Glucose-induced Insulin Secretion from Purified β-Cells

A ROLE FOR MODULATION OF Ca\(^{2+}\) INFLUX BY cAMP- AND PROTEIN KINASE C-DEPENDENT SIGNAL TRANSDUCTION PATHWAYS

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The effects of activation of cAMP- and protein kinase C-dependent signal transduction pathways were investigated on intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), cAMP content and insulin secretion from β-cells purified by fluorescence-activated cell sorting from normal rat islets. The secretion of insulin from suspensions of purified β-cells was dependent on glucose concentration and hormonal signals, including cAMP and activators of protein kinase C. Microfluorimetric measurement of [Ca\(^{2+}\)]\(_i\), with the fluorescent Ca\(^{2+}\) indicator fura-2 indicated that β-cells differed immensely in their individual responsiveness to glucose stimulation. An increase in [Ca\(^{2+}\)]\(_i\) occurred in ~70% of β-cells, whereas ~30% of β-cells were nonresponsive to a glucose stimulus. Elevation of Ca\(^{2+}\) levels by theophylline or glucagon transformed nonresponsive β-cells into cells which displayed marked increases in [Ca\(^{2+}\)]\(_i\), and β-cells which exhibited glucose-induced changes in [Ca\(^{2+}\)]\(_i\) showed further increases in [Ca\(^{2+}\)]\(_i\), and in the amplitude of Ca\(^{2+}\) oscillations. Carbachol and 12-O-tetradecanoylphorbol-13-acetate, activators of protein kinase C, did not induce any alterations in intracellular Ca\(^{2+}\) levels; nonetheless, these agents increased both the number of β-cells which exhibited glucose-induced changes in [Ca\(^{2+}\)]\(_i\), and the amplitude of oscillations. The ability of cAMP or activators of protein kinase C to increase [Ca\(^{2+}\)]\(_i\) in single β-cells was directly correlated with the ability of β-cells to secrete insulin in response to a glucose stimulus. These results suggest that both cAMP- and protein kinase C-dependent pathways may regulate Ca\(^{2+}\) entry into β-cells, possibly via voltage-dependent Ca\(^{2+}\) channels. Thus, this may represent a common mechanism whereby these different signal transduction pathways potentiate glucose-induced insulin secretion from β-cells.

Insulin secretion from pancreatic β-cells is regulated by interactions between a variety of nutrients, hormones, and neurotransmitters. Glucose is the predominant physiologic nutrient secretagogue, and its ability to elicit an insulin secretory response depends on the ability of the β-cell to metabolize glucose and generate intermediates, including ATP which results in membrane depolarization via the closing of ATP-dependent K\(^+\) channels (1-3). β-Cell depolarization is followed by Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels and subsequent bursts in electrical activity (4). In this series of signal transduction steps, intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\))\(^1\) has emerged as a major second messenger that regulates β-cell insulin secretion (3, 5). Evidence suggests that voltage-dependent Ca\(^{2+}\) channels are regulated by increases in [Ca\(^{2+}\)]\(_i\), and by other second messengers such as cAMP and possibly protein kinase C (6-8). It has been shown that cAMP potentiates insulin secretion and augments Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels and not via mobilization of stored Ca\(^{2+}\) from HIT cells, a hamster clonal-derived β-cell line (6, 7). Although the cellular mechanisms are unknown, the ability of cAMP to potentiate glucose-induced insulin secretion and Ca\(^{2+}\) influx may be mediated by phosphorylation of these Ca\(^{2+}\) channels.

Studies with β-cells purified by fluorescence-activated cell sorting (FACS) have emphasized a critical role for cAMP in stimulus secretion coupling (9-11). FACS-purified β-cells, in contrast to intact islets, do not secrete insulin even in the presence of elevated glucose concentrations. Previous studies have established that this property of β-cells is not a result of cellular damage due to purification, since insulin secretory activity is completely normalized in a biphasic manner by theophylline, glucagon, or recombination of β-cells with glucagon secreting α-cells (12). The facilitating effect of these conditions on glucose-induced insulin secretion by β-cells is believed to be mediated by an increase in cAMP and illustrates the importance of paracrine interactions among islet cells. Although cAMP-dependent processes affect other components in the signal transduction pathway, the modulation of voltage-dependent Ca\(^{2+}\) channels with the enhanced influx of Ca\(^{2+}\) may be a central mechanism whereby cAMP facilitates insulin secretion from β-cells.

