Heterologous Desensitization of the Glucagon-like Peptide-1 Receptor by Phorbol Esters Requires Phosphorylation of the Cytoplasmic Tail at Four Different Sites*

Christian Widmann, Wanda Dolci, and Bernard Thorens†
From the Department of Pharmacology and Toxicology, University of Lausanne, Rue du Bugnon 27, 1005 Lausanne, Switzerland

Glucagon-like peptide-1 (GLP-1) is an intestinal peptidic hormone that is secreted in the blood in response to nutrient ingestion. One of its major functions is to potentiate the glucose-dependent secretion of insulin by pancreatic β cells (1-3). This effect requires the binding of GLP-1 to a specific G-protein coupled receptor that is linked to the activation of the adenyl cyclase pathway. The effect of GLP-1 on insulin secretion depends on the presence of glucose concentrations equal to or greater than the normoglycemic value of ~5 mM. This hormone is thus a potentiator of the glucose signaling pathway, and its action is mediated by the production of cAMP and, probably, the activation of protein kinase A (4–6).

We previously demonstrated that the GLP-1 receptor-mediated cAMP response could undergo both homologous (following receptor exposure to its agonist) and heterologous (following the activation of PKC) desensitization (7). Both forms of desensitization were correlated with receptor phosphorylation and internalization. PMA-induced phosphorylation and desensitization but not that induced by GLP-1 were completely suppressed in the presence of the PKC inhibitor RO-318220. Removal of the last 33 amino acids of the carboxyl-terminal cytoplasmic tail of the receptor abolished both homologous and heterologous desensitization and phosphorylation.

Phosphorylation of G-coupled receptors by multiple protein kinases has been shown to be involved in both homologous and heterologous desensitization. For instance, for the β2-adrenergic receptor, heterologous desensitization can be induced by activation of protein kinase A or protein kinase C, an effect that requires the presence of a protein kinase A/PKC consensus site in the third intracellular loop of the receptor but not that present in the C-tail (8–11). On the other hand, homologous desensitization results from receptor phosphorylation by G-coupled receptor-specific kinases (12) that form a family of structurally related isoforms (13). This phosphorylation takes place in the serine/threonine-rich C-tail of the receptor (11) and induces association of the receptor with β-arrestins (14–17). This interaction prevents further activation of G-proteins following agonist binding to the receptor.

GLP-1 and phorbol ester-induced desensitization and phosphorylation of the GLP-1 receptor are additive, and both are suppressed by deletion of the last 33 amino acids of the receptor C-tail (7). This suggests that this receptor portion contains the amino acids that can be phosphorylated in both homologous and heterologous desensitization. These results are in contrast with those obtained with the β2-adrenergic receptor for which heterologous desensitization (protein kinase A- and PKC-mediated) requires phosphorylation in the third cytoplasmic loop, whereas β-adrenergic receptor kinases phosphorylate the C-tail of the receptor. In addition, phosphorylation of the C-tail by β-adrenergic receptor kinases, but not phosphorylation of the third cytoplasmic loop by protein kinase A or PKC, induces arrestin binding to the receptor (9), indicating different mechanisms by which phosphorylation causes desensitization. With the GLP-1 receptor both forms of desensitization correlate with phosphorylation of the same, relatively short, region of the receptor, suggesting that homologous and heterologous desensitization may be mediated by similar mechanisms.

To better understand the molecular mechanisms of hetero-
GLP-1 Receptor Phosphorylation and Desensitization

Phosphorylation of the GLP-1 receptor following activation of protein kinase C in intact cells. We demonstrate that four serine doublets located in the last 33 amino acids of the receptor become phosphorylated upon activation of PKC. Furthermore, we show that phosphorylation of at least three sites is required for desensitization of the receptor, which becomes maximal when all four sites are phosphorylated.

