The influence of the insulin secretagogues, carbachol and glucose, on protein kinase C activation in isolated pancreatic islets has been examined by determination of the phosphorylation state of an endogenous 80-kDa protein substrate of protein kinase C. The islet 80-kDa protein was identified as the myristoylated alanine-rich C kinase substrate previously described (Stumpo D. J., Graff, J. M., Albert, K. A., Greengard, P., and Blackshear, P. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4012-4016) by immunoprecipitation studies. The muscarinic agonist, carbachol (500 μM), induced insulin secretion and a time-dependent increase in the phosphorylation state of this protein in islets. This phosphorylation was maximal (220 ± 34% of control) at 5 min and was suppressed by the protein kinase C inhibitor, staurosporine. Concentrations of glucose (10 mM) which induce maximal insulin secretion did not induce a statistically significant increase in 80-kDa phosphorylation. The combination of carbachol and a submaximally stimulatory concentration of glucose (10 mM), when added simultaneously, exerted a marked synergistic effect on insulin secretion and a synergistic effect on the phosphorylation of the 80-kDa protein kinase C substrate. These data suggest that the activation of protein kinase C may play an important role in carbachol-induced insulin secretion and in the potentiation by carbachol of insulin secretion induced by glucose. However, the activation of protein kinase C does not appear to be a primary determinant of insulin secretion induced by glucose alone.

The possibility that protein kinase C activation may participate in glucose-induced insulin secretion from isolated pancreatic islets has generated considerable recent interest (for reviews see Refs. 1 and 2). Evidence in support of this possibility includes the observation that phorbol esters, potent activators of protein kinase C, promote insulin secretion both from isolated islets (3-7) and from insulin secreting clonal cell lines (8). Diacylglycerol, an endogenous activator of protein kinase C, also stimulates insulin secretion when added to isolated islets (9-11). Phorbol ester-induced insulin secretion is temporally associated with an increase in membrane-associated protein kinase C activity (7, 12) and is suppressed by pharmacologic inhibitors of protein kinase C (7, 13, 14). Glucose-induced insulin secretion from rat islets is also suppressed (60-100%) by such inhibitors suggesting a role of protein kinase C in this process (7, 12, 13). In contrast, glucose does not induce the translocation of protein kinase C activity to a membrane-associated state (7, 12). Furthermore, islets from which protein kinase C activity has been quantitatively depleted by prolonged (20-24 h) incubation with phorbol esters secrete insulin normally in response to glucose, although they no longer secrete insulin in response to further stimulation by phorbol ester (2, 15). The role of protein kinase C activation in glucose-induced insulin secretion is therefore controversial.

In a variety of cell types, the activation of protein kinase C is accompanied by the increased phosphorylation of an 80-kDa protein which is a widely distributed endogenous substrate for the enzyme (16, for reviews see Refs. 17-19). Recently, Stumpo et al. (20) have isolated and sequenced a cDNA clone encoding the gene for the bovine 80-kDa protein kinase C substrate and have determined that the molecular mass of this protein is in fact 31.9 kDa. This protein and the equivalent protein purified from fibroblasts were shown to be myristoylated and to contain a high proportion of alanine and acidic amino acid residues (21-23), and the 80-kDa protein has been designated the MARCKS' protein (20). This protein is rapidly phosphorylated following the activation of protein kinase C by such stimuli as growth factors (24, 25) and bombesin (26) in fibroblasts and by neurotransmitters in neuronal cells (27). It is also phosphorylated in numerous cell types in response to activation by phorbol esters and cell-permeable diacylglycerols (16, 18, 24-27). It is not phosphorylated, however, in cells that are stimulated with agonists, e.g. prostaglandin E1 and epidermal growth factor, that do not activate protein kinase C (18, 28, 29). The 80-kDa protein is therefore considered to be a selective endogenous substrate for protein kinase C whose phosphorylation state specifically reflects the activation of this enzyme in intact cells (17, 25, 30).