In the present study we have utilized FACS-purified β-cells to evaluate both the role of cAMP- and protein kinase C-dependent signal transduction pathways to concomitantly increase [Ca\(^{2+}\)]\(_i\), and induce insulin secretion. These results suggest that an increase in [Ca\(^{2+}\)]\(_i\) may be a common mechanism, whereby both cAMP and activators of protein kinase C facilitate glucose-induced insulin secretion from β-cells.

EXPERIMENTAL PROCEDURES

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 2-3 days prior to use. Collagenase (Type P) was obtained from Boehringer Mannheim. Tissue culture medium (CMRL-1066), penicillin, streptomycin,

1 The abbreviations used are: [Ca\(^{2+}\)], free intracellular Ca\(^{2+}\) concentration; TPA, 12-O-tetradecanoylphorbol-13-acetate; FACS, fluorescence-activated cell sorting; BSA, bovine serum albumin.
Hanks' buffer, heat-inactivated fetal bovine serum, and L-glutamine were obtained from GIBCO. Bovine serum albumin (BSA) was purchased from ICN Biomedicals (Costa Mesa, CA). d-Glucose was purchased from the National Bureau of Standards (Washington, D.C.). TPA, carbachol, theophylline, and glucagon were obtained from Sigma. cAMP radioimmunoassay kits were purchased from Du Pont-NEN. Fura-2 and the nigericin (a calcium ionophore) were obtained from ICN Molecular Probes (Eugene, OR) and Bay K 8644 from Calbiochem.

Preparation of Islets and Purified β-Cells—Pancreatic islets were isolated by the collagenase digestion procedure (13). Islets were cultured overnight at 37 °C in tissue culture medium CMRL-1066 containing 5.5 mM glucose, 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin under an atmosphere of 95% air, 5% CO2. After 24 h in culture, the islets were dispersed into individual islet cells by incubation with dispase (0.33 mg/ml) in a Ca2+- and Mg2+-free Hanks' solution at 31 °C for 15 min (14). The dispersed islet cells were then incubated for 45-60 min in CMRL-1066 culture medium at 37 °C before purification. Islet β-cells were obtained by FACS as described previously with a purity of β-cells >95% (15).

Insulin Secretion—After FACS separation, purified cells were cultured overnight in CMRL-1066 medium in Petri dishes (60 x 15 mm) at 37 °C under an atmosphere of 95% air, 5% CO2. For complete equilibration of the perifusion chamber at this flow rate (2.5-5 x 10-6 ml/ min) was sedimented, supernatant collected for analysis of insulin secretion. Isolated islets were incubated for 30 min and then centrifuged at 250 g for 2 min and then resuspended in 1 ml of KRB medium containing theophylline, glucagon, or TPA. The β-cell suspensions were mixed and transferred to Petri dishes and then incubated at 37 °C under an atmosphere of 95% air, 5% CO2 for 3 h. After incubation, the β-cell suspensions were collected and sedimented as described above. A portion of the supernatant (100 μl) was removed for insulin determination by radioimmunoassay.

Measurement of Intracellular Ca2+—β-Cells (1 x 106) contained in Microfuge tubes were placed in a shaking incubator at 37 °C for 30 min and then centrifuged at 250 g for 2 min. The supernatant was removed and the pellet was resuspended in 0.5 ml of the medium supplemented as described under “Results.” The β-cells were then incubated for 30 min at 37 °C under an atmosphere of 95% air, 5% CO2. At the end of the incubation period, the β-cell suspension was sedimented, supernatant collected for analysis of insulin secretion, and the Ca2+ content of the cells was measured by radioimmunoassay after trichloroacetic acid extraction and acetylation (16).

