Glucagon-like Peptide 1 Activates Protein Kinase C through Ca\(^{2+}\)-dependent Activation of Phospholipase C in Insulin-secreting Cells*

Revised for publication, May 4, 2006, and in revised form, July 21, 2006. Published, JBC Papers in Press, July 26, 2006. DOI 10.1074/jbc.M604291200

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Received for publication, May 4, 2006, and in revised form, July 21, 2006. Published, JBC Papers in Press, July 26, 2006. DOI 10.1074/jbc.M604291200

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Although the stimulatory effect of glucagon-like peptide 1 (GLP-1), a CAMP-generating agonist, on Ca\(^{2+}\) signal and insulin secretion is well established, the underlying mechanisms remain to be fully elucidated. We recently discovered that Ca\(^{2+}\) influx alone can activate conventional protein kinase C (PKC) as well as novel PKC in insulin-secreting (INS-1) cells. Building on this earlier finding, here we examined whether GLP-1-evoked Ca\(^{2+}\) signaling can activate PKC\(\alpha\) and PKC\(\epsilon\) at a substimulatory concentration of glucose (3 mM) in INS-1 cells. We first showed that GLP-1 translocated endogenous PKC\(\alpha\) and PKC\(\epsilon\) from the cytosol to the plasma membrane. Next, we assessed the phosphorylation state of the PKC subfamily, myristoylated alanine-rich C kinase substrate (MARCKS), by using MARCKS-GFP. GLP-1 translocated MARCKS-GFP to the cytosol in a Ca\(^{2+}\)-dependent manner, and the GLP-1-evoked translocation of MARCKS-GFP was blocked by PKC inhibitors, either a broad PKC inhibitor, bisindolylmaleimide I, or a PKC\(\epsilon\) inhibitor peptide, antennapedia peptide-fused pseudosubstrate PKC\(\epsilon\)-(149–164) (antp-PKC\(\epsilon\)) and a conventional PKC inhibitor, Gö-6976. Furthermore, forskolin-induced translocation of MARCKS-GFP was almost completely inhibited by U73122, a putative inhibitor of phospholipase C. These observations were verified in two different ways by demonstrating 1) forskolin-induced translocation of the GFP-tagged C1 domain of PKC\(\gamma\) and 2) translocation of PKC\(\epsilon\)-DsRed and PKC\(\epsilon\)-GFP. In addition, PKC inhibitors reduced forskolin-induced insulin secretion in both INS-1 cells and rat islets. Thus, GLP-1 can activate PKC\(\epsilon\) and PKC\(\epsilon\), and these GLP-1-activated PKCs may contribute considerably to insulin secretion at a substimulatory concentration of glucose.

Glucagon-like peptide 1 (GLP-1)\(^2\) is an insulinotropic peptide that is known as “incretin,” a gastrointestinal hormone released from the intestinal L cell in response to a meal or an oral glucose challenge. Upon binding to its receptor, GLP-1 increases cAMP levels via G-protein-coupled activation of adenylate cyclase, leading to activation of protein kinase A (PKA) (1, 2). One of the mechanisms by which GLP-1 potentiates glucose-induced insulin secretion from the pancreatic \(\beta\)-cells is to modulate Ca\(^{2+}\) signaling in several ways: 1) GLP-1 induces membrane depolarization by the closure of ATP-sensitive K\(^+\) channels (K\(_{ATP}\) channels) and/or by the opening of cAMP-operated nonselective cation channels, eliciting Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCCs) (3); 2) PKA-dependent phosphorylation of L-type VDCCs increases the integrated Ca\(^{2+}\) current by slowing the time course of inactivation and augmenting the Ca\(^{2+}\) current (2, 4); and 3) GLP-1 promotes Ca\(^{2+}\) mobilization from Ca\(^{2+}\) stores (5). In addition to the processes mentioned above, which increase the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), other roles of GLP-1 as a Ca\(^{2+}\) modulator are gradually being elucidated.