The abbreviations used are: MARCKS, myristoylated alanine-rich C kinase substrate; TPA, 12-O-tetradecanoylphorbol 13-acetate; EGTA, [ethylenebis(oxyethylenenitri])tetraacetic acid; TBS, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino]ethanesulfonic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, 4-morpholinoethanesulfonic acid; MeSO, dimethyl sulfoxide; PDBu, phorbol 12,13-dibutyrate; ANOVA, analysis of variance.
In an attempt to clarify the role of protein kinase C in glucose-induced insulin secretion, the phosphorylation of the 80-kDa protein has been utilized as an indicator of the activation of protein kinase C in intact islets. This experimental approach has been used to compare the effects of three distinct insulin secretagogues on phosphorylation of the 80-kDa protein with its effects on insulin secretion. The compounds studied are the fuel secretagogue D-glucose, the muscarinic agonist carbachol (carbachol), and the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA).

**EXPERIMENTAL PROCEDURES**

**Materials**—Male Sprague-Dawley rats (160-180 g) were purchased from Sasco (O'Fallon, MO) and maintained on Rodent Chow 5001 (Ralston Purina, St. Louis, MO) *ad libitum* for at least 7 days prior to use. The sources of materials are as previously described (7). Normal rabbit serum was obtained from Sigma. Pansorbin (Cells) was obtained from Calbiochem (San Diego, CA). Antibody raised to an amino-terminal peptide of the MARCKS protein was a generous gift of Dr. Terry Blackshear. In addition, [3H]phorbol 12,13-dibutyrate was purchased from New England Nuclear Research Products, and [3H]phorbol 12,13-dibutyrate was obtained from Amersham Corp.

**Isolation and Culture of Islets**—Pancreatic islets were isolated aseptically from male rats as previously described (31). Islets were cultured overnight at 24 °C in tissue culture medium CMRL-1066 containing 5.5 mM glucose, 1% L-glutamine, 10% heat-inactivated fetal bovine serum, 0.5% penicillin, and 0.5% streptomycin under an atmosphere of 95% air, 5% CO2.

**Preparation of a Heat-Stable Protein Fraction from Isolated Islets**—Islets obtained from six to eight rats were homogenized by 13 passes in a Teflon/glass homogenizer in buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 2.5 mM CaCl2, 1 mM EDTA, 150 mM sucrose, 2.5 mM EGTA, 10% glycerol, 10% fetal bovine serum, 0.5% penicillin, and 0.5% streptomycin under an atmosphere of 95% air, 5% CO2.

**Phosphorylation of Islet 80-kDa Protein in Vitro**—Islets obtained from six rats (~1000 islets) were disrupted by 13 passes of a Teflon/glass homogenizer in 400 μl of ice-cold buffer containing 25 mM TES-NaOH, pH 7.4, 1 mM EGTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 250 μg/ml bovine serum albumin (BSA), and 100 μg/ml leupeptin. The homogenate was centrifuged (Beckman SW50.1 rotor, 135,000 × g, 60 min, 4 °C) and the supernatant retained as a source of protein kinase C.

In *vitro* phosphorylation of islet cytosolic proteins was performed in a total of 10 μl of assay medium containing 10 mM TES-NaOH, pH 7.4, 5 mM MgCl2, 1 mM EGTA ± 1.3 mM Ca2+ (free Ca2+ concentration ~300 μM), ±125 μg/ml phosphatidylserine, and ±2.4 μg/ml 1,2-diolein. To each tube was added 30 μl of the heat-stable protein preparation (~1-3 μg of protein) and 10 μl of islet cytosol extract. After a preincubation period of 2 min, the reaction was initiated by the addition of [γ-32P]ATP (10 μl, final concentration, 20 μM, ~30-40 μCi/tube). The incubation was terminated at 1 min by the addition of 25 μl of 50% trichloroacetic acid (final concentration 10-15%, w/v). The tubes were then vortexed and placed on ice. After 30 min, the samples were centrifuged (Beckman microfuge 12,000 × g, 5 min) and the supernatant aspirated. The tubes were then washed with ice-cold acetone (1 ml) and the tubes allowed to air dry. The samples were then prepared for two-dimensional SDS-PAGE electrophoresis as described below.

**Phosphorylation of the Islet 80-kDa Protein in Situ**—For phosphorylation studies, isolated islets were washed three times in Krebs-Ringer-Hepes buffer (25 mM Hepes, pH 7.4, 115 mM NaCl, 24 mM NaHCO3, 5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2) containing 3 mM glucose, and 0.1% BSA (basal medium). One-hundred (100) islets were individually selected under a stereomicroscope and placed in polycarbonate tubes (Beckman, Palo Alto, CA). The bathing medium was then removed and replaced with a drawn-out Pasteur pipette and replaced with basal medium (300 μl) containing 32P [200 μCi, (200 μM)]. The islets were then exposed to 100 mM glucose for 30 min in a 37 °C water bath with gentle shaking. This medium was then gassed with air and replaced with basal medium with or without carbachol (500 μM) or glucose (16.5 mM). After 5 min, this medium was aspirated, and its insulin content was subsequently measured by radioimmunoassay.