MATERIALS AND METHODS

Cells and Cell Culture—COS cells and Chinese hamster lung (CHL) fibroblasts were cultured as described (5). Clone 5 cell is a CHL fibroblast stably transfected with the rat GLP-1 receptor cDNA (5, 18). Transformation of COS cells and generation of stable CHL transformants were performed as described earlier (5), except that when the cDNAs were inserted in the pcDNA-3 vector (see below), co-transformation of the fibroblasts with the pWLneo plasmid was not required.

Mutagenesis—The different GLP-1 receptor mutants used in this study are described in Fig. 2. The deletion mutant ΔCT431 was described previously (7). The other mutations were generated by polymerase chain reaction amplification as described (19), and each mutant was verified by DNA sequencing. The mutant GLP-1 receptor cDNAs were subcloned in the pcDNA-3 vector (Invitrogen, Leek, The Netherlands) and in the pmlMTIii vector (7). The cDNAs subcloned in the pcDNA-3 and pmlMTIii vectors are under the control of the cytomegalovirus and metallothionein promoters, respectively.

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Phosphorylation Experiments on Intact Cells—PMA-induced phosphorylation of the wild-type or mutant GLP-1 receptors expressed by transiently transfected COS cells was assessed as described earlier (7). When phosphorylation was induced by dioctanoyl-sn-glycerol (Sigma), this compound was added at a concentration of 30 μM for 10 min. The bands corresponding to the phosphorylated GLP-1 receptors were analyzed by densitometry scanning. The extent of phosphorylation (F) of the wild-type and mutant GLP-1 receptors was calculated as follows from the densitometry scanning measures (Dm in arbitrary units) corrected for the cellular content of the culture wells (Pm in μg) and for the cell surface receptor expression (Sm in cpm, derived from saturation binding experiments):

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GLP-1 Receptor Phosphorylation and Desensitization

In Vitro Phosphorylation of Fusion Proteins Containing Wild-type or Mutated Carboxyl-terminal GLP-1 Receptor Sequences—Residues 412–463 of the wild-type GLP-1 receptors and of the last 6 mutant GLP-1 receptors described in Fig. 2 and residues 412–430 of mutant ΔCT431 were fused to the glutathione S-transferase protein encoded by plasmid pGEX-1 (22). This was accomplished by polymerase chain reaction amplification of the cDNAs encoding the wild-type and the mutant GLP-1 receptors using the antisense nucleotide SP6/EcoRI (GGCGAGCT431). Both sense oligonucleotides contain an EcoRI site and the sense oligonucleotide EcoRI-1250 (CAGATGGATTCCGGAGAAGCTGGGAG) or 416EcoRI (CAGATGGATTCCCCAGGGCTTCGAGGCG). The amplified fragments were digested with EcoRI and thus will generate mutation S416A when used (see Fig. 2). The amplified fragments were digested with EcoRI and subcloned into the EcoRI site of the pGEX-1 vector. Each construction was verified by sequencing. The expression and purification of the fusion proteins were performed with a slightly modified version of Smith and Johnson’s method (22). Overnight cultures of Escherichia coli transformed with parental or recombinant pGEX-1 plasmid were diluted 1/10 in 100 ml of LB medium (10 g/liter NaCl, 10 g/liter Tryptone; 5 g/liter yeast extract, pH 7.4) and grown for 1 h at 37°C before adding isopropyl-β-D-thiogalactopyranoside to 0.1 mM. After a further 4–5 h of growth, bacteria were pelleted and resuspended in 10 ml of sonication buffer (50 mM Tris, pH 8, 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml DNase I, 1% Triton X-100) and incubated 10 min at 4°C on a rotating platform. The suspension was then sonicated (1 min, position 7 using the sonicator B-12 (Branson sonic power company, Danbury, Connecticut)), and pelleted at 11,200 × g for 10 min at 4°C. The supernatant was incubated twice with 100 μl of 50% glutathione-agarose beads (G-4510, Sigma, Buchs, Switzerland) for 15 min at 4°C on a rotating platform. The beads were then pooled and washed twice with 10 ml NETN (0.5% Nonidet P-40, 20 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA) and incubated twice with 3 × 5 min washes with 100 μl of elution buffer (20 mM reduced glutathione, 100 mM Tris, pH 8, 120 mM NaCl) and kept at −20°C before use. In vitro PKC-induced phosphorylation of the fusion proteins was performed as described (23), except that 2–3 μg of fusion proteins and 540 nM [γ-32P]ATP were used.

