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Pituitary Adenylate Cyclase-Activating Peptide, Carbachol, and Glucose Stimulate Insulin Release in the Absence of an Increase in Intracellular Ca^{2+}

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SUMMARY

Insulin secretion from the pancreatic β cell line HIT-T15 was examined under conditions in which the elevation of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was inhibited by nitrendipine or diazoxide or by severe Ca^{2+} deprivation. Glucose-induced insulin release was completely abolished under these conditions. However, in the presence of 12-*O*-tetradecanoylphorbol-13-acetate or forskolin, 10 mM glucose significantly enhanced insulin release, even in the presence of 5 μM nitrendipine or 150 μM diazoxide. The $[Ca^{2+}]_i$ was not increased under these conditions. Even under Ca^{2+}-deprived conditions, achieved by 60-min preincubation in Ca^{2+}-free buffer containing 1 mM ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), glucose in the complete absence of extracellular Ca^{2+} significantly enhanced insulin release when the cells were treated also with 12-*O*-tetradecanoylphorbol-13-acetate and forskolin. Because of these findings, additional studies were performed with pituitary adenylate cyclase-activating peptide (PACAP) and carbachol to see whether physiological stimulation via receptor activation could stimulate insulin release in the absence of a rise in $[Ca^{2+}]_i$. Under normal Ca^{2+}-containing conditions, PACAP and carbachol stimulated insulin release and markedly potentiated glucose-stimulated release. In the presence of nitrendipine and thapsigargin, glucose failed to stimulate insulin release. Also, neither glucose in combination with PACAP nor glucose with carbachol was able to stimulate release. However, under the same conditions, the combination of glucose, PACAP, and carbachol did stimulate release while being unable to elevate $[Ca^{2+}]_i$. Thus, simultaneous activation of the β cell by PACAP, carbachol, and glucose can stimulate insulin release even when $[Ca^{2+}]_i$ is not elevated.

It is well known that elevation of $[Ca^{2+}]_i$ has an important role in stimulus-secretion coupling for hormone and neurotransmitter release. In the pancreatic β cell, an elevation of $[Ca^{2+}]_i$ in response to many secretagogues has been considered crucial for stimulus/secretion coupling (1). There is a consensus that glucose, one of the most important physiological secretagogues for the β cell, elevates $[Ca^{2+}]_i$ as follows (2–6). An increase in the glucose concentration is followed by a prompt equilibration across the cell membrane due to the abundant glucose transporter GLUT 2. Intracellular glucose is metabolized by glucokinase, and hexokinase to a lesser extent, and cascades of subsequent reactions. The metabolism of glucose increases the ATP/ADP ratio, and this is thought to contribute to closure of the K_{ATP} channels and membrane depolarization. Depolarization activates the L-VDCCs, causing Ca^{2+} influx and an elevation of $[Ca^{2+}]_i$. The elevated $[Ca^{2+}]_i$ triggers Ca^{2+}-dependent insulin secretion by mechanisms that are largely unknown but which almost certainly involve Ca^{2+}-binding proteins (7–10). In addition to the insulinotropic effects of glucose via $[Ca^{2+}]_i$, glucose powerfully augments Ca^{2+}-induced secretion (11, 12). Thus, there are at least two pathways of glucose signaling. These have been termed the K_{ATP} channel-dependent and -independent pathways (13–18). The mechanisms by which augmentation is achieved are unknown. Nevertheless, when glucose acts alone as a secretagogue, the elevation of $[Ca^{2+}]_i$ is mandatory for secretion and for the augmentation (11, 12). We demonstrated recently that glucose could stimulate, or augment, insulin secretion in rat pancreatic islets in the absence of extracellular Ca^{2+} under conditions of severe Ca^{2+} depletion and in the absence of any increase in $[Ca^{2+}]_i$ (19). This Ca^{2+}-independent stimulation of secretion by glucose was achieved by the simultaneous activation of protein kinases A and C. The stimulation was concentration dependent. Glucose metabolism was necessary, and the stimulated release

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ABBREVIATIONS: PACAP, pituitary adenylate cyclase-activating peptide; K_{ATP} channel, ATP-sensitive K^+ channel; L-VDCC, L-type voltage-dependent Ca^{2+} channel; KRB, Krebs-Ringer bicarbonate; AM, acetoxymethyl ester; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

could be inhibited completely by the physiological inhibitor norepinephrine. Because of the importance of this finding in understanding the mechanisms underlying the stimulation of insulin release by glucose and because the simultaneous activation of protein kinases A and C occurs physiologically under combined stimulation by agents such as acetylcholine, cholecystokinin, glucagon-like peptide-1, and PACAP, we broadened the study to additional cell types. The purpose of the current work was to determine whether the Ca^{2+}-independent effect of glucose is a property of the β cell, as distinct from the multicellular rat islets; to provide a homogeneous population of β cells for studies on the underlying mechanisms; and to study the effect of physiological stimulation by agents that activate protein kinases A and C via receptor occupancy. The data provide further evidence that the physiological regulators of insulin secretion, glucose, PACAP (20), and acetylcholine (21), are strongly involved at distal sites in stimulus/secretion coupling and can stimulate insulin release without elevation in $[Ca^{2+}]_i$.