Measurement of Calcium—The basic method of measurement of [Ca2+]i was performed as described previously (17). In brief, FACS purified β-cells (1 x 106 cells) were plated onto 25-mm diameter glass coverslips and incubated in CMRL-1066 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 24-h culture, CMRL-1066 culture medium in Petri dishes was replaced with KRB medium, and β-cells were loaded with fura-2 (5 μM) for 45 min in KRB medium containing 3 mM glucose and 0.1% BSA. The coverslips were rinsed and used as the bottom of an open perfusion chamber. For perfusion, two injection metal tubes were inserted on opposite sides of the wall of the chamber. A two-channel peristaltic pump was connected both to the chamber for inflow and outflow, and the flow rate was maintained at 1 ml/min. The lag time for complete equilibration of the perfusion chamber at this flow rate based on dye calibrations was 120 s. The chamber was placed on the stage of an inverted Nikon microscope and maintained at 37 °C. Dual excitation wavelength (340 and 380 nm) microfluorimetry (emission monitored at 500-530 nm) and data calibrations were performed as described previously with a Photon Technology International Inc. Delta Scan instrument (17, 19). The HeNe laser was used to excite the fura-2 dye. The fura-2 fluorescence was measured using a Photometrics Instruments (model HN-S) at 250 g for 2 min and then resuspended in 1 ml of KRB medium containing theophylline, glucagon, or TPA. Results were presented as the mean ± S.E. from six separate experiments. p < 0.01 for 3 versus 20 mM glucose in all conditions except in the absence of theophylline, glucagon, carbachol, or TPA.

RESULTS

Restoration of Glucose-induced Insulin Secretion from Purified β-Cells by Activation of cAMP and Protein Kinase C Signal Transduction Pathways—The effects of activation of cAMP and protein kinase C signal transduction pathways were initially examined on glucose-induced insulin secretion by suspensions of FACS-purified β-cells. As shown in Fig. 1, 20 mM glucose, which is sufficient to stimulate insulin secretion from intact islets by 5–10-fold, did not significantly (p > 0.05) increase insulin secretion from purified β-cells compared with basal conditions of 3 mM glucose. Incubation of β-cells under conditions which elevate intracellular Ca2+ by either using the phosphodiesterase inhibitor theophylline, or glucagon, induced a significant increase in insulin secretion in the presence of stimulatory (20 mM) but not basal (3 mM) glucose concentrations. These results are in agreement with previous studies demonstrating a requirement for a threshold level of

cAMP levels or by activators of protein kinase C, indicating that these were normal viable β-cells. A second category of purified β-cells (~20%) exhibited only a monophasic increase in [Ca2+], with a rapid and sustained return to basal levels, and a third category (~50%) exhibited a biphasic type of response in which changes in [Ca2+], during the second phase were either above basal levels or displayed a variety of Ca2+ oscillations. In these three categories of responses which represented the majority of all β-cells examined, dramatic changes in [Ca2+], were effected by agents which either increased cAMP levels or by activators of protein kinase C. In order to validate sequential studies with a single β-cell serving as its own control, a series of experiments was performed in which individual β-cells were exposed to the same stimulus for three periods with removal of the stimulus (glucose, theophylline, or K+) by washing to obtain basal levels of [Ca2+], prior to each exposure. These control experiments demonstrated that purified β-cells responded in an identical manner following three separate applications of the same stimulus in this sequential design.

The ability to quantitate glucose stimulated insulin secretion from an individual β-cell is technically difficult based on present methodologies (4). In the present studies, insulin secretion was determined from β-cell suspensions obtained from the same preparations used to measure changes in [Ca2+]i in individual β-cells. Statistics—Student’s t test for unpaired data was used to determine statistical significance at p < 0.05.