RESULTS

PKC-induced activation of PKC can increase the phosphorylation of the GLP-1 receptor but not that of a truncated mutant lacking the last carboxy-terminal 33 amino acids. To determine which amino acids of the C-tail are phosphorylated following PKC stimulation, fibroblasts expressing the wild-type receptor were labeled with [32P]orthophosphate and were either left untreated (−) or stimulated for 15 min with 400 nM PMA (+). Phosphorylated pGEX-1 receptors were then digested as described in the legend to Fig. 1. All four mutant receptors expressing only one serine doublet were phosphorylated in response to PMA, indicating that each doublet is a phosphoamino acid analysis of the purified receptor revealed that only phosphoserines are present (Fig. 1, right).

The last 33 carboxy-terminal amino acids of the GLP-1 receptor contain 10 serine residues, 8 of which are present as serine doublets. To determine whether the residues phosphorylated in response to PMA may be found within these 4 serine doublets, we have generated a series of receptor mutants in which one or more of the serine doublets were replaced with alanine residues (see Fig. 2). The mutant receptors were then expressed transiently in COS cells and tested for their ability to be phosphorylated in response to PMA. Fig. 3A shows that mutation of all the 4 serine doublets led to the suppression of receptor phosphorylation. In Table I, we present the quantitative determination of the extent of phosphorylation of receptor mutants with substitution to alanines of either single, pairs, or the four serine doublets. These quantitations take into account the efficiency of immunoprecipitation and the cell surface expression of the transfected mutant receptors. These data show that mutation of each serine doublet reduces the extent of phosphorylation by −50% except for the S431A/S432A (Fig. 2, 431AA) mutant for which reduction is −25%. When any pair of serine doublets were substituted with alanines there was a 70–80% reduction in the extent of phosphorylation. Finally, elimination of all 4 serine doublets, either by truncation of the receptor C-tail (ΔCT431) or by substitution of serines with alanine residues, prevented phosphorylation of the receptor.

These data suggest that each serine doublet is phosphorylated following activation of PKC by phorbol esters. To further explore this point, we generated mutants in which only one doublet of serines was left intact. All four mutant receptors expressing only one serine doublet were phosphorylated in response to PMA, indicating that each doublet is a PKC-induced phosphorylation site. In the bottom part of the figure is the average phosphorylation of two different phosphorylation experiments. Error bars represent half of the range of the two experiments.
from amino acids 412 to 463 and containing different replacements of serines to alanines. These were then used in an in vitro PKC phosphorylation assay, and the level of phosphorylation was determined. Fig. 4 shows that the glutathione S-transferase protein was not phosphorylated by PKC (lane 1), whereas the fusion protein containing the wild-type receptor C-tail sequence was phosphorylated (lane 2). When all four serine doublets were substituted with alanines, the fusion protein was still phosphorylated, although to a much lower extent as compared with the wild-type protein (lane 5). A similar situation was observed with the truncated C tail of ΔCT431 (lane 3). Serine 416, which is in a consensus PKC site (see Fig. 2), was responsible for this phosphorylation as demonstrated by the abolition of the phosphorylation when this residue was mutated to alanine together with all four serine doublets (lane 4). Thus, serine 416 is an in vitro PKC phosphorylation site but is not phosphorylated in intact cells in response to PMA (see Table I, ΔCT431 and the quadruplet mutant). To assess the ability of individual serine doublets to be phosphorylated by purified PKC, we have thus mutated serine 416 and combinations of 3 serine doublets to alanine residues so that the resulting fusion proteins bear only one potential site of phosphorylation. As expected, serines 431/432 were very efficiently phosphorylated by PKC (Fig. 4, lane 9). In contrast, the other serine doublets were not (441/442) or only poorly (444/445 and 441/442) phosphorylated by PKC (lanes 5-7). Thus, the 4 serine doublets are phosphorylated in response to PMA in intact cells, whereas only serines 431/432 and 416 are efficiently phosphorylated in vitro by purified PKC.