Experimental Procedures

HIT-T15 cells culture. HIT-T15 cells were maintained in monolayer culture in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 units/ml penicillin at 37° in a 95% air/5% CO_2 atmosphere. Cells at passages 72–78 were used.

Insulin release. Insulin release was measured in static incubation and perifusion experiments as described previously (10, 22). Briefly, in static incubation experiments, the cells were incubated at 37° for 60 min in KRB buffer (129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.0 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.2 mM glucose, 0.1% bovine serum albumin, 10 mM HEPES, pH 7.4; preincubation). At the end of preincubation, the solutions were replaced with fresh KRB buffer containing test agents and incubated for 30 or 60 min. In some experiments, nitrendipine, diazoxide, or nitrendipine plus thapsigargin was present throughout. In Ca^{2+}-depletion experiments, KRB buffer devoid of Ca^{2+} with 1 mM EGTA (Ca^{2+}-free/EGTA buffer) was used. To achieve the depletion of Ca^{2+}, the cells were washed and incubated with the Ca^{2+}-free/EGTA buffer at 37° for 60 min before the experimental incubations were started. The preincubation solutions were replaced with Ca^{2+}-free/EGTA KRB buffer containing test agents, and the cells were incubated at 37° for an additional 60 min. The supernatants were collected for radioimmunoassay of insulin. In perifusion experiments, the cells, dispersed in a Ca^{2+}-free solution with EDTA plus trypsin, were loaded in each perifusion chamber (8×10^6 cells/0.7-ml chamber) and perifused with the Ca^{2+}-free/EGTA buffer containing 1 μM thapsigargin at 37° at a flow rate of 1 ml/min. The experiments were started after an equilibration period of 60 min of perifusion. Samples were collected every 2 min, and insulin in the perifusate was measured by radioimmunoassay.

Measurement of $[Ca^{2+}]_i$ concentration. $[Ca^{2+}]_i$ was measured by two methods: in cell suspensions and in single cells. First, the Ca^{2+}-sensitive fluorescent dye Fura-2 was used. Details of the measurement of $[Ca^{2+}]_i$ have been described previously (10, 20, 22–24). For Fura-2 loading, the cell suspension was centrifuged, resuspended in KRB buffer containing 0.25 mM sulfinpyrazone with 1 μM Fura-2/AM at 4×10^6 cells/ml, and incubated with continuous shaking at 37° for 30 min. The Fura-2-loaded cells thus obtained were washed and resuspended in KRB buffer containing 0.25 mM sulfinpyrazone at 1.5×10^6 cells/ml, and 3 ml of the cell suspension was placed in each quartz cuvette. During experiments and in the spectrofluorometer (model LS-5, Perkin-Elmer Cetus, Norwalk, CT), the cell suspensions were continuously stirred with small magnetic bars within the cuvettes. The temperature of the cell suspension was maintained at 36–37° by circulation of warm water through the cuvette holder. When needed, 5 μM nitrendipine, 150 μM diazoxide, or 5 μM nitrendipine plus 2 μM thapsigargin was present throughout the experiments except for the period of Fura-2 loading. An excitation wavelength of 340 nm and an emission wavelength of 510 nm were used, and $[Ca^{2+}]_i$ was calculated using the following equation: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, where K_d is the effective dissociation constant for Ca^{2+} binding to Fura-2 (224 nM), and F, F_{min}, and F_{max} are the fluorescence values at a time of interest and at zero and saturated $[Ca^{2+}]_i$, respectively, after correction for extracellular Fura-2 and autofluorescence of the cells. In experiments under Ca^{2+}-deprived conditions, results are shown by representative tracings from the dual excitation wavelengths of 340 and 380 nm rather than by calculated values of $[Ca^{2+}]_i$, because of the 1 mM EGTA in the cell suspension buffer, which hampers calculation for the absolute value of $[Ca^{2+}]_i$.

Second, HIT cells were incubated on 35-mm glass coverslips in RPMI cell culture medium and maintained at 37° before use in single-cell studies. Cells were loaded with 1 μM Indo-1 AM in KRB buffer containing 250 μM sulfinpyrazone supplemented with 0.2 mM glucose for 45–60 min, washed, and placed in a 1-ml Teflon chamber. The chamber was placed in a Narishige microincubation system mounted on the stage of a Nikon Diaphot 200 inverted epifluorescence microscope. Cells were excited at 360 nm using a xenon lamp, and emission was monitored at 405 and 485 nm using a photometer (Photon Technology International, S. Brunswick, NJ). The ratio of detected light (405 nm/485 nm) was calculated and displayed using FELIX software (Photon Technology) and a Dell Optiplex 433/L computer.