Fig. 1. Effects of theophylline, glucagon, carbachol, and TPA on insulin secretion from purified β-cells. β-Cells were cultured overnight in CMRL-1066 medium at 37 °C and then preincubated for 30 min at 37 °C in KRB medium supplemented with 3 mM glucose and 0.1% BSA. Insulin secretion was measured after 3 h incubations in the presence of glucose (3 or 20 mM) with the addition of agents as described under “Experimental Procedures.” Results are presented as the mean ± S.E. from six separate experiments. p < 0.001 for 3 versus 20 mM glucose in all conditions except in the absence of theophylline, glucagon, carbachol, or TPA, where p > 0.05 (open columns). p > 0.05 in comparing 3 mM glucose with 3 mM glucose in the presence of theophylline, glucagon, carbachol, or TPA.
cAMP to facilitate glucose-induced insulin secretion from FACS-purified β-cells (9).

To determine whether the activation of the protein kinase C signal transduction pathway could also facilitate insulin secretion from β-cells, the effects of carbachol, a muscarinic agonist, which induces diacylglycerol accumulation and phospholipase C activation in islets, and TPA, an activator of protein kinase C, were also evaluated. Carbachol (500 nM) enhanced insulin secretion at a glucose concentration of 20 mM but had no effect on basal insulin secretion at 3 mM glucose (Fig. 1). Exposure of β-cells to TPA (10 nM) also resulted in enhanced insulin secretion in the presence of 20 mM glucose, whereas no detectable changes were observed in the presence of 3 mM glucose. These results suggest that activation of either cAMP or protein kinase C signal transduction pathways restores glucose-induced insulin secretion to FACS-purified β-cells.

Effects of Theophylline, Glucagon, and Activators of Protein Kinase C on cAMP Levels in Purified β-cells—Studies were performed to verify that incubation of β-cells in the presence of theophylline or glucagon increased cAMP and to determine if the ability of carbachol and TPA to facilitate glucose-induced insulin secretion was also mediated by an increase in intracellular cAMP. In Fig. 2A β-cells were incubated with either 5 mM theophylline or 10 nM glucagon in the presence of 3 or 20 mM glucose. Both theophylline and glucagon significantly increased intracellular cAMP from a basal level of 39.6 ± 8.9 to 126.1 ± 7.0 and 118.1 ± 16.7 fmol/1000 cells/30 min, respectively. There was no significant difference (p > 0.05) between the effects of 3 or 20 mM glucose on cAMP levels in the presence of theophylline or glucagon. As shown in Fig. 2B, carbachol (500 nM) or TPA (10 nM) did not induce any changes in cAMP levels by β-cells incubated in the presence of 20 mM glucose. However, the addition of theophylline in the presence of carbachol or TPA increased cAMP levels. These results indicate that the potentiating effects of activators of protein kinase C on insulin release are not mediated by elevation of cAMP.

In separate experiments, the Ca2+ channel agonist Bay K 8644 (5 μM) in the presence of 3 or 20 mM glucose and in the absence of the phosphodiesterase inhibitor theophylline exerted no detectable effects on insulin secretion from purified β-cells (n = 4, data not shown). However, supra-physiological increases in [Ca2+]i, by pharmacologic maneuvers, such as K+ depolarization that stimulate Ca2+ and calmodulin-dependent adenylate cyclase activity (22, 23), are accompanied by cAMP-dependent insulin secretion only in the presence of 20 mM glucose from FACS-purified β-cells (data not shown).

Heterogeneity of Glucose Effects on [Ca2+]i in β-cells—In initial experiments, glucose concentrations were increased from 3 to 20 mM, and changes in [Ca2+]i by purified β-cells were measured for at least 20 min. Glucose stimulation of β-cells resulted in marked heterogeneity both in the variety of responses and in overall sensitivity of β-cells to glucose stimulation. Fig. 3A illustrates a typical response observed in ~30% of β-cells (12 out of 40 cells) in which glucose stimulation resulted in a barely detectable increase in [Ca2+]i. In contrast, intracellular responses observed in Fig. 3B were characterized by a rapid increase in [Ca2+]i followed by a return to near basal levels in ~20% of β-cells (8 out of 40 cells). In Fig. 3C, 20 mM glucose also caused a rapid monophasic increase in [Ca2+]i, followed by smaller secondary increases in [Ca2+]i. This type of response occurred in ~50% of β-cells (20 out of 40 cells). These data illustrate that the patterns of [Ca2+]i, including both the frequency and amplitude, vary immensely among β-cells and in many instances β-cells appear nonresponsive to glucose stimulation.