To understand the role of PKC-dependent phosphorylation in receptor desensitization, we expressed the receptor mutants in different cellular systems: transiently transfected COS cells that express ~10^6 receptors/cell, CHL fibroblasts expressing the GLP-1 receptor under the control of the metallothionein promoter, and CHL fibroblasts stably transfected with GLP-1 receptor cDNAs under the control of the metallothionein promoter. Low expressor fibroblasts are CHL fibroblasts stably transfected with GLP-1 receptor cDNAs under the control of the metallothionein promoter.

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**Table I**

| Mutations | Phosphorylation* of wild-type receptor |
|-----------|---------------------------------------|
| Wild type | 100                                   |
| ΔCT431    | 1 ± 3 (n = 7)                         |
| S416A     | 95 ± 8 (n = 4)                        |
| S451A     | 51 ± 17 (n = 3)                       |
| S444A     | 48 ± 2 (n = 3)                        |
| S441A     | 45 ± 7 (n = 5)                        |
| S431A     | 75 ± 12 (n = 7)                       |
| S431A/S444A | 20 ± 4 (n = 3)                      |
| S431A/S444A | 33 ± 3 (n = 3)                      |
| S431A/S451A | 30 ± 9 (n = 4)                      |
| S441A/S444A | 26 ± 2 (n = 3)                      |
| S431A/S444A/S451A | 2 ± 4 (n = 3)          |

*The extent of phosphorylation of the mutant GLP-1 receptors, calculated as described under "Materials and Methods," was normalized to the corresponding value obtained for the wild-type receptor. The results are expressed as the means ± S.E. for the indicated number of experiments.

**Table II**

| Mutations | Clones (number) | Receptor number per cella | V_m1 of PMA-treated cellsb |
|-----------|-----------------|---------------------------|-----------------------------|
| Wild type | MTI-GLPR (3)    | 2,500 ± 300                | 46.8 ± 1.4                  |
| ΔCT431    | MTI-ΔCT431(28)  | 3,000 ± 800                | 35.9 ± 3.3                  |
| S451A     | MTI-451AA (3)   | 2,300 ± 400                | 61.9 ± 2.2                  |
| S444A     | MTI-444AA (4)   | 3,300 ± 400                | 50.4 ± 5.1                  |
| S441A     | MTI-441AA (8)   | 1,300 ± 200                | 68.0 ± 2.8                  |
| S431A     | MTI-431AA (2)   | 2,400 ± 100                | 65.9 ± 4.6                  |
| S441A/S444A | 5,300 ± 300    | 71.9 ± 4.2                  |
| S431A/S444A | 11,200 ± 900   | 98.9 ± 5.0                  |
| S431A/S441A | 6,600 ± 400    | 100.0 ± 0.3                 |
| S431A/S451A | 4,200 ± 600    | 107.9 ± 9.3                 |
| S431A/S441A | 1,300 ± 600    | 75.9 ± 3.4                  |
| S431A/S444A | 4,500 ± 400    | 91.7 ± 1.8                  |
| S431A/S441A | 7,800 ± 3,900  | 85.7 ± 13.7                 |
| S431A/S451A | 3,100 ± 400    | 88.6 ± 13.2                 |
| S431A/S51AA | 1,700 ± 400    | 96.5 ± 5.1                  |

aGLP-1 receptor cell surface expression was determined by saturation binding experiments. The results are expressed as the average of two experiments. Error values are the half range of the two experiments.