All cells were incubated under static conditions at 37° in KRB buffer with or without 2 μM thapsigargin and 5 μM nitrendipine for 20 min before data acquisition. Fluorescence was recorded at 405 and 485 nm for ~10 min before the addition of 10 μl of a stock solution containing PACAP, carbachol, and glucose to give a final chamber concentration of 10 nM PACAP, 0.5 mM carbachol, and 20 mM glucose.

Materials. Diazoxide, TPA, 4α-phorbol-12,13-didecanoate, forskolin, 1,9-dideoxyforskolin, Fura-2/AM, sulfinpyrazone, and carbachol were obtained from Sigma Chemical (St. Louis, MO). Indo-1/AM was purchased from Molecular Probes (Eugene, OR). Nitrendipine and thapsigargin were obtained from Research Biochemicals (Natick, MA). PACAP-27-NH_2 was purchased from Peninsula Laboratory (Belmont, CA). [^{125}I]Insulin (80–120 μCi/μg) was purchased from DuPont-New England Nuclear (Boston, MA).

Statistical analysis. Results are presented as mean ± standard error. Statistical analysis was performed by one-way analysis of variance with pairwise comparison with the Bonferroni method. A value of $p < 0.05$ was considered statistically significant

Results

Fig. 1 illustrates the results of insulin secretion studies under static incubation conditions in normal Ca^{2+}-containing KRB buffer. As shown in Fig. 1A, 10 mM glucose caused a 4-fold increase in insulin release over basal values. Exposure to 500 nM TPA, an activator of protein kinase C, and 30 μM forskolin, an activator of adenylyl cyclase, produced 2- and 2.7-fold increases in basal insulin release, respectively. Simultaneous stimulation with glucose and TPA or with glucose and forskolin elicited large increases in insulin release (Fig. 1A, *bars 4* and *6*). In assessing the influence of the elevation of $[Ca^{2+}]_i$ under these conditions, similar secretion experiments were performed in the presence of 5 μM nitrendipine, a blocker of L-VDCC, or in the presence of 150 μM diazoxide, a K_{ATP} channel activator (Fig. 1, B and C). Under both of these conditions, the stimulatory effect of glucose was

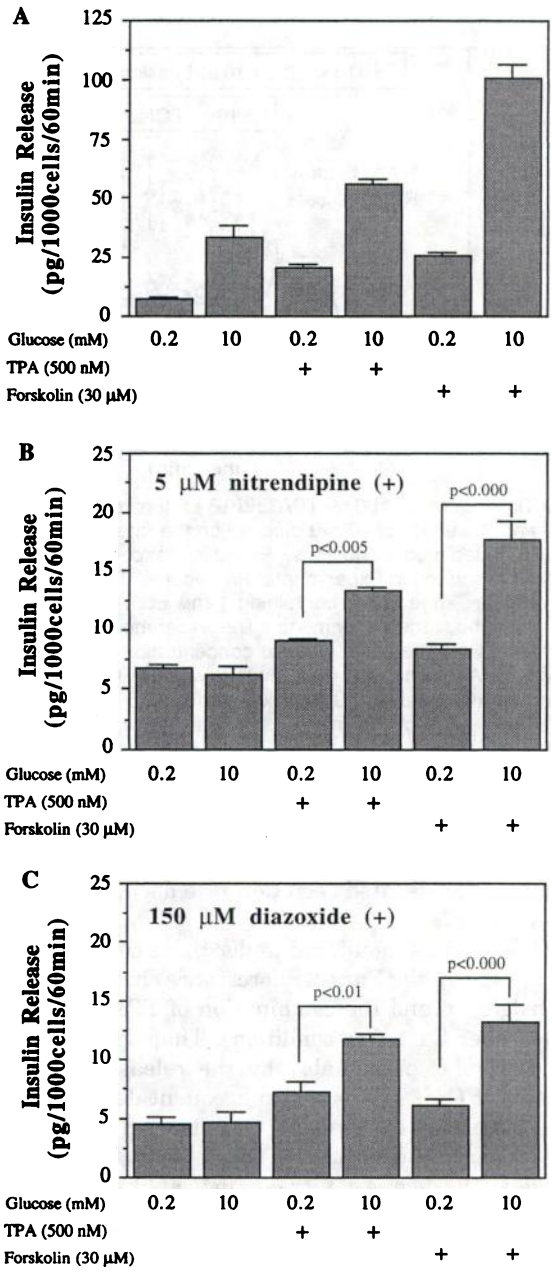

Fig. 1. Insulin release from the HIT-T15 cells performed under static incubation conditions. Insulin release was measured as described in Experimental Procedures. Incubation conditions are shown at the bottom of each figure. A, Insulin release in 1 mM Ca^{2+}-containing KRB buffer. B, Insulin release in the presence of 5 μM nitrendipine in the Ca^{2+}-containing KRB buffer. C, Insulin release in the presence of 150 μM diazoxide in the Ca^{2+}-containing KRB buffer. Values represent the mean ± standard error of four determinations.

abolished. However, although TPA and forskolin increased insulin release slightly (but without reaching statistical significance), the combination of glucose and either TPA or forskolin strongly augmented release. As neither 500 nM 4α-phorbol-12,13-didecanoate, an inactive analog of TPA, nor 30 μM 1,9-dideoxyforskolin, an inactive analog of forskolin, increased the rates of insulin release (data not shown), it seems unlikely that the effects observed with TPA or forskolin were due to nonspecific actions.