Effects of Agents Which Elevate Intracellular cAMP on [Ca2+]i—The effects of raising intracellular cAMP on [Ca2+]i, were examined both in the presence of basal (3 mM) and insulin stimulatory concentrations of glucose (20 mM). Fig. 4A illustrates that 5 mM theophylline under basal conditions of 3 mM glucose exerted no effect on [Ca2+]i, in any of the β-cells tested (0 of 8). In the recording shown in Fig. 4B, β-cells which were nonresponsive to increases in glucose (20 mM) produced a robust increase in [Ca2+]i, in the presence of theophylline (7 of 7 β-cells). Fig. 4C shows another example in which a β-cell is responsive to glucose stimulation with an initial increase in [Ca2+]i, followed by a slightly sustained increase of [Ca2+]i, over basal values. After the addition of theophylline, a significant increase in [Ca2+]i, was observed with a subsequent oscillatory pattern of [Ca2+]i, from all β-cells examined (12 of 12).

Glucagon produced effects on β-cell [Ca2+]i, similar to those observed with theophylline. In Fig. 5A glucagon (10 nM) had no effect on [Ca2+]i, in the presence of 3 mM glucose, whereas glucagon activated the responsiveness of β-cells to 20 mM glucose in all β-cells tested (20 of 20). In Fig. 5B, 1 nM glucagon was ineffective in restoring glucose sensitivity, whereas 10 nM glucagon resulted in a marked increase in [Ca2+]i, from this same β-cell (representative of 8 β-cells). Thus, 10 nM glucagon was comparable with 5 mM theophylline in stimulating increases in [Ca2+]i, by β-cells in the presence
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Fig. 3. Heterogeneous responses of \([\text{Ca}^{2+}]_i\) by β-cells exposed to a glucose stimuli. β-Cells were cultured overnight in CMRL-1066 medium at 37°C and then loaded with fura-2 AM (6 μM) for 45 min in KRB medium containing 3 mM glucose and 0.1% BSA. Increases in glucose concentration from 3 to 20 mM in the KRB perfusion medium at 37°C are indicated by the solid line. Three types of responses of \([\text{Ca}^{2+}]_i\), by single β-cells to glucose stimulation are illustrated (A-C). Each graph is a representative experiment of at least five separate experiments with 40 β-cells examined.

A typical of 20 mM glucose. Fig. 5C demonstrates the ability of glucagon (10 nM) to convert a monophasic pattern of \([\text{Ca}^{2+}]_i\), by a β-cell into a series of slow-wave \([\text{Ca}^{2+}]_i\), oscillations (representative of 12 β-cells).

Effects of Carbachol and TPA on \([\text{Ca}^{2+}]_i\). As illustrated in Fig. 6A β-cells responded to carbachol (500 μM) with an increase in \([\text{Ca}^{2+}]_i\), when incubated in the presence of 3 mM glucose. This transitory increase in \([\text{Ca}^{2+}]_i\), under basal conditions of 3 mM glucose is believed to be mediated primarily by the mobilization of intracellular \([\text{Ca}^{2+}]_i\) by inositol trisphosphate. In contrast, Fig. 6B shows that carbachol in the presence of 20 mM glucose provoked a sustained increase in \([\text{Ca}^{2+}]_i\), followed by a series of oscillations of \([\text{Ca}^{2+}]_i\), in a β-cell previously nonresponsive to glucose alone. This response was typical of all 6 β-cells tested. In Fig. 6C, 20 mM glucose caused an increase in \([\text{Ca}^{2+}]_i\), in 9 out of 15 tested β-cells and the subsequent addition of carbachol resulted in a further rise in \([\text{Ca}^{2+}]_i\), which was characterized by an increase in amplitude from 305 ± 41 to 750 ± 60 nM (n = 9).