bDose-response curves such as those presented in figure 5, middle panels were analyzed with the KaleidaGraph v2.1 software (Abelbeck Software) and fitted with \( y = m_1/(x + m_2) \) where \( y \) = CAMP production; \( x \) = GLP-1 concentration; \( m_1 \) = estimated maximal increase in CAMP production; \( m_2 \) = estimated EC50 of the curve; \( m_3 \) = estimated basal response. The parameter \( m_1 \) was defined as the maximal agonist stimulation (Vmax). The results are expressed as the maximal GLP-1-induced CAMP production in treated cells relative to untreated cells average of two experiments ± half range of the two experiments.

cFrom Ref. 7.
EC_{50}. On the other hand, as expected for cells expressing low levels of receptors, the desensitization in low expressor fibroblasts results mainly in the reduction in the maximal GLP-1-mediated cAMP response. For DCT431 receptor mutant expressed in all three cell types, no desensitization could be induced by PMA (Fig. 5, lower panels). These findings indicate that PMA-induced desensitization of the GLP-1 receptor could be measured both in permanent and transient expression systems. Thus, we further characterized the desensitization properties of the phosphorylation site mutants expressed in fibroblasts (low expressors) and in COS cells.

The desensitization of the GLP-1-mediated cAMP production was tested in low expressor fibroblasts for each mutant receptor using two different clones to limit the effect of clonal variations. Table II shows for each clone the cell surface receptor expression and the remaining maximal cAMP production induced by GLP-1 (V_{max}) after PMA preincubation. In wild-type-expressing cells, PMA induced a 60% reduction in the V_{max} as compared with untreated cells. The reduction was less marked (30–50%) in cells expressing receptors with single serine doublet substitution. When mutant receptors with two substituted doublets were studied, desensitization could no longer be observed, as was the case for the DCT431 mutant that lacked all 4 serine doublets.

Fig. 6 shows representative experiments performed with transiently transfected COS cells to determine the dose-response curves for PMA-induced desensitization of the wild-type receptor (top panel), the receptor with mutation of residues 431/432 to alanines (middle panel), and a double mutant (431AA/451AA, lower panel). Desensitization of the wild-type receptor is measured as a shift in the EC_{50} of 2.4 ± 0.2-fold (mean ± S.E., n = 3). When single mutants were tested, the shift was still about 2-fold (mutant S431A, 2.3-fold (Fig. 6); S444A, 1.11-fold; S451A, 2.4-fold). With mutants carrying two doublet substitutions, the shift in EC_{50} was reduced to about 1.3-fold (mutant S431A/S451A, 1.2-fold (Fig. 6); range 1.2- to 1.4-fold for four other different double mutants tested). These results indicated that at least three phosphorylation sites must be present in order to induce a measurable desensitization of the receptor and that further mutation of any other serine doublet led to receptor that could not be desensitized. These results are in agreement with the experiments performed in fibroblasts.

To determine whether physiological activators of protein kinase C were able to induce receptor phosphorylation and desensitization, we performed two kinds of experiments. First, we transiently transfected COS cells with the GLP-1 receptor and exposed the [32P]orthophosphate labeled cells to dioctanoyl-sn-glycerol for 10 min. Following immunoprecipitation, the receptor was analyzed by gel electrophoresis as described above. Fig. 7 shows that diacylglycerol induced wild-type receptor phosphorylation and that phosphorylation was abolished when all the identified PKC phosphorylation sites were mutated to alanines. Secondly, we showed that activation of the thrombin receptor, which is coupled to activation of the phospholipase C pathway (5), led to a marked desensitization of the GLP-1-dependent cAMP response in GLP-1 receptor-transfected fibroblasts (Fig. 8). Together these data demonstrate that activation of PKC in intact cells with a cell-permeable diacylglycerol or by an agonist of a phospholipase C-coupled receptor, reproduces the effects observed with PMA.
DISCUSSION

GLP-1 binding to its pancreatic β cells receptor potentiates glucose-induced insulin secretion by stimulation of the adenylyl cyclase pathway. We have previously demonstrated that the GLP-1-mediated cAMP response could be desensitized in an agonist-dependent manner as well as following the activation of protein kinase C by PMA. Both homologous and heterologous desensitization were correlated with phosphorylation of the GLP-1 receptor carboxyl-terminal tail.