Protein kinase C (PKC) plays a role in insulin secretion that is equally important as that of cAMP/PKA signaling. Among 10 identified PKCs, conventional PKC (cPKC; PKC\(\alpha\), PKC\(\beta\), PKC\(\beta\)II, and PKC\(\gamma\)) is activated by Ca\(^{2+}\) and diacylglycerol (DAG), and novel PKC (nPKC; PKC\(\delta\), PKC\(\epsilon\), PKC\(\eta\), and PKC\(\theta\)) is activated by DAG in a Ca\(^{2+}\)-independent manner (6–8). In general, DAG is thought to be a product of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) hydrolysis. This hydrolysis is caused by activation of phospholipase C (PLC) following agonist binding to a G-protein-coupled receptor. We have recently demonstrated a new mechanism by which Ca\(^{2+}\) influx alone, via VDCCs, can generate DAG (9). This occurs through Ca\(^{2+}\)-dependent PLC activation, leading to activation of PKC\(\alpha\) and PKC\(\theta\) as representatives of cPKC and nPKC in INS-1 cells, which are an insulin-secreting cell line. An additional line of evidence shows that GLP-1 increases not only levels of cAMP but also levels of inositol 1,4,5-trisphosphate (IP\(_3\)), the other product of PIP\(_2\) hydrolysis (2, 5, 10). These observations prompted us to investigate phospholipase C; VDCC, voltage-dependent Ca\(^{2+}\) channel; IP\(_3\), inositol 1,4,5-trisphosphate; GFP, green fluorescent protein; MARCKS, myristoylated alanine-rich C kinase substrate; DIC\(_{\alpha}\), 1,2-diacylglyceryl-3athyloxyl-5-acyl; BIS, bisindolylmaleimide I; ACh, acetylcholine; 8-bromo-cAMP, 8-bromoadenosine 3’5’-cyclic monophosphate; TIRFM, total internal reflection fluorescence microscopy; KRb, Kreb’s-Ringer buffer; 2-APB, 2-aminoethoxydiphenyl borate.
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whether GLP-1 can activate both cPKC and nPKC in a Ca$^{2+}$-dependent manner.

Recent advances in the use of fluorescent proteins, such as green fluorescent protein (GFP) and red fluorescent protein (DsRed), have allowed us to investigate PKC activity in intact living cells by monitoring translocation of GFP-tagged PKCs and related proteins (11–14). Using this approach, we have established the following fusion proteins as markers of PKC activity in INS-1 cells: 1) fluorescent protein-tagged PKCs, PKCα-GFP (DsRed), and PKCe-GFP; 2) the C1 domain of PKCγ-GFP (C1γ-GFP) for DAG binding as a DAG biosensor; and 3) myristoylated alanine-rich C kinase substrate (MARCKS)-GFP as a substrate of PKC. These markers enable us to probe many aspects of the mechanisms of GLP-1-evoked PKC activation using epifluorescence microscopy and total internal reflection fluorescence microscopy (TIRFM).

The present study was conducted to examine whether GLP-1 activates PKCα and PKCe at a substimulatory concentration of glucose. Among the multiple PKC isoforms expressed in pancreatic β cells, these two proteins are likely to play a dominant role in glucose-induced insulin secretion (15, 16). The roles of PKCα and PKCe in forskolin-induced insulin secretion were also evaluated in INS-1 cells and rat islets. Here we provide fresh evidence that GLP-1 can activate PKCα and PKCe through Ca$^{2+}$-dependent activation of PLC, suggesting that GLP-1-evoked PKC activation contributes significantly to basal insulin secretion.

EXPERIMENTAL PROCEDURES

Plasmid Construction

PKCα-pEGFP and pDsRed1-N1 were obtained from Clontech (Palo Alto, CA). pEGFP of PKCα-pEGFP was replaced with pDsRed1-N1. The plasmids encoding PKCe-GFP, MARCKS-GFP, and C1γ-GFP were prepared as described previously (9, 17).

Cell Culture and Transfection

Insulin-producing INS-1 cells were a gift from Dr. Sekine (Tokyo University) (18). The cells were grown in 100-mm culture dishes at 37 °C and 5% CO$_2$ in a humidified atmosphere. The culture medium was RPMI 1640 (Sigma) with 10 mM glucose supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1 mM L-glutamine, and 50 μM mercaptoethanol. For fluorescence imaging, the cells were cultured on a 35-mm glass bottom dish (Asahi Techno Glass, Japan) at 50% confluence 2 days before transfection. A plasmid encoding the GFP or red fluorescent protein (GFP) and red fluorescent protein (DsRed), and PKC inhibitors, bisindolylmaleimide I (BIS) and Gö-6976, U73122, U73433, and ionomycin, 1,2-dioctyl-sn-glycerol (DiC$_8$), 8-bromo-cAMP, and forskolin were purchased from Sigma. Fura2-AM (hereafter termed Fura2) was from Molecular Probes, Inc. (Eugene, OR). Glucagon-like peptide 1 (human, 7–36 amide) was obtained from the Peptide Institute, Inc. (Osaka, Japan). Anti-PKCα (H-7) and anti-nPKCe (C-15) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PKC inhibitors, bisindolylmaleimide I (BIS) and Gö-6976, U73122, U73433, and 2-aminoethoxydiphenyl borate (2-APB) were from Calbiochem. All other chemicals were from Sigma. A PKC-ε inhibitor peptide, antennapedia-PKC-(149–164) (antp-PKCe) (RMKW KKERM RPRKR QGAVR RRV), was synthesized by Takara Shuzo Co., Ltd. (Tokyo, Japan). It is a tandemly synthesized peptide comprising peptides from the third α-helix of the homeodomain of antennapedia (residues 52–58, known as penetratin) (19, 20) and the PKC-ε pseudosubstrate peptide (residues 149–164 (21, 22). Antennapedia (antp), used as a control, was also synthesized by Takara Shuzo.