The remaining islets were disrupted by 13 passes of a Teflon/glass homogenizer in ice-cold buffer containing 25 mM TES-NaOH, pH 7.4, 250 mM sucrose, 2.5 mM EGTA, 2.5 mM Mg acetate, 2.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 100 μg/ml leupeptin. The homogenate was centrifuged (Beckman SW50.1, 135,000 × g) for 1 h at 4 °C. The resulting supernatant was retained in a Teflon/glass homogenizer in buffer containing 25 mM TES-NaOH, pH 7.4, 5 mM MgCl2, 1 mM EGTA ± 1.3 mM Ca2+ (free Ca2+ concentration ~300 μM), ±125 μg/ml phosphatidylserine, and ±2.4 μg/ml 1,2-diolein. To each tube was added 30 μl of the heat-stable protein preparation (~1-3 μg of protein) and 10 μl of islet cytosol extract. After a preincubation period of 2 min, the reaction was initiated by the addition of [γ-32P]ATP (10 μl, final concentration, 20 μM, ~30-40 μCi/tube). The incubation was terminated at 1 min by the addition of 25 μl of 50% trichloroacetic acid (final concentration 10-15%, w/v). The tubes were then vortexed and placed on ice. After 30 min, the samples were centrifuged (Beckman microfuge 12,000 × g, 5 min) and the supernatant aspirated. The tubes were then washed with ice-cold acetone (1 ml) and the tubes allowed to air dry. The samples were then prepared for two-dimensional SDS-PAGE electrophoresis as described below.
for the determination of specific binding activity for the radiolabeled ligand ([3H]PDBu) by cytosolic protein kinase C. The pellet was resuspended in homogenization buffer (300-400 μl) and used for the determination of binding activity by membrane-associated protein kinase C.

Specific [3H]PDBu binding to these subcellular fractions (20-50 μl) was determined as described by Jaken (34). A concentration of [3H]PDBu between 20-40 nM was employed and nonspecific binding was measured in the presence of 100 μM unlabeled PDBu. The binding assay was performed at 4 °C for 1 h.

**Insulin Secretion**—In static secretion experiments, islets were counted (20 or 25/tube) into 12 × 75-mm siliconized borosilicate tubes and preincubated for 30 min at 37 °C with gentle shaking in basal medium containing 0.1% BSA (200 μl) under an atmosphere of air/CO₂ (95/5%). The medium was replaced with fresh basal medium alone or supplemented with glucose (10 mM) and carbachol (500 μM) or a combination of glucose and carbachol. The incubation was then continued for a further 30 min and was terminated by the removal of medium. Insulin content of this supernatant medium was determined by radioimmunoassay. In protein kinase C inhibition experiments, staurosporine (100 nM) was present during both the preincubation and incubation periods.

In the perifusion experiments, islets were placed in chambers (100 islets/chamber) and perfused with basal medium containing 0.1% BSA for 30 min at 37 °C as previously described (35, 36). During the last 5 min of this preincubation period staurosporine (final concentration 100 nM) or vehicle (0.1% Me₃SO) was added. The chambers were then perfused with the same medium supplemented with glucose (10 mM) and carbachol (600 μM) and containing either vehicle or staurosporine (100 nM) for a further 40 min. The insulin content of the perfusion effluent was measured by radioimmunoassay.

**Proteins Estimation**—Protein concentration was estimated by the method of Bradford et al. (37) using bovine serum albumin as standard.

**RESULTS**

**Phosphorylation of Islet 80-kDa Protein Is Stimulated by Ca²⁺ and Phospholipid in Vitro**

The phosphorylation of an endogenous 80-kDa protein has been used as an intracellular marker of the activation of protein kinase C in numerous cell types. The initial aim of the present study was to determine whether the phosphorylation of a similar 80-kDa protein reflected the activation of protein kinase C in islets. Fig. 1 (A and B) illustrates an autoradiogram of two-dimensional SDS-PAGE analysis of proteins phosphorylated by a cell-free extract of islet cytosol. This figure demonstrates that an acidic protein of Mr ~ 80 kDa was phosphorylated in response to the activation of protein kinase C in islet cytosol by the addition of the cofactors of protein kinase C (Ca²⁺, phosphatidylinerine, and diacylglycerol). The isoelectric point of this phosphorylated protein was 4.0-4.5 pH units and thus was very similar to that reported for the 80-kDa protein kinase C substrate in other cell types (16, 24-26).