Here, using a set of receptor mutants expressed in COS cells, we have identified the sites of the GLP-1 receptor that are phosphorylated following activation of PKC. We determined that four serine doublets located in the C-tail of the receptor at positions 431/432, 441/442, 444/445, and 451/452 were phosphorylated in intact cells following PMA treatment of the cells. This was inferred from two sets of observations. First, mutation to alanines of any single serine doublet markedly reduced the extent of phosphorylation, indicating that each doublet was phosphorylated. Second, each of the receptor mutants with a single serine doublet left could be phosphorylated after activation of PKC. Interestingly, the level of phosphorylation was not strictly correlated with the number of phosphorylation sites present, because removal of a single serine doublet led to a reduction in phosphate incorporation by about 50%. This therefore suggests some cooperativity in the phosphorylation process, as reported previously for several other proteins (25).

Among the four different phosphorylation sites only one, serines 431/432, is a classical phosphorylation site for PKC. To determine whether the identified phosphorylation sites are actually PKC substrates, we performed in vitro phosphorylation reactions on fusion proteins containing the different mutated forms of the receptor. These experiments indicated that the in vitro phosphorylation sites are at least in part different. This may result from the existence of different isoforms of PKC in COS cells as compared with the bovine brain kinase preparation. Alternatively, the conformation of the native receptor prevents phosphorylation of consensus sites by PKC such as serine 416. Still another possibility is that PKC may activate other kinases for which the receptor is a substrate. In that respect, it has recently been

FIG. 6. Desensitization of wild-type and mutant GLP-1 receptors transiently transcribed in COS cells. COS cells were transiently transfected with plasmid encoding the indicated receptor forms. A dose-response curve for GLP-1-induced cAMP production was generated after treatment of the cells with or without 400 nM PMA. The shift in EC_{50} was then determined and was 2.6-fold for wild-type GLP-1 receptor, 2.3-fold for the S431A mutant and 1.2-fold for the S431A/S451A mutant. Similar data were obtained with the other single and double receptor mutants described in the legend to Fig. 2.

FIG. 7. Activation of protein kinase C by dioctanoyl-sn-glycerol induces receptor phosphorylation. COS cells transfected with plasmids encoding the indicated GLP-1 receptors (w.t. indicates wild-type receptor; 4x indicates all four serine doublets mutated to alanines) were labeled with [32P]orthophosphate and were either left untreated (-) or stimulated for 10 min with 30 μM dioctanoyl-sn-glycerol (DiC8) (+). The cells were then lysed and immunoprecipitated with receptor-specific antibodies. The immunoprecipitated proteins were detected following gel electrophoresis and autoradiography.

FIG. 8. Desensitization of the GLP-1 receptor expressed in fibroblasts by thrombin. Fibroblasts expressing the wild-type GLP-1 receptor (clone M71-GLPR number 3) were exposed or not for 10 min to 10 units of thrombin. The cells were then stimulated with the indicated concentrations of GLP-1, and the accumulation of cAMP was measured as described in the legend to Fig. 5. Pre-exposure of the cells to thrombin induced a reduction in the maximal production of cAMP by 28%.

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demonstrated that the β-adrenergic receptor kinase-1 can be activated by phosphorylation by protein kinase C (26). However, for β-adrenergic receptor kinase-1 to phosphorylate the receptor, the receptor has to be in agonist-bound form. In our conditions, receptor phosphorylation after treatment with phorbol esters is observed independently of agonist binding, suggesting that if another kinase is activated by PKC it may be different from β-adrenergic receptor kinase-1.