Imaging Experiments

Epifluorescence Microscopy—Fluorescence images were captured at 5-s intervals using an Olympus inverted microscope (numerical aperture = 1.35, ×40, oil immersion objective) equipped with a cooled (−20 °C) coupled charge device digital camera (ORCA-ER, Hamamatsu Photonics, Hamamatsu, Japan) and recorded and analyzed on an Aquacosmos imaging station (Hamamatsu Photonics). The excitation light source was a 150-watt xenon lamp with a high speed scanning polychromatic light source (C7773; Hamamatsu Photonics). GFP fluorescence was excited at 488 nm, whereas Fura2 for Ca$^{2+}$ measurement was excited at wavelengths alternating between 340 and 380 nm. The emitted light was collected through a 535/45-nm band pass filter with a 505-nm dichroic mirror, and a short pass filter of 330–495 nm was used to reduce background fluorescence between the dichroic mirror and the emission filter, allowing simultaneous measurement of GFP and Fura2 fluorescence. We measured the fluorescence intensity of the GFP-tagged proteins in the cytosol of the cell, excluding the nucleus as a marker of translocation. These values (F) were normalized to each initial value (F$_0$) so that the relative fluorescence change was referred to as F/F$_0$. The cells transiently expressing GFP-tagged proteins were loaded with 2 μM Fura2 in the standard extracellular solution for 30 min at room temperature. We previously confirmed that we can distinguish...
between GFP and Ca\(^{2+}\) signals under this experimental condition (9). The cells were washed twice and used within 2 h. The Fura2 ratio was calibrated by exposure to 10 μM ionomycin and 10 mM Ca\(^{2+}\) or 10 mM EGTA in the Fura2-loaded cells that were not transfected with the GFP-tagged proteins (\(F_{\text{max}} = 4.75, F_{\text{min}} = 0.47, \beta = 9\)). A dissociation constant of 150 nM for Ca\(^{2+}\) and Fura2 at room temperature was used (23).

**TIRFM or Evanescent Wave Microscopy**—To obtain a high signal-to-noise ratio as compared with conventional epifluorescence microscopy, we installed a TIRFM unit (Olympus) into the same imaging system described above. The incident light was introduced from the objective lens for TIRFM (Olympus; numerical aperture = 1.45, ×60) to generate the electromagnetic zone or so-called “evanescent field.” The evanescent wave selectively excites fluorophores within 100 nm of the glass-water interface, which enabled us to monitor fluorescent proteins at and/or beneath the plasma membrane of a cell. GFP and DsRed were excited by a 488-nm laser, and the light emitted was collected through 535/45- and 605/50-nm emission filters, respectively. For simultaneous measurement of the relative fluorescence intensity changes of PKCα-DsRed and PKCe-GFP, the signals from GFP and DsRed excited by a 488-nm laser were collected simultaneously through an emission splitter (W-view; Hamamatsu Photonics) equipped with a 550-nm dichroic mirror and two emission band pass filters, 535/45 and 605/50 nm.

**Immunocytochemistry**

INS-1 cells cultured on a coverslip at about 80% confluence were preincubated with KRB containing 3 mM glucose for 1–2 h and then either not treated (as a control) or treated with GLP-1 in buffer for 10 min. They were fixed with 3% paraformaldehyde in PBS for 30 min and permeabilized by 0.1% Triton X-100 for 10 min. The cells were blocked with 2% bovine serum albumin in buffer for 10 min. They were fixed with 3% paraformaldehyde and then either not treated (as a control) or treated with GLP-1

**Measurement of Insulin Secretion**

Male Wistar rats (200–250 g) were obtained from Imai Animal Company (Saitama, Japan). Pancreatic islets were isolated by digestion with collagenase (Wako Pure Chemical Industries, Tokyo, Japan) (24). Islets were detected by inspection under a microscope. Insulin secretion from pancreatic islets was measured in a static incubation system as described previously (25). Insulin secretion in INS-1 cells was measured using an enzyme-linked immunosorbent assay insulin kit (Seikagaku Corp., Tokyo, Japan). INS-1 cells were subcultured in 35-mm dishes and grown up to 80–90% confluence for 3–4 days. INS-1 cells and freshly isolated islets for each experimental group (5 size-matched islets/group) were preincubated in KRB buffer containing 3 mM glucose at 37 °C in a humidified incubator. The solution was then replaced with KRB alone or KRB containing various test agents. BIS and Gö-6976 were added directly with secretion solution, whereas antp-PKCe was added 1 h prior to the insulin secretion experiment. The stimulation time was carefully adjusted to standardize the times for solution changing and sample collection. The experiments were terminated by withdrawal of the supernatant solution after 1 h of incubation. The supernatant was then placed on an ice bath. Samples were kept at −20 °C until the insulin assay was performed. All samples were assayed in duplicate. Insulin concentration in rat islets was determined using a time-resolved immunofluorometric assay system as described previously (26).