The effects of TPA, a direct activator of protein kinase C, were evaluated on changes in \([\text{Ca}^{2+}]_i\), from β-cells in a manner similar to that of carbachol. As shown in Fig. 7A, TPA (10 nM) in the presence of basal glucose (3 mM) produced no change in \([\text{Ca}^{2+}]_i\), in any β-cells examined (0 of 8). This lack of an effect of TPA on \([\text{Ca}^{2+}]_i\) at basal glucose concentrations differs from that observed with carbachol (Fig. 6A) under these same conditions and indicates that TPA is ineffective in mobilizing intracellular \([\text{Ca}^{2+}]_i\) from purified β-cells. Fig. 7B illustrates a β-cell which was nonresponsive to 20 mM glucose; TPA (10 nM) induced a robust increase in \([\text{Ca}^{2+}]_i\) (representative of 7 β-cells). In Fig. 7C, 20 mM glucose caused an increase in \([\text{Ca}^{2+}]_i\), from 8 different β-cells; however, TPA was still capable of further potentiating this response above that of glucose alone.

DISCUSSION
This study has examined the effects of conditions which increase cAMP levels or activate protein kinase C on both \([\text{Ca}^{2+}]_i\), and insulin secretion from populations of β-cells purified from normal rat islets by FACS. Insulin secretion in response to glucose stimulation was markedly amplified by
FIG. 5. Effect of glucagon on $[\text{Ca}^{2+}]_{i}$, by $\beta$-cells. $\beta$-Cells were treated as described in the legend to Fig. 3. A indicates the effect of glucagon in the presence of 3 mM glucose on $[\text{Ca}^{2+}]_{i}$ by single $\beta$-cells. In B and C glucagon was introduced into the KRB perfusion medium containing 20 mM glucose as indicated. Each graph is a representative experiment of at least six separate experiments with the individual number of $\beta$-cells indicated for each condition under "Results."

agents that elevate intracellular cAMP levels such as theophylline or glucagon. These results support previous studies that have examined the effects of cAMP on insulin secretion by FACS-purified $\beta$-cells (9-12). It was also found in the present study that activators of protein kinase C, including carbachol and TPA, increased the responsiveness of $\beta$-cells to glucose stimulation. This effect was, however, independent of changes in cAMP. Both the effects of increased cAMP levels and of activators of protein kinase C were glucose-dependent, i.e. insulin secretion occurred only at elevated glucose concentrations even in the presence of activators of protein kinase C or agents which elevate $\beta$-cell cAMP. These results indicate that glucose-induced insulin secretion from $\beta$-cells may be restored not only by increases in cAMP but also independently of cAMP by activation of protein kinase C.

Striking changes in $[\text{Ca}^{2+}]_{i}$ were observed when $\beta$-cells were incubated in the presence of agents that increased intracellular cAMP. $\beta$-Cells which were nonresponsive to glucose stimulation (~30%) showed marked increases in $[\text{Ca}^{2+}]_{i}$, when subsequently stimulated with glucose (20 mM) in the presence of agents that elevated intracellular cAMP levels. In addition, $\beta$-cells which responded with modest changes in $[\text{Ca}^{2+}]_{i}$ upon glucose stimulation (~70%) displayed significantly amplified increases of $[\text{Ca}^{2+}]_{i}$, when exposed to agents which increased cAMP. The ability of such agents to increase $[\text{Ca}^{2+}]_{i}$ was glucose-dependent, because neither theophylline nor glucagon alone affected $\beta$-cell $[\text{Ca}^{2+}]_{i}$. In single $\beta$-cells obtained from the ob/ob mouse, glucagon has been shown to induce increases in cAMP, and these increases in cAMP result in a sustained elevation of $[\text{Ca}^{2+}]_{i}$, with fast oscillations imposed on top of large amplitude oscillations (24-26). These studies identified cAMP as a determinant for glucose-induced Ca$^{2+}$ oscillations and also emphasized paracrine interactions between $\beta$ and $\alpha$-cells of the islet for the maintenance of critical levels of cAMP. Recently, the effects of cAMP on $[\text{Ca}^{2+}]_{i}$, have also been studied in HIT cells. In this insulinoma-derived cell line, cAMP elevates $[\text{Ca}^{2+}]_{i}$ by increasing Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels (6, 7). Although the mechanism for this effect is unknown, it is suspected that cAMP-dependent phosphorylation of voltage-dependent Ca$^{2+}$ channels may be involved. There is also recent evidence of cAMP-dependent phosphorylation and regulation of cardiac myocyte dihydropyridine-sensitive Ca$^{2+}$ channels (27).