To confirm that the presence of nitrendipine or diazoxide did indeed prevent the secretagogue-stimulated elevations of $[Ca^{2+}]_i$ under these conditions, changes in $[Ca^{2+}]_i$ in the absence and presence of nitrendipine and diazoxide were monitored. The results are shown in Fig. 2. Nitrendipine at 5 μM and diazoxide at 150 μM, both of which slightly reduced the basal $[Ca^{2+}]_i$, completely inhibited the elevations of $[Ca^{2+}]_i$ induced by 10 mM glucose (Fig. 2A), 10 mM glucose plus 500 nM TPA (Fig. 2B), and 10 mM glucose plus 30 μM forskolin (Fig. 2C). These data are in accord with the elimination of the insulin release response to glucose alone but demonstrate that the release seen in the presence of glucose and nitrendipine (Fig. 1B) or glucose and diazoxide (Fig. 1C) is occurring in the absence of any rise in $[Ca^{2+}]_i$.

To further investigate the nature of the insulinotropic actions taking place without an elevation of $[Ca^{2+}]_i$, we next

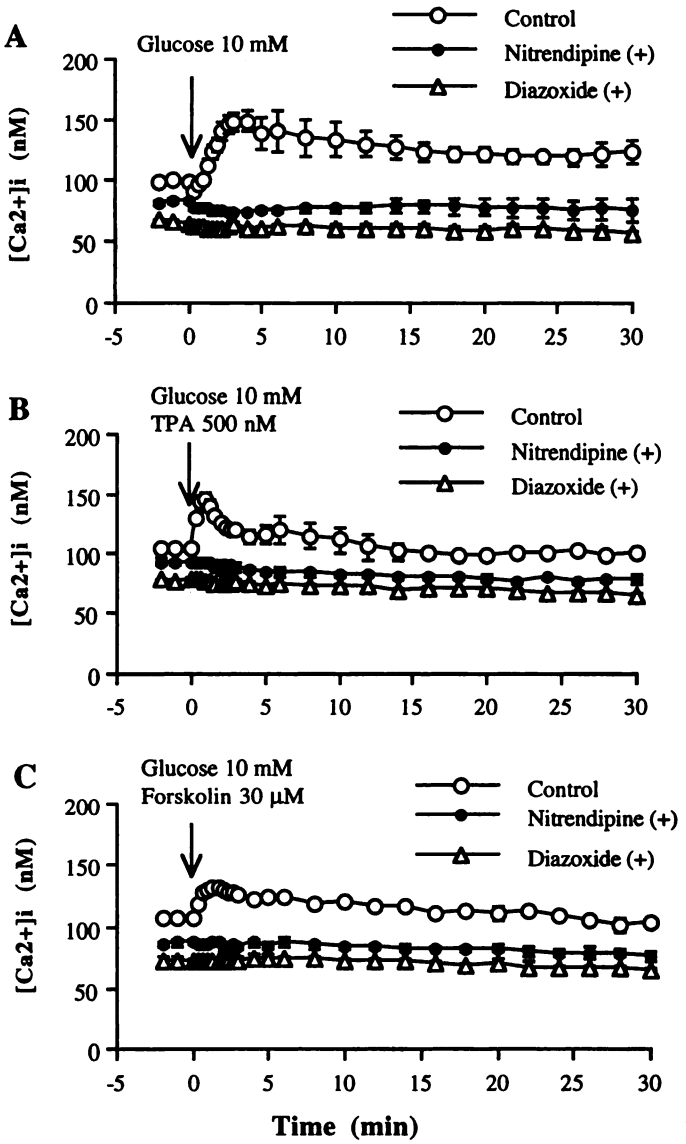

Fig. 2. Effects of nitrendipine or diazoxide on changes in $[Ca^{2+}]_i$ in the HIT-T15 cells. $[Ca^{2+}]_i$ was monitored by Fura-2, and $[Ca^{2+}]_i$ was calculated as described in Experimental Procedures. A, Effect of 10 mM glucose on $[Ca^{2+}]_i$ with or without 5 μM nitrendipine or 150 μM diazoxide. B, Effect of 10 mM glucose and 500 nM TPA on $[Ca^{2+}]_i$ with or without 5 μM nitrendipine or 150 μM diazoxide. C, Effect of 10 mM glucose and 30 μM forskolin on $[Ca^{2+}]_i$ with or without 5 μM nitrendipine or 150 μM diazoxide. Values represent the mean ± standard error of four determinations.