**Statistical Analysis**

Data are given as means ± S.E. Statistical significance was evaluated using Student’s t test for paired observations.

**RESULTS**

GLP-1 Triggers as Well as Amplifies Ca\(^{2+}\) Oscillation at a Substimulatory Concentration of Glucose—We first examined the temporal profile of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in response to GLP-1 (100 nM) in Fura2-loaded INS-1 cells. To reduce the effect of glucose on GLP-1 receptor-mediated signal transduction as much as possible, we used a standard extracellular solution containing 2.5 mM Ca\(^{2+}\) and 3 mM glucose, which is a substimulatory concentration for electrical activity and insulin secretion. In this condition, GLP-1 (100 nM) triggered and amplified [Ca\(^{2+}\)]\(_i\), oscillations in, respectively, more than 20 and 50% of the Fura2-loaded INS-1 cells (\(n = 107\)) (Fig. 1 and Table 1). This result is consistent with observations that GLP-1 alone can generate a Ca\(^{2+}\) signal at a substimulatory concentration of glucose in βTC cells and MIN6 cells (3, 27) and that GLP-1 induces action potentials in electrically quiescent rat β cells following a 10-min exposure to a glucose-free solution (4). Application of 10 μM forskolin (\(n = 122\)) and 1 mM 8-bromo-cAMP (\(n = 150\)) yielded similar results (data not shown), confirming that GLP-1-initiated Ca\(^{2+}\) signaling occurs downstream of adenylate cyclase in INS-1 cells.
Translocation of Endogenous PKCα and PKCe from the Cytosol to the Plasma Membrane in Response to GLP-1—We have recently demonstrated that depolarization-evoked Ca^{2+} influx via VDCCs can activate cPKC and nPKC through Ca^{2+}-dependent PLC in INS-1 cells (9). This prompted us to investigate whether GLP-1-induced Ca^{2+} signaling activates cPKC as well as nPKC. Thus, we examined translocation of endogenous PKCα and PKCe as representatives of cPKC and nPKC. Among the multiple PKC isoforms expressed in pancreatic β cells, PKCα and PKCe are likely to play a dominant role in glucose-induced insulin secretion (15, 16, 28). An immunocytochemical assay clearly showed that 100 nM GLP-1 in the presence of 3 mM glucose induced translocation of endogenous PKCα and PKCe from the cytosol to the plasma membrane; this translocation is a marker of PKC activation (Fig. 2), indicating that GLP-1 can activate the two PKCs at a substimulatory concentration of glucose.

GLP-1 Translocates MARCKS-GFP from the Plasma Membrane to the Cytosol—We next employed GFP-tagged MARCKS (MARCKS-GFP), a putative substrate for PKC (29), as another temporal marker of PKC activation in order to substantiate our above finding of GLP-1-induced activation of PKC in a living cell. When activated PKC phosphorylates plasma membrane-anchored MARCKS, the phosphorylated form of MARCKS translocates from the plasma membrane to the cytosol (30). This translocation is accompanied by reciprocal changes in the fluorescence intensities of MARCKS-GFP in the cytosol and at the plasma membrane (9). Thus, we measured the relative fluorescence change of MARCKS-GFP in the cytosol and at the plasma membrane; this translocation is a marker of PKC activation (Fig. 2), indicating that GLP-1 can activate the two PKCs at a substimulatory concentration of glucose.

**TABLE 1**

Effect of GLP-1, forskolin, and 8-bromo-cAMP on [Ca^{2+}]_{i} at a substimulatory concentration of glucose

| Agonist         | Fraction of INS-1 cells responding to GLP-1, forskolin, and 8-bromo-cAMP |
|-----------------|--------------------------------------------------------------------------|
| GLP-1 (100 nM)  | 24 of 107 (20.5%) 57 of 107 (54%)                                      |
| Forskolin (10 μM) | 21 of 122 (17.2%) 80 of 122 (65.6%)                                   |
| Br-cAMP (1 mM)  | 21 of 150 (14%) 114 of 150 (76%)                                      |

The base-line level of [Ca^{2+}]_{i}, was 110 ± 3 nM (n = 33 from three different experiments, mean ± S.E.). Only cells exhibiting an increase of ≥90 nM over the base-line level were counted as amplifications. Forskolin and 8-bromo-cAMP results were similar, indicating that the GLP-1-initiated Ca^{2+} signal originates downstream of adenylyl cyclase.