Quantitative analysis of autoradiograms of these in vitro experiments demonstrated that the activation of protein kinase C by the simultaneous addition of Ca²⁺, phosphatidylinerine, and diacylglycerol resulted in a 9-fold increase in 80-kDa protein phosphorylation (Table I). Phosphorylation of the islet 80-kDa protein by rat islet cytosol was not significantly influenced by Ca²⁺ alone (free concentration ~300 μM). The modest, nonsignificant activation that was observed may reflect the presence of small amounts of phospholipids in the cytosol fraction prepared from islets. In contrast, addition of Ca²⁺ alone did promote, to a significant degree, the phosphorylation of a protein(s) of molecular mass 54-57 kDa (Fig. 1B, Table I). Proteins of this molecular weight include the subunits of Ca²⁺-calmodulin-dependent protein kinase (38) and tubulin (39), and their phosphorylation is known to be stimulated by Ca²⁺ and calmodulin. Since the islet cytosol preparation used in these assays contains a high concentration of calmodulin, the phosphorylation of this 54-57 kDa protein(s) is likely due to the action of a Ca²⁺-calmodulin-dependent protein kinase. The data in Table I, therefore, demonstrate that the 80-kDa protein in islets is selectively phosphorylated by the activation of protein kinase C in vitro and that its phosphorylation state is not increased under conditions in which the Ca²⁺-calmodulin-dependent protein kinase is activated.

As demonstrated in Table II, protein kinase C-mediated phosphorylation of the islet 80-kDa protein was suppressed by the inclusion of the protein kinase C inhibitors, staurosporine (7, 40) and K252b (41). Staurosporine, at a concen-

**Fig. 1. Protein kinase C-mediated phosphorylation of an 80-kDa protein by islet cytosol in vitro and in intact islets in situ.** Two-dimensional SDS-PAGE of samples prepared as described under "Experimental Procedures" was performed, and autoradiographs are shown here. Quadrants A and B are samples of islet cytosol extract which had been incubated in vitro in the absence (A) or presence (B) of Ca²⁺, phosphatidylinerine (PS), and 1,2-diolein (DAG). Quadrants C and D are samples of 100 islets which had been incubated in situ for 15 min in the presence of carrier alone (0.1% Me₃SO) (C) or 100 nM TPA (D). The position of the 80-kDa protein in both conditions is indicated by the arrow. The position of a 54-57 kDa protein(s) visible in the in vitro incubation using islet cytosol is indicated by the arrowhead (see Table I). The pH range used in isoelectrofocusing gels was 7.5 (at the right-hand side of the gel as represented) to 3.5 on the left (as shown above panels A and B). The positions of the molecular mass markers (kDa × 10⁻¹) run on the same gels are shown on the right.

**TABLE I**

<table>
<thead>
<tr>
<th>Additions</th>
<th>80 kDa</th>
<th>54-57 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(P) incorporation</td>
<td>(P) incorporation</td>
</tr>
<tr>
<td>None</td>
<td>0.69 ± 0.05</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.41 ± 0.41</td>
<td>5.78 ± 0.33</td>
</tr>
<tr>
<td>Ca²⁺, PS, DAG</td>
<td>6.40 ± 0.87</td>
<td>3.33 ± 0.37</td>
</tr>
</tbody>
</table>
Protein was also increased by treating islets with TPA (Fig. 1). Phosphorylation of the 80-kDa protein was clearly the most significant (p = 0.007 and 0.015, respectively). Phosphorylation site of the 80-kDa protein in intact islets was inhibited by >95%. K252b is an inhibitor which shows considerable specificity toward protein kinase C relative to other kinases (41). At concentrations of K252b which inhibit islet protein kinase C activity (assayed by histone phosphorylation) by >95% (data not shown), this compound also completely prevented the phosphorylation of the 80-kDa protein by islet cytosol.