examined insulin secretion after depletion of the cells of Ca^{2+} and under Ca^{2+}-free conditions. To deprive the cells of Ca^{2+}, they were washed with Ca^{2+}-free/EGTA buffer (no added Ca^{2+} plus 1 mM EGTA) and then incubated with the Ca^{2+}-free/EGTA buffer at 37° for 60 min. Subsequently, insulin secretion was measured during an additional 60-min incubation in fresh Ca^{2+}-free/EGTA buffer containing various test agents. Insulin secretion under these Ca^{2+}-deprived conditions is shown in Fig. 3. As expected, 10 mM glucose had no effect on the basal rate of insulin secretion. Neither the TPA- nor the forskolin-evoked increases in insulin release reached statistical significance. Glucose did not significantly augment secretion in the presence of TPA or forskolin. However, simultaneous stimulation with TPA and forskolin produced a 4-fold increase in insulin secretion in the presence of 0.2 mM glucose (Fig. 3, *bar 7*), and 10 mM glucose in the presence of TPA and forskolin caused a significant augmentation of this release. Thus, as was shown in rat pancreatic islets (19), the HIT-T15 β cell responds to glucose in the absence of extracellular Ca^{2+}, after a long period of Ca^{2+} deprivation, and in the absence of a rise in $[Ca^{2+}]_i$.

Because of the novelty and potential importance of this Ca^{2+}-independent stimulation of insulin release by glucose, we performed perifusion experiments to confirm the effect and to determine its temporal profile. These experiments were performed under even more stringent conditions of Ca^{2+} deprivation; i.e., the cells were placed in a perifusion chamber and perifused with Ca^{2+}-free/EGTA buffer containing 1 μM thapsigargin, an inhibitor of microsomal Ca^{2+}-ATPase, which depletes the intracellular Ca^{2+} stores (25). As shown in Fig. 4, after a 60-min period of Ca^{2+} depletion, the basal rates of insulin secretion were stable under the Ca^{2+}-free conditions. On the addition of 100 nM TPA and 10 μM forskolin to both chambers at 10 min, the rates of insulin secretion increased slowly for ~15 min. Subsequently, the elevated rates of insulin secretion remained at a plateau until the TPA and forskolin were washed out at 60 min. The rate of secretion then declined to basal values. One set of chambers received 10 mM glucose at 30 min, in addition to the TPA and forskolin, while still under the Ca^{2+}-depleted conditions. This increased the rate of insulin secretion even further over the next 10 min to a higher plateau, at which the secretion rate remained until the secretagogues were washed out. Again, the elevated secretion rate declined to basal values.

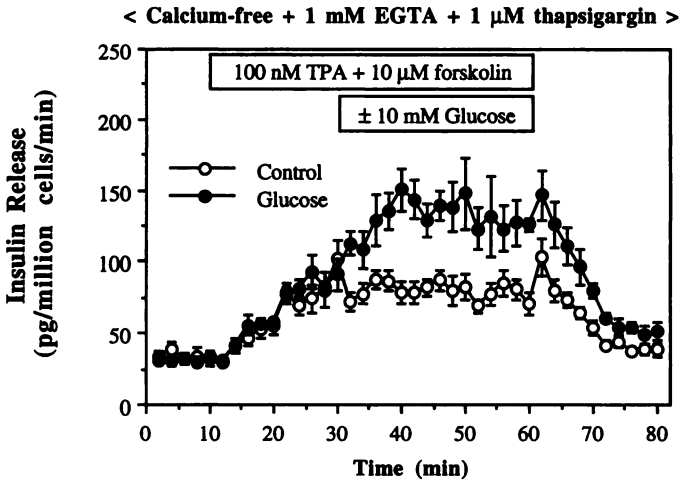

Fig. 4. Time course of 100 nM TPA and 10 μM forskolin-induced insulin release and the effect of 10 mM glucose on the rates of insulin release under Ca^{2+}-depleted conditions. Perifusion experiments were performed as described in Experimental Procedures. The cells were perifused with Ca^{2+}-free buffer containing 1 mM EGTA and 1 μM thapsigargin throughout the experiments. The experiments began after 60 min of perifusion. The basal glucose concentration was 0.2 mM. *Horizontal bar*, TPA (100 nM) and forskolin (10 μM) were introduced. One set of chambers (●) received 10 mM glucose from 30–60 min (*horizontal bar*). Values represent the mean ± standard error of five determinations.