In addition to cAMP, agents that activate protein kinase C
also potentiate glucose-induced insulin secretion from pancreatic islets (28-30). The potentiating effects of muscarinic receptor agonists on insulin secretion from islets are believed to be mediated by the mobilization of Ca\textsuperscript{2+} from intracellular stores (31, 32), increased entry of Ca\textsuperscript{2+} from extracellular sources (33-36), and activation of protein kinase C (29, 30). Our previous studies have demonstrated that both carbachol and TPA activate protein kinase C from islets as evidenced by phosphorylation of the MARCKS protein (29) and similar results have been obtained with FACS purified β-cells.\footnote{J. L. Wang, J. A. Corbett, C. A. Marshall, and M. L. McDaniel, unpublished observations.}

In the present study, both carbachol and TPA restore the insulin secretory response of β-cells to glucose stimulation and also markedly influence β-cell Ca\textsuperscript{2+} oscillatory behavior. Carbachol and TPA not only increased [Ca\textsuperscript{2+}], in β-cells but also converted totally nonresponsive β-cells to responsive cells in a manner analogous to the effects produced by increases in cAMP. The cellular mechanisms responsible for the stimulatory effects of carbachol and TPA on [Ca\textsuperscript{2+}], are different, since carbachol raises diacylglycerol levels and also promotes increases in [Ca\textsuperscript{2+}], by generation of inositol triphosphate, whereas TPA mediates its action on β-cells by direct stimulation of protein kinase C. Our results with FACS-purified β-cells obtained from rat islets differ from studies performed with the ob/ob mouse. In this latter model, exposure of β-cells to TPA transforms glucose-induced oscillations into a sustained increase of [Ca\textsuperscript{2+}], but the levels of [Ca\textsuperscript{2+}], are lower than those achieved with glucose alone (25). It is believed that TPA mediates the lowering of [Ca\textsuperscript{2+}], from the β-cells of ob/ob mice by an undefined extrusion process (37).

The observations that acetylcholine-induced insulin secretion depends on extracellular Ca\textsuperscript{2+} have suggested that increases in Ca\textsuperscript{2+} influx may also be important in mediating the secretory effects of muscarinic agonists (33-36). The ability of acetylcholine to stimulate Ca\textsuperscript{2+} influx has been documented in mouse islets by \textsuperscript{45}Ca\textsuperscript{2+} isotopic flux measurements (33, 34) and also in a clonal β-cell line (HIT-T15) by measurements of changes in [Ca\textsuperscript{2+}], in cell suspensions with the fluorescent Ca\textsuperscript{2+} indicator quin-2 (36). Evidence has also been reported that carbachol produces a transient increase in Ca\textsuperscript{2+} influx based on intracellular electrical activity measurements of mouse islets (35).