Phosphorylation of the 80-kDa Protein Is Stimulated by Phorbol Esters in Intact Islets

The phorbol ester TPA is a potent activator of protein kinase C, and therefore its ability to increase the phosphorylation site of the 80-kDa protein in intact islets was assessed. As shown in Fig. 1 (C and D), the [32P]P incorporation into an acidic protein of M, ~80 kDa (indicated by the arrow) was significantly increased in islets incubated in the presence of TPA. This phosphoprotein found in intact islets corresponded on two-dimensional SDS-PAGE to the protein phosphorylated by the activation of protein kinase C in vitro with respect to isoelectric point, size, and shape (cf. Fig. 1B). The incubation of islets (15 min) with TPA (100 nM or 1 μM) increased the phosphorylation of islet 80-kDa protein (by 222 and 320%, respectively, as expressed as percentage of the control at 3 mM glucose). Both increases were statistically significant (p = 0.007 and 0.015, respectively). Phosphorylation of the 80-kDa protein stimulated by TPA (100 nM) was significantly suppressed (~75%) by staurosporine (100 nM; 125 ± 22% versus 199 ± 44%, expressed as a percentage of basal). Phosphorylation of the 80-kDa protein was clearly the most prominent 1TPA-sensitive phosphorylation. Although other TPA-sensitive phosphorylations were observed (see Fig. 1) they were both less intense and less consistent.

Immunoprecipitation of Islet 80-kDa Protein by Antibody Raised to the MARCKS Protein

In order to confirm the identity of the islet 80-kDa protein as the MARCKS protein, an antibody raised to amino-terminal peptide of the MARCKS protein was obtained from Dr. Perry Blackshear. This antibody was used to immunoprecipitate phosphoproteins from islet homogenates previously labeled with [32P]. As illustrated in Fig. 2, this antibody immunoprecipitated a phosphoprotein equivalent to the islet 80-kDa protein phosphorylated by protein kinase C with respect to molecular mass, acidic isoelectric point, and distinctive shape (cf. Fig. 1). Furthermore, the phosphorylation of this protein was also increased by treating islets with TPA (Fig. 2B cf. Fig. 2A). This protein was not precipitated by normal rabbit serum. These observations indicate that the 80-kDa protein is in fact the MARCKS protein previously described from other tissues and validates its use as an indicator of the activation of islet protein kinase C.

Secretagogue-induced Phosphorylation of the Endogenous 80-kDa Protein Kinase C Substrate in Intact Islets

Effects of Carbachol—Carbachol is known to promote cellular responses by muscarinic receptor-mediated activation of phospholipase C and the subsequent production of inositol phosphates (including inositol triphosphate (42) and diacylglycerol (11, 52). Inositol 1,4,5-triphosphate is known to stimulate the release of Ca²⁺ from the islet endoplasmic reticulum (35) and thus to increase the intracellular Ca²⁺ concentration (43). It is well established that diacylglycerol is an endogenous activator of protein kinase C (44). Carbachol would therefore be expected to promote phosphorylation of the endogenous 80-kDa protein kinase C substrate in islets. As demonstrated in Fig. 3, carbachol (500 μM) did in fact induce a marked increase in the [32P]P incorporation into the islet 80-kDa protein. This effect was transient, with maximal incorporation at 5 min (219 ± 22% of control, p = 0.015). By 10 min, the [32P]P incorporation had decreased to 157 ± 19% of control (p = 0.005). Under similar conditions, carbachol (500 μM) induced approximately a 2.6-fold increase in insulin secretion over a 5-min incubation period (Table II). Carbachol-induced phosphorylation of the islet endogenous 80-kDa protein kinase C substrate was suppressed in a dose-dependent manner by the protein kinase C inhibitors K252b and staurosporine (100 μM). These data indicate that carbachol induces the time-dependent activation of protein kinase C in intact islets and suggest that this mechanism may participate in the stimulation of insulin secretion by this muscarinic agonist.

Effect of Glucose—As shown in Fig. 3, a concentration of glucose (28 mM) that maximally stimulates insulin secretion from isolated islets also promoted phosphorylation of the islet endogenous 80-kDa protein kinase C substrate. Glucose-induced incorporation of [32P], into this 80-kDa protein was maximal at 5 min (128 ± 20% of control, p = 0.25) and was reduced to 111 ± 12% of control by 10 min. This time course mimicked that observed with carbachol, but the effect of glucose was small and did not achieve statistical significance as assessed by two-way analysis of variance (ANOVA) (Fig. 2). It has been proposed (45) that protein kinase C preferentially regulates the second phase of insulin secretion. The effect of glucose at later time points was therefore examined. Glucose (28 mM) was not found to induce a significant increase in the phosphorylation of the 80-kDa protein in islets after a 30-min incubation (116 ± 16% of control), although second phase insulin secretion is well established at this time.