The $[Ca^{2+}]_i$ was monitored under these conditions also. In Fig. 5 are shown the Fura-2 fluorescence changes in response to thapsigargin and the combination of TPA, forskolin, and glucose under Ca^{2+}-free conditions. Thapsigargin (1 μM) increased $[Ca^{2+}]_i$, presumably by the release of Ca^{2+} from intracellular Ca^{2+} stores and subsequent depletion of Ca^{2+}. In this particular experiment, thapsigargin was introduced 15 min after the exposure of the cells to Ca^{2+}-free/EGTA conditions. The degree of increase in $[Ca^{2+}]_i$ induced by thapsigargin was diminished over time after exposure to the Ca^{2+}-free/EGTA conditions. After 60 min of exposure to

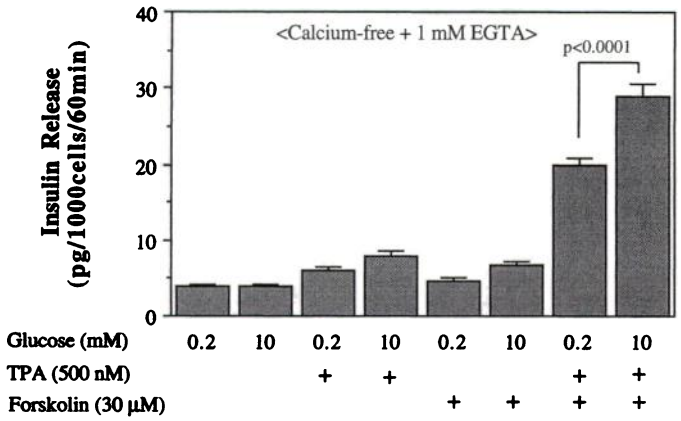

Fig. 3. Insulin release from the HIT-T15 cells in static incubation under Ca^{2+}-deprived conditions. Insulin release was measured as described in Experimental Procedures. The cells were washed with the Ca^{2+}-free KRB buffer containing 1 mM EGTA and incubated with the same buffer for 60 min (preincubation). After the preincubation, experimental incubations for 60 min were performed in fresh Ca^{2+}-free KRB buffer containing test substances (*bottom*). Values represent the mean ± standard error of nine determinations.

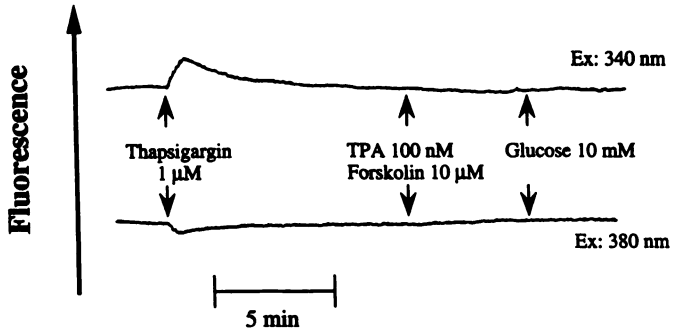

Fig. 5. Effect of thapsigargin and lack of effect of TPA and forskolin and subsequent addition of glucose on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. A pair of representative tracings from three similar experiments of Fura-2 fluorescences is shown. The fluorescence traces were obtained as described in Experimental Procedures. An increase in the fluorescence from an excitation wavelength of 340 nm accompanied by a decrease in the fluorescence from an excitation wavelength of 380 nm indicates an elevation of $[Ca^{2+}]_i$.

these conditions, thapsigargin failed to elevate $[Ca^{2+}]_i$ because long term exposure to the Ca^{2+}-free/EGTA buffer also caused depletion of Ca^{2+} stores. Under these Ca^{2+}-deprived conditions, neither the combination of TPA and forskolin nor a subsequent exposure to glucose elevated $[Ca^{2+}]_i$.

To extend the significance of these findings into physiological relevance, we next examined insulinotropic effects of physiological regulators of insulin secretion such as PACAP and carbachol. As shown in Fig. 6, the simultaneous stimulation with carbachol (0.5 mM) and PACAP (10 nM) dramatically enhanced insulin release in a glucose-dependent manner. One possible mechanism of this strong augmentation of glucose-induced insulin release might be due to the enhancement of glucose-induced elevation of $[Ca^{2+}]_i$ because carbachol is known to stimulate Ca^{2+} release from the intracellular Ca^{2+} store via activation of phospholipase C (21) and because PACAP has been reported to increase $[Ca^{2+}]_i$ in a glucose-dependent manner (20, 26). To test this possibility, changes in $[Ca^{2+}]_i$ in response to 20 mM glucose alone and combined stimulation with 20 mM glucose, 10 nM PACAP, and 0.5 mM carbachol were examined. As shown in Fig. 7, both stimulations produced almost identical patterns of increase in $[Ca^{2+}]_i$ except for the initial 2 min of stimulation. Glucose increased $[Ca^{2+}]_i$ after a 1-min latent period, whereas the simultaneous stimulation with glucose, PACAP, and carbachol elicited a rapid increase in $[Ca^{2+}]_i$ without a measurable latent period. Similar rapid responses were observed in the stimulation with glucose plus TPA or glucose plus forskolin, as shown in Fig. 2, B and C. This finding implies that the insulinotropic actions of PACAP plus carbachol occurred mainly through mechanisms that are independent of an increase in $[Ca^{2+}]_i$ because glucose-induced insulin release was strongly enhanced by PACAP plus carbachol with similar degrees of elevation of $[Ca^{2+}]_i$.