**FIGURE 2.** GLP-1 induces translocation of endogenous PKCα and PKCe from the cytosol to the plasma membrane in INS-1 cells. This immunocytochemical study was performed using antibodies against PKCα and PKCe with stimulation of GLP-1 (100 nM) for 10 min in KRB containing 3 mM glucose. The distribution of PKCα and PKCe was observed under control conditions (left panel) and after GLP-1 stimulation (right panel). GLP-1-induced translocation of the two PKCs (PKCα (upper panel) and PKCe (lower panel)) to the plasma membrane is shown. Scale bar, 10 μm. Representative images are shown from six independent experiments.

**PKCα control**

**PKCα GLP-1**

**PKCe control**

**PKCe GLP-1**

MARCKS-GFP (referred to as either M5 or M6 cells), more than 80% of which responded to GLP-1. Fig. 3B shows that the amplitude of MARCKS translocation induced by GLP-1 was comparable with that elicited by either acetylcholine (ACh; 100 μM) or a depolarizing concentration of potassium (40 mM K^{+}) (n = 28), both of which might produce sufficient DAG to activate PKC (9). First, we tested whether a broad PKC inhibitor, BIS (1 μM), blocks GLP-1-evoked MARCKS translocation. In contrast to the data in Fig. 3B, neither GLP-1 nor ACh induced MARCKS translocation, despite the [Ca^{2+}]_{i} elevation in the BIS-treated M5 cells (n = 20; control n = 24) (Fig. 3C). We then plotted the GLP-1-evoked increases in the relative fluorescence intensities from MARCKS-GFP in the cytosol (df_{MAR}) against the peak values of [Ca^{2+}]_{i} in the absence or presence of BIS. Fig. 3D clearly shows that GLP-1-evoked df_{MAR} increased with the peak [Ca^{2+}]_{i} elevation, whereas there was little change in GLP-1-evoked df_{MAR}, irrespective of the peak [Ca^{2+}]_{i} elevation in the BIS-treated M5 cells. We further examined the effect of two isoform-specific PKC inhibitors, Gő-6976 (31), an inhibitor of conventional PKC, and antp-PKCe (14), an inhibitor of PKCe, on GLP-1-evoked df_{MAR}; antp-PKCe enables the pseudosubstrate to be loaded into intact cells. These two inhibitors, Gő-6976 and antp-PKCe, have been previously shown to inhibit phosphorylation of MARCKS at 1 and 75 μM, respectively (14). Fig. 3E shows that neither 1
GLP-1-induced MARCKS translocation was synchronous with 
Ca$$^{2+}$$, the relative fluorescence intensity of MARCKS-GFP (△)
was translocated from the plasma membrane to the cytosol, the relative fluorescence intensity of MARCKS-GFP (∙) increased.

FIGURE 3. GLP-1 translocates MARCKS-GFP from the plasma membrane to the cytosol, and GLP-1-evoked translocation of MARCKS is inhibited by PKC inhibitors. Simultaneous monitoring of MARCKS (closed circles) translocation as a marker of PKC activation and [Ca$$^{2+}$$], (open circles) is shown. Images were taken at the times indicated by the arrows in each image. The region of interest in the cytosol is indicated (white boxes). When MARCKS-GFP was translocated from the plasma membrane to the cytosol, the relative fluorescence of MARCKS-GFP in the cytosol increased. Scale bar, 10 μm. A, GLP-1-induced MARCKS translocation was synchronous with [Ca$$^{2+}$$]. In Fura2-loaded INS-1 cells transiently expressing MARCKS-GFP (n = 15), B, relative change in the fluorescence intensity of MARCKS-GFP in response to GLP-1 (100 nM), ACh (100 μM), and 40 mM K$$^{+}$$ in stable transfectants expressing MARCKS-GFP (M5) (n = 28, seven independent experiments). C, M5 cells were pretreated with Fura2 and 1 μM BIS, a broad PKC inhibitor. GLP-1-evoked MARCKS translocation was greatly suppressed despite the increase in [Ca$$^{2+}$$] in the presence of 1 μM BIS. D and E, scattered plots of the GLP-1-evoked increase in the relative fluorescence intensity of MARCKS-GFP (△) in the cytosol versus the peak value of [Ca$$^{2+}$$]. M5 cells were preincubated with Fura2 and 1 μM BIS (n = 24); asterisk, BIS (n = 20); control (n = 14) blocked it. These observations suggest that a GLP-1-mediated PKC signaling pathway exists.