Secretagogue-induced Translocation of Protein Kinase C

To confirm the results of the phosphorylation studies, another approach was also used to examine the influence of both carbachol and of glucose on protein kinase C activation in islets. This approach involved the assessment of the effect of these insulin secretagogues on the translocation of protein kinase C to a membrane-associated state. Since different protein kinase C isoenzymes may exist in islet β-cells (46) and may have distinct functions and/or different substrate specificities (47), a phorbol ester binding procedure was used to detect the mass of protein kinase C associated with cytosolic and particulate (membranous) fractions of islets stimulated with carbachol or glucose. The radiolabeled phorbol ester [3H]PDBu was found to bind either to cytosolic or to...
2. Immunoprecipitation of islet 80-kDa protein by MARCKS protein antibody. Immunoprecipitation was performed on islets incubated in the presence of vehicle (0.1% Me$_2$SO) (panel A) or 200 nM TPA (panel B) as described under "Experimental Procedures." Shown above are autoradiograms of two-dimensional SDS-PAGE analyses performed on these samples under conditions identical to those described in the legend to Fig. 1.

3. Carbachol- and glucose-induced phosphorylation of the 80-kDa protein in islets. Islets (100/tube) were labeled with $^3$P, as described under "Experimental Procedures." The islets were then incubated in the presence of carbachol (500 µM) or glucose (28 mM) for the time indicated. The samples were analyzed by two-dimensional SDS-PAGE and the $^3$P incorporated into the 80-kDa protein quantitated by laser densitometry. The data is expressed as a percentage of P incorporated in the presence of 3 mM glucose (basal conditions). *p < 0.02, **p < 0.002, ***p < 0.0001 versus control, two-way analysis of variance (ANOVA). The effect of carbachol as compared to glucose was also significant at 1 min (p = 0.029) and 5 min (p = 0.0005).

Synergy between Glucose and Carbachol on Insulin Secretion and Phosphorylation of the 80-kDa Protein

It is known that the insulin secretagogue effect of carbachol requires the presence of at least basal glucose concentrations (3-5 mM) and is potentiated in the presence of submaximal concentrations of glucose (e.g. 10 mM) (1). This observation has been confirmed in the present study (Fig. 5A). Glucose (10 mM) or carbachol (500 µM) induced a 6- or 3-fold increase in insulin secretion, respectively, as expressed as a percentage of control (Fig. 5A). The simultaneous addition of these secretagogues promoted a 30-fold increase in insulin secretion compared with basal glucose concentrations (3 mM) over a 30 min period. It was of interest, therefore, to examine whether glucose influenced the carbachol-induced phosphorylation of the 80-kDa protein. As shown in Fig. 5B, and consistent with data shown in Fig. 3, glucose (10 mM) alone did not increase the phosphorylation state of the 80-kDa protein under conditions in which carbachol (500 µM) promoted a 1.74-fold increase compared with islets that had been incubated in basal medium (p = 0.0097). The simultaneous addition of glucose (10 mM) and carbachol (500 µM) did, however, significantly increase the phosphorylation (277 ± 35%) of the 80-kDa protein either compared with basal phosphorylation levels (p < 0.0001, ANOVA) or compared with phosphorylation induced by carbachol alone (p = 0.0007, ANOVA). Experiments performed with the phorbol ester binding assay indicated that the simultaneous addition of glucose (10 mM) and carbachol (500 µM) did not significantly increase $[^3]$H$^3$PDBu binding by membranous fractions over that induced by carbachol alone (data not shown).