The simultaneous stimulation with PACAP and carbachol would allow glucose to induce its concentration-dependent insulin release, as is the case with TPA and forskolin, under the conditions in which the rise in $[Ca^{2+}]_i$ is blocked provided that the combined stimulation with PACAP and carbachol enhances glucose-induced insulin release in a Ca^{2+} rise-independent manner. We therefore examined insulin release and changes in $[Ca^{2+}]_i$ in the presence of nitrendipine and thapsigargin. As shown in Fig. 8, glucose-induced, concentration-dependent insulin release was completely abolished by the presence of 5 μM nitrendipine and 2 μM thapsigargin as expected. However, when glucose was added with 10 nM PACAP and 0.5 mM carbachol simultaneously, glucose induced concentration-dependent increases in insulin release in the presence of 5 μM nitrendipine and 2 μM thapsigargin. The amount of the released insulin exceeded the amount of insulin release induced by glucose alone. To emphasize the importance of the simultaneous activation of the PACAP and carbachol pathways, studies were performed under the same conditions of nitrendipine and thapsigargin treatment but with PACAP and carbachol used separately with glucose.

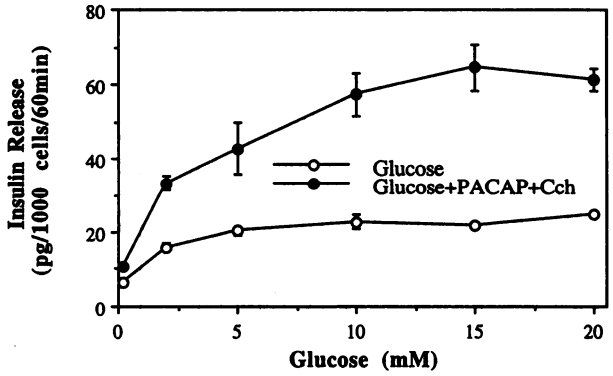

Fig. 6. Insulin release from the HIT-T15 cells in static incubation conditions as a function of glucose concentration. Insulin release was measured as described in Experimental Procedures. ○, Insulin release induced by glucose alone. ●, Insulin release induced by glucose stimulation combined with 10 nM PACAP and 0.5 mM carbachol (*Cch*). Values represent the mean ± standard error of four determinations.

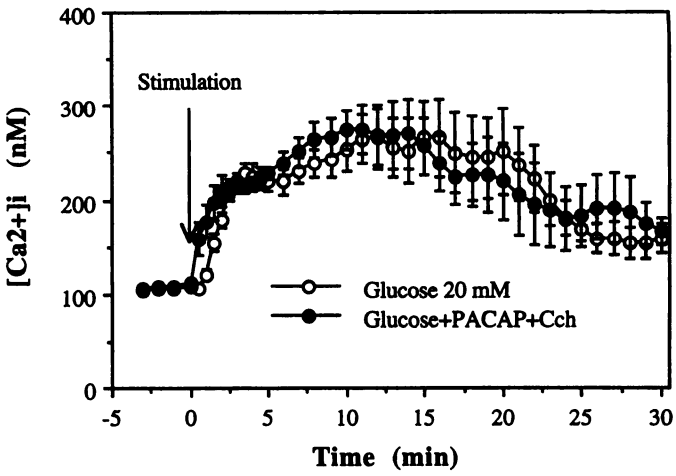

Fig. 7. Comparison of the effect of 20 mM glucose alone and 20 mM glucose combined with 10 nM PACAP and 0.5 mM carbachol on changes in $[Ca^{2+}]_i$ of the HIT-T15 cells. $[Ca^{2+}]_i$ was monitored and calculated as described in Experimental Procedures. ○, Changes in $[Ca^{2+}]_i$ in response to 20 mM glucose. ●, Changes in $[Ca^{2+}]_i$ in response to 20 mM glucose, 10 nM PACAP, and 0.5 mM carbachol (*Cch*). *Arrow*, Time of stimulation. Values represent the mean ± standard error of five determinations.