Approaches to determine the cellular mechanisms by which muscarinic agonists stimulate Ca\textsuperscript{2+} influx into β-cells have proven difficult. Thus, attempts to demonstrate that TPA, an activator of protein kinase C, increases Ca\textsuperscript{2+} influx has produced conflicting results (36-38). Pretreatment of HIT cells with TPA for 22-24 h to deplete endogenous protein kinase C activity had no effect on either glucose or acetylcholine-induced changes in [Ca\textsuperscript{2+}], (36). In another study TPA increased the effects of tolbutamide on \textsuperscript{45}Ca\textsuperscript{2+} efflux and insulin secretion without changing \textsuperscript{45}Ca\textsuperscript{2+} efflux, or electrical activity (38). It was concluded that cAMP but not activation of protein kinase C increases Ca\textsuperscript{2+} influx triggered by primary insulin secretagogues (38). In contrast, application of the whole cell configuration of the patch-clamp technique to Rin-m5F cells demonstrated that acute exposure to 10 nM TPA induced increases in inward Ca\textsuperscript{2+} currents with no changes in Na\textsuperscript{+} currents (39). In support of these findings, acute exposure to TPA also enhanced Ca\textsuperscript{2+} currents in Aplysia neurons (40). These disparate effects of TPA to alter Ca\textsuperscript{2+} influx may be explained by the variety of cell preparations employed (e.g. islets versus clonal-derived β-cell lines), the different techniques used to quantitate [Ca\textsuperscript{2+}], (e.g. \textsuperscript{45}Ca\textsuperscript{2+} isotopic fluxes, the Ca\textsuperscript{2+} fluorescent indicator quin-2, and patch-clamp techniques), and also the presence of different types of Ca\textsuperscript{2+} channels.

Our results support previous studies indicating that muscarinic agonists increase Ca\textsuperscript{2+} influx into β-cells. The ability of TPA, a direct activator of protein kinase C, to mimic the effects produced by carbachol on changes of [Ca\textsuperscript{2+}], and insulin secretion further suggest that activation of protein kinase C may mediate, in part, the effects of carbachol on both Ca\textsuperscript{2+} influx and insulin secretion by FACS-purified β-cells. The fact that TPA fails to increase intracellular [Ca\textsuperscript{2+}], under basal glucose conditions (3 mM) supports the conclusion that TPA mediates its effects via modulation of Ca\textsuperscript{2+} entry into β-cells.

The effects of carbachol and TPA on [Ca\textsuperscript{2+}], and insulin secretion by purified β-cells are independent of any changes in cAMP. A previous study has indicated that carbachol and TPA cause an increase in intracellular cAMP content in mouse islets and suggested that the effects of carbachol and TPA on insulin secretion may be due to increases in intracellular cAMP levels (41). The results from the present study,
however, clearly indicate that neither carbachol nor TPA induces an increase in cAMP accumulation by FACS purified β-cells in the presence of 3 or 20 mM glucose. The explanation for these conflicting results may lie in the experimental models, e.g. purified β-cells versus intact islets. Based on patch-clamp studies, it has been proposed that activators of protein kinase C affect insulin secretion by closure of ATP-sensitive K+ channels, followed by β-cell depolarization and increases in [Ca2+]i. (42). Results from the present study are consistent with the hypothesis that β-cell voltage-dependent Ca2+ channels may be modulated by activators of protein kinase C, although this possibility was not directly examined in our studies.

In summary these results indicate that the nutrient secretagogue, glucose, requires a critical threshold level of cAMP to facilitate both increases in [Ca2+]i and insulin secretion from β-cells. Increases in cAMP are necessary but insufficient by themselves to allow β-cells to secrete insulin unless β-cells are concomitantly exposed to elevated glucose concentrations. In contrast, the receptor-mediated agonist carbachol, and TPA, a direct activator of protein kinase C, facilitate changes in both [Ca2+]i and insulin secretion independent of cAMP but also require the presence of elevated glucose concentrations. This common requirement of elevated glucose concentrations, in addition to increases in [Ca2+]i, for purified β-cells to secrete insulin may reflect the demand for increased rates of ATP synthesis and other metabolites. Overall, these results indicate that both activation of cAMP- and protein kinase C-dependent signal transduction pathways exert a facilitating effect on insulin secretion from purified β-cells. The common mechanism responsible for this increased sensitivity of β-cells to nutrient and receptor-mediated agonists may be due, in part, to modulation of Ca2+ influx.

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REFERENCES