The hypothesis that the increased activation of protein kinase C might mediate the synergistic insulin secretory response to the addition of the combination of glucose and carbachol was tested by examining whether staurosporine was able to suppress insulin secretion under these conditions. As shown in Fig. 6B, insulin secretion was inhibited in a dose-dependent manner by staurosporine. Maximal inhibition (~31%) was achieved at 100 nM staurosporine. In islet peri-
fusion secretion experiments staurosporine was found not to influence the first phase of secretion (Fig. 6A). In some experiments staurosporine actually potentiated first phase secretion slightly. In contrast, staurosporine profoundly suppressed second phase secretion (Fig. 6A). This selective inhibition of second phase insulin secretion by staurosporine likely explains the observation that staurosporine only partially inhibits insulin secretion in a static secretion model carried out over a period of 30 min. Both first and second phase secretion contribute to the total insulin secreted in such static experiments. Since staurosporine inhibits only second phase, and not first phase insulin secretion, total insulin secretion is only partially inhibited by maximally effective concentrations of staurosporine in static experiments. This phenomenon also suggests that protein kinase C may play an important role in the regulation of second phase insulin secretion.

**DISCUSSION**

Insulin secretion from islets can be stimulated by secretagogues of at least two general classes. The muscarinic neurotransmitter acetylcholine, induces insulin secretion by interaction with β-cell plasma membrane receptors and is thought to be involved in the cephalic phase of insulin secretion (48). D-Glucose, the major physiological insulin secretagogue, is an example of a “fuel secretagogue,” so-called because its metabolism is required to induce insulin secretion (1). The present study has assessed the role of protein kinase C in these mechanisms by comparing the effects of these secretagogues on the phosphorylation of the endogenous 80-kDa protein kinase C substrate in islets.

The phosphorylation of this endogenous 80-kDa protein has been used as an indicator of the activation of protein kinase C in numerous cell types in response to a variety of agonists. The advantage of this approach is that an assessment of protein kinase C activity can be obtained in intact cells. This reduces the possibility of artifacts which have been suggested to occur in disrupted cell assays (7, 17). The use of the phosphorylation of the 80-kDa protein in islets as an indicator of the activation of protein kinase C was validated in several different ways. 1) It was demonstrated that the islet 80-kDa protein was phosphorylated in intact islets as an indicator of the activation of protein kinase C was validated in intact islets (7, 17). The use of this phosphorylating activity could be totally eliminated by the inclusion of the protein kinase C inhibitors staurosporine and K252b. 2) Second, the islet 80-kDa protein was phosphorylated in intact islets in situ in response to TPA, which activates protein kinase C in islets (7, 12). This event was also prevented by staurosporine. 3) Immunoprecipitation studies using the anti-MARCKS antibody were performed to demonstrate that the islet 80-kDa protein is closely related to or identical to the MARCKS protein. This protein has been shown to be a selective substrate for protein kinase C (24–25, 28, 30). The cellular function of this protein is not yet established but recent studies have demonstrated that it is myristoylated and has the capacity to bind and release calmodulin in the non-phosphorylated and phosphorylated forms, respectively (49). These observations suggest that the MARCKS protein may have an important role in the regulation of acute cellular responses (50).

Carbachol was found to induce the phosphorylation of this

**TABLE III**

<table>
<thead>
<tr>
<th>Condition</th>
<th>No.</th>
<th>[3H]PDBu bound</th>
<th>Insulin secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytosol</td>
<td>Particulate</td>
</tr>
<tr>
<td>0 mM glucose</td>
<td>6</td>
<td>0.505 ± 0.051</td>
<td>0.137 ± 0.019</td>
</tr>
<tr>
<td>16.5 mM glucose</td>
<td>5</td>
<td>0.530 ± 0.043</td>
<td>0.138 ± 0.039</td>
</tr>
<tr>
<td>3 mM glucose</td>
<td>6</td>
<td>0.498 ± 0.049</td>
<td>0.199 ± 0.016*</td>
</tr>
<tr>
<td>+ 500 μM carbachol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05 versus 3 mM glucose

**Fig. 4.** Effect of staurosporine on carbachol-induced phosphorylation of islet 80-kDa protein (A) and insulin secretion (B). A, islets (1000–1500) were labeled with 32P and incubated with basal medium alone (in the presence of 0.1% Me2SO), or basal medium supplemented with carbachol (CCh) (500 μM, 0.1% Me2SO) or carbachol in the presence of staurosporine (1–100 nM). The samples were analyzed by two-dimensional SDS-PAGE and the 80-kDa protein quantitated by densitometry. B, islets (25/tube) were preincubated in basal medium containing vehicle (0.1% Me2SO) or staurosporine (100 nM) for 30 min at 37 °C. The islets were then incubated with basal medium or basal medium containing carbachol (500 μM) in the absence and presence of staurosporine (100 nM). The data is expressed as a delta (Δ) of insulin secretion induced by carbachol less that secreted in basal medium. Staurosporine had no effect on basal insulin secretion.

