15344
10 Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N. and Rosenfeld, M. G. (1987) Nature (London) **328**, 820–823
11 Glenney, J. R., Jr., Chen, W. S., Lazar, C. S., Walton, G. M., Zokas, L. M., Rosenfeld, M. G. and Gill, G. N. (1988) Cell **52**, 675–684
12 Livneh, E., Reiss, N., Bevent, E., Ullrich, A. and Schlessinger, J. (1987) EMBO J. **6**, 2669–2676
13 Honegger, A. M., Dull, T. J., Felder, S., Van Obberghen, E., Bellot, F., Szapary, D., Schmidt, A., Ullrich, A. and Schlessinger, J. (1987) Cell **51**, 199–209
14 Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. **257**, 7847–7851
15 Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. and Ullrich, A. (1986) Science **233**, 853–859
16 Grunberger, G. and Gorden, P. (1982) Am. J. Physiol. **243**, E319–E324
17 Kirsch, D., Obermaier, B. and Haring, H. U. (1985) Biochem. Biophys. Res. Commun. **128**, 824–832
18 Hachiya, H. L., Takayama, S., White, M. F. and King, G. L. (1987) J. Biol. Chem. **262**, 6417–6424
19 Rouis, M., Thomopoulos, P., Postel-Vinay, M. C., Testa, U., Guyda, H. J. and Posner, B. I. (1984) Mol. Physiol. **5**, 123–130
20 Beguinot, 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
21 May, W. S. and Tyler, G. (1987) J. Biol. Chem. **262**, 16710–16718
22 Fallon, R. J. and Schwartz, A. L. (1986) J. Biol. Chem. **261**, 15081–15089
23 Takayama, S., White, M. F., Lauris, V. and Kahn, C. R. (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 7797–7801
24 Haring, H., Kirsch, D., Obermayer, B., Ermel, B. and Machicao, F. (1986) J. Biol. Chem. **261**, 3869–3875
25 Takayama, S., White, M. F. and Kahn, C. R. (1988) J. Biol. Chem. **263**, 3440–3447
26 Jacobs, S., Sahyoun, N. E., Saltier, A. R. and Cuatrecasas, P. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 6211–6213
27 Bollag, G. E., Roth, R. A., Beaudoin, J., Mochly-Rosen, D. and Koshland, D. E., Jr. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 5822–5824
28 Fantus, I. G., Saviolakis, G. A., Hedo, J. A. and Gorden, P. (1982) J. Biol. Chem. **257**, 8277–8283
29 Leach, K. L., James, M. L. and Blumberg, P. M. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 4208–4212
30 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
31 Hubbard, A. L. and Cohn, Z. A. (1975) J. Cell Biol. **64**, 438–460
32 Taylor, S. I. and Marcus–Samuels, B. (1984) J. Clin. Endocrinol. Metab. **58**, 182–186
33 Torossian, K., Freedman, D. and Fantus, I. G. (1988) J. Biol. Chem. **263**, 9353–9359
34 Holloway, P. W. (1973) Anal. Biochem. **53**, 304–308
35 Sando, J. J., Hilfiker, M. C., Salomon, D. S. and Farrar, J. J. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 1189–1193
36 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. **51**, 660–672
37 Hannun, Y. A. and Bell, R. M. (1987) Science **235**, 670–674
38 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
39 Aggarwal, B. B. and Eessalu, T. E. (1987) J. Biol. Chem. **34**, 16450–16455
40 Rouis, M., Goldstein, S., Thomopoulos, P., Berthelier, M., Henry, C. and Testa, U. (1984) J. Cell. Physiol. **121**, 540–546
41 Iacopetta, B., Carpentier, J.-L., Pozzan, T., Lew, D. P., Gorden, P. and Orci, L. (1986) J. Cell Biol. **103**, 851–856
42 Smith, R. M., Seely, B. C., Shah, N., Olefsky, J. M. and Jarett, L. (1991) J. Biol. Chem. **266**, 17522–17530
43 Jacobs, S. and Cuatrecasas, P. (1986) J. Biol. Chem. **261**, 924–939
44 Davis, R. J. and Meisner, H. (1987) J. Biol. Chem. **262**, 16041–16047
45 Nishizuka, Y. (1984) Nature (London) **308**, 693–698
46 Ballester, R. and Rosen, O. M. (1985) J. Biol. Chem. **260**, 15194–15199
47 Blackshear, P. J., Nemenoff, R. A., Hovis, J. G., Halsey, D. L., Stumpo, D. J. and Huang, J. K. (1987) Mol. Endocrinol. **1**, 44–52
48 Lewis, R. E., Cao, L., Perregaux, D. and Czech, M. P. (1990) Biochemistry **29**, 1807–1813
49 Anderson, C. M. and Olefsky, J. M. (1991) J. Biol. Chem. **266**, 21760–21764
50 Thies, R. S., Webster, N. J. and McClain, D. A. (1990) J. Biol. Chem. **265**, 10132–10137
51 Rajagopalan, M., Neidigh, J. L. and McClain, D. A. (1991) J. Biol. Chem. **266**, 23086–23073
52 Backer, J. M., Kahn, C. R. and White, M. F. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3209–3213
53 Marshall, S. and Monzon, R. (1987) Endocrinology **121**, 1116–1122
54 Chu, D. T. W., Stumpo, D. J., Blackshear, P. J. and Granner, D. K. (1987) Mol. Endocrinol. **1**, 53–59
55 Klip, A. and Ramlal, T. (1987) Biochem. J. **242**, 131–136
56 Staendaert, M. L., Farese, R. V., Cooper, D. R. and Pollet, R. J. (1988) J. Biol. Chem. **263**, 8696–8705
57 Cooper, D. R., de Ruiz Galaretta, C. M., Fanjul, L. F., Mojsilovic, L., Staendaert, M. L., Pollet, R. J. and Farese, R. V. (1987) FEBS Lett. **214**, 122–126

Received 19 June 1992/1 September 1992; accepted 11 September 1992