Our findings provide strong evidence that phorbol ester-induced phosphorylation is strictly related to desensitization of the GLP-1 receptor expressed both in fibroblasts and COS cells. This was shown, in fibroblasts, by the fact that removal of any pair of serine doublets created receptors that were totally resistant to the desensitization, whereas mutation of any one of the four serine doublets led to a receptor that was still desensitized, although to a reduced extent as compared with the wild-type receptor. Because the receptor mutants lacking one and two serine doublets are phosphorylated to Ϫ50 and Ϫ25%, respectively, of the wild-type receptor, it appears that a threshold of phosphorylation comprised between 25 and 50% of the maximal phosphorylation is required to mediate the desensitization induced by PMA.

In COS cells, the results were similar to those obtained in fibroblasts. Deletion of any pair of serine doublets led to receptors that were no longer desensitized. Mutation of a single phosphorylation sites led to a small reduction in the shift of EC50, although it is difficult, with the small variations in EC50 obtained, to have very significant values between those of the wild-type receptor and those of the desensitization-resistant mutants. However, these data confirm that maximal desensitization is obtained only when all the four serine doublets can be phosphorylated and that phosphorylation on two sites is not sufficient for desensitization to be observed.

Importantly, we demonstrated that physiological activators of protein kinase C such as the diacylglycerol dioctanoyl-sn-glycerol or thrombin can induce phosphorylation and desensitization of the receptor to a similar extent as observed with PMA. Our present data using PMA as an activator of PKC are thus relevant to more physiological situations in which a GLP-1 receptor-expressing cells is subjected to activation of PKC by agonists of phospholipase C-coupled receptors.

We previously reported that agonist-induced desensitization of the receptor also involves phosphorylation of the receptor on the same last 33-amino acid segment of the receptor C-tail. Furthermore, PKC and agonist-induced desensitization and phosphorylation were additive, and the phosphorylation induced by PMA was blocked by the RO-318220 inhibitor, whereas that induced by agonist binding was not impaired. This suggests that the phosphorylation sites involved in homologous versus heterologous desensitization are different. In the present work we have not determined which individual serine of the different doublets were phosphorylated in response to PKC activation. It is possible that different residues on each doublet are phosphorylated in homologous and heterologous desensitization. The C-tail of the receptor also contains two more serine residues at position 461 and 463, which could potentially be phosphorylated. It will thus be important to determine which serine residues are phosphorylated by the agonist-activated kinase and whether this kinase is one of the already characterized G-coupled receptor-specific kinases (27).

There are a number of hormones and neurotransmitters, such as acetylcholine, that can activate PKC in pancreatic β cells and thus stimulate insulin secretion. We have previously suggested that these secretagogues induce desensitization of the GLP-1 receptor in order to prevent overstimulation of insulin secretion and thus hypoglycemia that could occur in the combined presence of these secretagogues and GLP-1. However, our data indicate that at least three serine doublets must be phosphorylated to induce some desensitization but that maximal desensitization is achieved only when all four doublets are phosphorylated. What could be the biological significance of these observations? Glucose-induced secretion is a very tightly regulated mechanism, and insulin oversecretion should not occur. Thus when an agonist only moderately activates the phospholipase C pathway and, consequently, mildly stimulates insulin secretion, no desensitization of the GLP-1 receptor is needed and partial phosphorylation of the receptor leaves the receptor fully active. Only in conditions of strong stimulation of the PLC pathway will the receptor be fully phosphorylated and desensitized to prevent overstimulation of insulin secretion by increasing GLP-1 plasma levels. This finely regulated phosphorylation and desensitization of the GLP-1 receptor may thus have evolved to tightly control insulin secretion by a variety of hormones that are key in the complex control of glucose homeostasis.