The data is expressed as a delta (Δ) of insulin secretion induced by carbachol less that secreted in basal medium. Staurosporine had no effect on basal insulin secretion.
Protein Kinase C and Insulin Secretion

FIG. 5. Synergistic effect of glucose and carbachol on insulin secretion (A) and 80-kDa protein phosphorylation (B) in islets. A, islets (20/tube) were incubated for 30 min at 37 °C in the presence of glucose, carbachol (CCh), or both (see “Experimental Procedures”). The insulin content of the incubation medium was determined by radioimmunoassay. B, the phosphorylation experiments were performed as described in the legend to Fig. 2. Prelabelled islets were incubated with secretagogues for 5 min. *p = 0.0097 versus control, **p = 0.0007 versus carbachol, ANOVA.

80-kDa protein in islets in a time-dependent manner reflecting the activation of protein kinase C. This result is consistent with the prevailing view that the cellular responses of this muscarinic agonist are mediated by receptor-coupled stimulation of phospholipase C-catalyzed hydrolysis of membrane phospholipids (1). Carbachol has been shown to induce the formation of inositol phosphates (including inositol trisphosphate) in islets (42, 51, 52). More recently, it has been shown that carbachol induces the accumulation of [3H]diacylglycerol from [3H]glycerol labeled islets and that the fatty acid composition of this diacylglycerol is consistent with its derivation in part from membrane phosphoinositides (9, 53). It is striking that the time course of carbachol-induced phosphorylation of the islet 80-kDa protein corresponds very closely to the time course of carbachol-induced accumulation of [3H]diacylglycerol in islets prelabeled with [3H]glycerol (9). These observations support the interpretation that carbachol-induced phosphorylation of the islet 80-kDa protein is mediated by the activation of protein kinase C induced by the accumulation of endogenous diacylglycerol. The data from the [3H]PDBu binding assays indicate that carbachol-induced activation of islet protein kinase C is accompanied by its translocation to a membrane-associated state and agree with preliminary observations (11, 54). Furthermore, the 2.2-fold increase in 80-kDa phosphorylation and the 2.6-fold stimulation of insulin secretion in carbachol-stimulated islets are quantitatively similar. Finally, the temporal correspondence of diacylglycerol accumulation and insulin secretion observed previously (9) in carbachol-stimulated islets suggest a role for protein kinase C in carbachol-induced insulin secretion. The fact that staurosporine only partially inhibits carbachol-induced insulin secretion suggests that the activation of protein kinase C is not the sole mechanism mediating the insulin secretory response of islets to this agonist.

By contrast, stimulatory concentrations of glucose alone did not induce a significant increase in the phosphorylation of the endogenous islet 80-kDa protein kinase C substrate. This indicates that glucose alone does not strongly activate protein kinase C in islets. This interpretation is supported by the observation that glucose, despite inducing an insulin secretory response even more robust than that induced by carbachol, did not induce the translocation of protein kinase C mass to an activated, membrane-associated state as assessed by [3H]PDBu binding (Table III). These observations are consistent with previous reports the glucose does not induce the translocation of protein kinase C mass to an activated, membrane-associated state as assessed by histone phosphorylation assay (7, 12). Furthermore, it has recently been shown that glucose-stimulated islets do not accumulate an increased mass of diacylglycerol as determined by a diacylglycerol kinase assay (11). The sum of these accu-
mulated observations indicates that glucose-induced insulin secretion is mediated principally by mechanisms independent of the activation of protein kinase C.

The combination of carbachol and a submaximally effective concentration of glucose (10 mM) was, however, found to act synergistically to increase the phosphorylation of the endogenous 80-kDa protein kinase C substrate in islets, and there was a corresponding synergistic effect of the combination of carbachol and glucose to increase insulin secretion. These observations suggest that the synergistic effect of these secretagogues on insulin secretion might be mediated by the activation of protein kinase C. Consistent with this hypothesis, insulin secretion induced by the combination of these two secretagogues was found to act synergistically.

In summary, the phosphorylation of the 80-kDa protein in carbachol-induced insulin secretion provides further evidence that the activation of protein kinase C. Consistent with this hypothesis, consistent with this hypothesis, consistent with this hypothesis, consistent with this hypothesis, consistent with this hypothesis.
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