GLP-1-mediated Ca$$^{2+}$$ Influx and Ca$$^{2+}$$ Mobilization from Intracellular Ca$$^{2+}$$ Stores Contribute to MARCKS Translocation—It is well established that GLP-1 elicits an increase in [Ca$$^{2+}$$], which is derived from Ca$$^{2+}$$ influx and Ca$$^{2+}$$ mobilization from the intracellular Ca$$^{2+}$$ store (5). We evaluated the relative contributions of these Ca$$^{2+}$$ sources to GLP-1-mediated PKC signaling. Fig. 4A shows a representative experiment.
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of this type (n = 45; control, n = 44). Upon removal of extracellular Ca^{2+}, inhibition of the GLP-1-mediated Ca^{2+} influx led to a decrease in the two values: dF_{MAR} and peak Ca^{2+} ratio (Fig. 4B). In addition to removal of the extracellular Ca^{2+}, Ca^{2+} buffering in the cytosol by loading 20 μM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetylomethyl ester (n = 18) resulted in the marked suppression of these two parameters as compared with control cells (Fig. 4B), indicating that GLP-1 induces Ca^{2+}-signal-dependent translocation of MARCKS. We also examined the mechanism by which GLP-1 elicits Ca^{2+} mobilization from intracellular Ca^{2+} stores in the absence of extracellular Ca^{2+}. Simultaneous monitoring of MARCKS translocation and [Ca^{2+}]], revealed that GLP-1-evoked MARCKS translocation and [Ca^{2+}]], elevation took place in 40% of the Fura2-loaded M5 cells examined (Fig. 4A), whereas none of these cells responded to GLP-1 in the presence of 100 μM 2-aminoethoxydiphenyl borate, an IP_{3} receptor antagonist (n = 55; data not shown) (32). This observation supports the idea that, other than Ca^{2+}-induced Ca^{2+} release, the Ca^{2+} that is released in response to GLP-1 is most likely derived from IP_{3}-induced Ca^{2+} release.

**U73122, a Putative PLC Inhibitor (33), Blocks GLP-1-evoked MARCKS Translocation**—GLP-1 increases cAMP via G_{s}-protein-coupled activation of adenylyl cyclase. However, the results obtained above indicated that the main mechanism by which GLP-1 activates the PKC signaling pathway is by mediating signals downstream of AC. To identify other mechanisms by which a cAMP-generating agonist might activate the PKC signaling pathway, we chose to employ forskolin, a direct activator of adenylyl cyclase (34), in these experiments. Fig. 5 shows that, despite the peak in the Ca^{2+} ratio, forskolin-evoked MARCKS translocation was markedly inhibited in U73122-pretreated cells but not in cells treated with U73433, an inactive analog of U73122. This result is entirely consistent with the inhibitory effect of BIS on GLP-1-evoked MARCKS translocation (Fig. 3C), indicating a mechanism of cAMP-mediated activation of PLC.

**Temporal Profiles of PKCα and PKCe Translocation in Response to Forskolin in the Presence or Absence of Ca^{2+} using TIRFM.** PKCα-DsRed and PKCe-GFP were co-transfected into INS-1 cells. A and B, representative data (n = 8) for the relative change in fluorescence intensity (F/F_{0}) of PKCα-DsRed (closed circles) and PKCe-GFP (open circles) at the plasma membrane, showing their translocation in response to forskolin in the presence of Ca^{2+}. A, the entire time course of translocation of the two PKCs in response to forskolin, ACh, and 40 mM K^{+}. Translocation of PKCe induced by ACh was comparable with that in response to 40 mM K^{+}, whereas the F/F_{0} of PKCα in response to 40 mM K^{+} was far larger than that induced by ACh. B, expansion of the area outlined by the dashed box in A. Repetitive translocation of the two PKCs took place in response to 10 μM forskolin, C, forskolin- and ACh-evoked translocation of the two PKCs in Ca^{2+}-free extracellular solution containing 0.2 mM EGTA. Representative data (n = 10) show that transient translocation of PKCe, but not PKCα, was induced by forskolin.
8-bromo-cAMP (n = 3) resulted in similar observations (data not shown). These results indicate that a cAMP-generating agonist can activate the two PKCs. We next tested the effect of forskolin-evoked Ca\(^{2+}\) release on translocation of the two PKCs in the absence of extracellular Ca\(^{2+}\). Five of 10 experiments of this type (Fig. 6C) showed that transient forskolin-evoked translocation of PKCe took place without concomitant transfer of PKCa, whereas translocation of both PKCs occurred in two of 10 experiments. This suggests that, although the two PKCs can be activated in the absence of Ca\(^{2+}\), higher [Ca\(^{2+}\)]\(_i\) is required for activation of PKCa even in the presence of DAG.

Single-cell Measurement of the Forskolin-evoked Increase in DAG Content—We measured the increase in DAG induced by forskolin in single INS-1 cells expressing C1\(_2\)-GFP using TIRFM. An extracellular solution containing the DAG analogue DiC\(_8\) was introduced at the end of each experiment, following stimulation with 10 \(\mu\)M forskolin. Fig. 7 shows a representative experiment in which application of three different concentrations of DiC\(_8\) (1, 3, and 10 \(\mu\)M) resulted in different quasi-steady-state levels of C1\(_2\)-GFP translocation. Because the concentration of DiC\(_8\) inside the cell, at each level, is thought to equilibrate with that outside the cell, the forskolin-evoked increase in DAG concentration was estimated (from the calibration curves of the three experiments) to be 1.44 \(\pm\) 0.48 \(\mu\)M (mean \(\pm\) S.E., \(n = 20\)).

Effect of PKC Inhibitors on Forskolin-stimulated Insulin Secretion in INS-1 Cells and Rat Islets—We examined the effect of 1 \(\mu\)M BIS in INS-1 cells and of Go\ spender 6976 and antp-PKCe in rat islets on forskolin-stimulated insulin secretion at a substimulatory concentration of glucose (3 mM). At a concentration of 1 \(\mu\)M, BIS blocked MARCKS translocation irrespective of an increase in [Ca\(^{2+}\)]\(_i\) (Fig. 3, C and D). As shown in Fig. 8A, forskolin-stimulated insulin secretion was significantly inhibited in BIS-treated cells as compared with control cells. We further tested the isoform-specific roles of the two PKCs in forskolin-stimulated insulin secretion in rat islets by using Go\ spender 6976 and antp-PKCe. These inhibitors did not significantly affect insulin secretion per se in rat islets at 3 mM glucose in our previous report (see Figs. 4E and 5, A and C, in Ref. 14). Forskolin-stimulated insulin secretion at 3 mM glucose was significantly reduced by Go\ spender 6976 and was markedly reduced by 75 \(\mu\)M antp-PKCe (Fig. 8B). These results indicate that a PKC signaling pathway mediated by a cAMP-generating agonist plays an important role in insulin secretion at a substimulatory concentration of glucose.

**DISCUSSION**

**Additional Mechanisms by Which GLP-1 Elicits [Ca\(^{2+}\)]\(_i\) Elevation**—We confirmed that GLP-1-initiated Ca\(^{2+}\) signaling occurs downstream of adenylate cyclase in INS-1 cells (Table 1 and Fig. 1), and that GLP-1 alone can elicit [Ca\(^{2+}\)]\(_i\) elevation at a substimulatory concentration of glucose (2, 3). There seem to be two additional mechanisms available by which GLP-1 may elicit [Ca\(^{2+}\)]\(_i\) elevation other than those described in the Introduction: 1) IP\(_3\) -induced Ca\(^{2+}\) release upon PIP\(_2\) hydrolysis.
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through PLC activation and 2) a change in membrane depolarization activity of K_ATP channels. Four observations support the idea that IP3-induced Ca2+ release occurs in response to GLP-1. First, GLP-1 elicited [Ca2+]i elevation with MARCKS translocation to the cytosol in the absence of extracellular Ca2+ (Fig. 4A), and this was completely blocked by 2-aminoethoxydiphenyl borate, an IP3 receptor antagonist. Second, U73122, a putative PLC inhibitor, inhibited forskolin-evoked translocation of MARCKS (Fig. 5). Third, if PIP2 hydrolysis induced by forskolin is followed by subsequent generation of DAG, an equivalent IP3 content is to be expected (Fig. 7). Fourth, we have observed GLP-1-evoked translocation of MARCKS without extracellular Ca2+ in MIN6 cells, a mouse insulin-secreting cell line.3 Taken together, these results indicate that the IP3-induced Ca2+ release pathway of PLC activation exists in both types of rodent. Tsuboi et al. (27) proposes that IP3-induced Ca2+ release mediated by GLP-1 accelerates mitochondrial ATP synthesis, thereby closing K_ATP channels. We can envisage a second mechanism as follows. Upon PIP2 hydrolysis through PLC, the decrease in PIP2 content and the increase in DAG content at the plasma membrane could change the activity of K_ATP channels with respect to membrane depolarization (35–37), thereby facilitating Ca2+ influx via VDCCs. According to a report from Baukrowiz el al. (36), the addition of 5 μM PIP2 to the cytoplasmic face of the plasma membrane in the inside-out mode for a patch clamp experiment dramatically reduces the ATP sensitivity of the K_ATP channel (the half-maximal inhibitory concentration of ATP increases from ~10 μM to more than 3 mM). The forskolin-evoked increase in DAG content (1.44 μM; Fig. 7) leads us to expect a stoichiometric decrease in PIP2 at the plasma membrane so that the second mechanism can be operative. Thus, the inhibitory effect of GLP-1 on K_ATP channels is probably partly due to a decrease in PIP2 content and increase in DAG content at the plasma membrane.

GLP-1-mediated Activation Mechanism of the PKC Signaling Pathway through Ca2+-dependent PLC Activation—We have demonstrated in this study 1) that GLP-1-induced translocation of MARCKS was inhibited by a variety of PKC inhibitors (Fig. 3, C and D) and 2) blocked by Ca2+ buffering (Fig. 4B); 3) that U73122 blocked forskolin-evoked translocation of MARCKS; 4) that application of GLP-1 and forskolin resulted in translocation of PKCα and PKCe (Fig. 2 and Fig. 6); and 5) that forskolin induced an increase in DAG. We also demonstrated in our previous report that Ca2+ influx via VDCCs can activate PLC (9). The simplest explanation for these collective observations is that cAMP-generating agonists activate PLC in a Ca2+-dependent manner, thereby leading to activation of the two PKCs. These observations also show that Ca2+ signaling is essential for GLP-1-evoked PKC activation. Although we do not know definitively at present whether GLP-1-mediated PKC signaling pathways require either a Ca2+ signal alone or a combined signal from Ca2+ and cAMP, the following mechanism may be envisaged. IP3 receptor sensitization induced by PKA phosphorylation triggers IP3-induced Ca2+ release (27), which in turn may activate a Ca2+-dependent PLC pathway. This is consistent with the two observations that 2-APB inhibited Ca2+ release induced by GLP-1 upon removal of the extracellular Ca2+ and that Ca2+ release alone can activate PKCe (Fig. 6C). Two PLC isoforms, PLCδ and PLCε, may be responsible for GLP-1-mediated PKC signaling pathways. Among all known PLCs, PLCδ isoforms have the highest sensitivity to Ca2+ (38), so that a GLP-1-evoked Ca2+ signal could be sufficient to trigger activation of PLCδ. Recent studies have shown that the action of cAMP on insulin secretion is mediated not only by PKA but also by cAMP-binding proteins designated as cAMP-regulated guanine nucleotide exchange factors (cAMP-GEF or Epac) (39). cAMP activates Epac1, which catalyzes activation of GTP loading on Rap 2B, leading to PLCε activation (40). Thus, PLCε may also be a potential candidate for GLP-1-mediated PKC signaling pathways. (However, in opposition to this hypothesis and our observations is a report that no inositol phosphate accumulation is detected in INS-1 cells after stimulation using either GLP-1 (100 nm) or an Epac-selective cAMP analog, 8-(4-chloro-phenylthio)-2′, 5′-methyladenosine-3′,5′-cyclic monophosphate (up to 300 μM) (41). We cannot clearly explain this discrepancy. It might be that we have shown that a cAMP-mediated PKC signaling pathway exists by using a single-cell-based analysis, whereas the other study measured inositol phosphate accumulation by a whole-cell population-based analysis using 3H-labeled inositol, which might not be sensitive enough to detect subtle changes in inositol phosphate content.

The Contribution of the PKC Signaling Pathway to Forskolin-stimulated Insulin Secretion Is Substantial at a Substimulatory Concentration of Glucose—GLP-1 is known to play a dual role in β cell function: 1) it stimulates insulin secretion per se at a substimulatory concentration of glucose (27), and 2) it renders β cells glucose–competent (42). We confirmed the first role of GLP-1 in this study. In addition, we have shown here that forskolin-induced insulin secretion is mediated partly by PKC signaling pathways (Fig. 8A). These results suggest that the relative contribution of GLP-1-mediated PKC signaling to GLP-1-induced insulin secretion is substantial at a substimulatory concentration of glucose. PKCe played a dominant role over PKCα in forskolin–induced insulin secretion of the rat islets (Fig. 8B). This result was reconciled with evidence of the dominant translocation of PKCe induced by GLP-1 irrespective of extracellular Ca2+ (Fig. 6, B and C). Several isoforms of PKC are expressed in insulin-secreting cells as well as the rat pancreatic islet (16, 28). When we monitored MARCKS translocation as a marker of PKC activation, BIS and U73122 blocked it, but neither Gö-6976 nor antp-PKCe did (Figs. 3, D and E, and 5). This is probably because of functional redundancy between the PKC isoforms. Among the PKC isoforms, however, PKCα and PKCe might be responsible for an agonist-stimulated insulin secretion (Fig. 8) (15, 16). The stimulatory role of GLP-1 on insulin secretion at a substimulatory concentration of glucose is very important, especially when one considers the effect of insulin secretion during a fasting period. GLP-1 is actually secreted not only during the postprandial period but also during fasting periods (43); therefore, it must be involved in basal insulin secretion mechanisms. Excessive secretion of GLP-1 might cause reactive hypoglycemia, as seen in dumping syndrome (43).

3 Y. Suzuki and H. Mogami, unpublished data.
Conclusion—In this study, we have shown that GLP-1, a cAMP-generating agonist, activates conventional PKCα and novel PKCe through Ca^{2+}-dependent PLC-mediated activation of INS-1 cells; in addition, we have measured the amount of DAG evoked by a cAMP-generating agonist in single living cells. In conclusion, GLP-1 may play a pivotal role in basal insulin secretion at substimulatory concentrations of glucose through the ternary signaling of a cAMP/PKA-Ca^{2+}-PKC pathway, and these signals cannot be segregated.

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