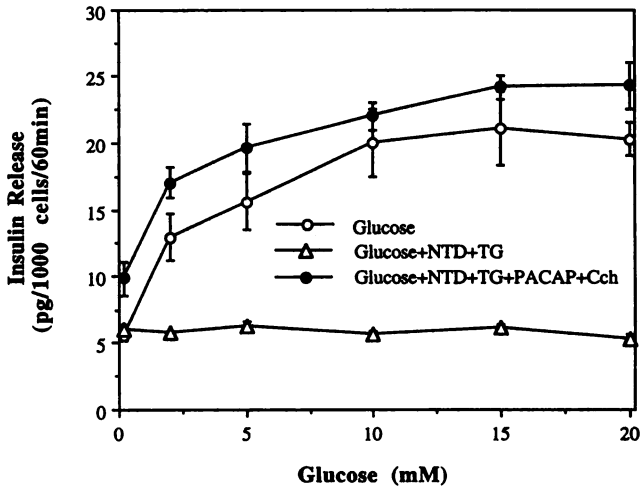

Fig. 8. Insulin release from the HIT-T15 cells in static incubation conditions as a function of glucose concentration. Insulin release was measured as described in Experimental Procedures. ○, Insulin release induced by glucose alone. △, Insulin release in the presence of 5 μM nitrendipine (*NTD*) and 2 μM thapsigargin (*TG*). ●, Insulin release induced by glucose stimulation combined with 10 nM PACAP and 0.5 mM carbachol (*Cch*) in the presence of 5 μM nitrendipine and 2 mM thapsigargin. Nitrendipine and thapsigargin were present throughout the experiments. Values represent the mean ± standard error of four determinations.

Under these conditions, neither PACAP and glucose nor carbachol and glucose were able to stimulate insulin release at any concentration of glucose, from 0.2 to 20 mM. These results are shown in Fig. 9.

The presence of nitrendipine and thapsigargin blocks two major mechanisms for the elevation of $[Ca^{2+}]_i$: Ca^{2+} influx through L-VDCCs and Ca^{2+} release from the intracellular Ca^{2+}-stores. Nevertheless, it was important to document whether the combined secretagogues could be increasing $[Ca^{2+}]_i$ by some other (unknown) mechanism. Consequently, two types of measurement of $[Ca^{2+}]_i$ were applied. In the first (Fig. 10), in the presence of 5 μM nitrendipine and 2 μM thapsigargin, the rise of $[Ca^{2+}]_i$ in response to 20 mM glucose, 10 nM PACAP, and 0.5 mM carbachol was shown to be completely inhibited. Subsequent studies were carried out using Indo-1, and measurements of $[Ca^{2+}]_i$ were made in 24 single cells to ensure that the secretion was not coming from a small subpopulation of cells that by some method increased the $[Ca^{2+}]_i$ under these conditions. In 12 cells studied in the absence of nitrendipine and thapsigargin, 9 (75%) responded to PACAP, carbachol, and glucose with a large increase in $[Ca^{2+}]_i$. In stark contrast, in the 12 cells studied in the presence of nitrendipine and thapsigargin, none of them responded. Representative experiments illustrating these results are presented in Fig. 11. These findings clearly show that simultaneous stimulation with PACAP and carbachol, as well as stimulation with TPA and forskolin, allows glucose to stimulate insulin release in the absence of a rise in $[Ca^{2+}]_i$.

Discussion

Intracellular Ca^{2+} in the β cell plays an important role in glucose-stimulated insulin release, and elevation of $[Ca^{2+}]_i$ has been described as "critical" and "essential" for stimulation. That this is true can be seen from the results of many studies and from the current study, in which by blocking glucose-stimulated Ca^{2+} entry and preventing a rise in

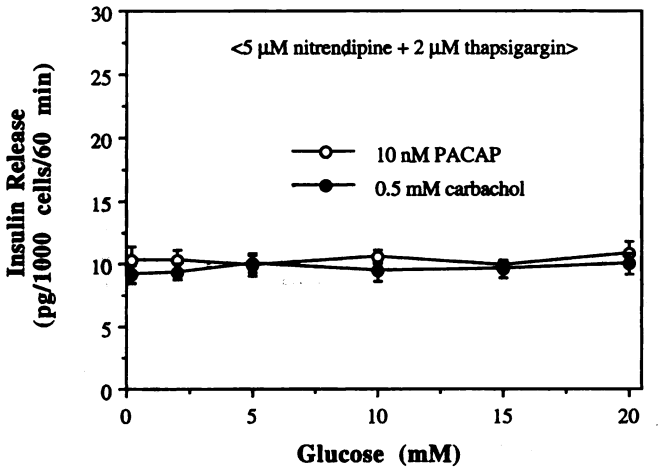

Fig. 9. Insulin release from HIT-T15 cells in static incubation conditions in the presence of either 10 nM PACAP or 0.5 mM carbachol as a function of glucose concentration in the presence of 5 μM nitrendipine and 2 μM thapsigargin. ○, Insulin release in the presence of 10 nM PACAP and the different glucose concentrations. ●, Insulin release in the presence of carbachol and the different glucose concentrations. Nitrendipine and thapsigargin were present throughout the experiments. Values represent the mean and standard error of eight determinations.

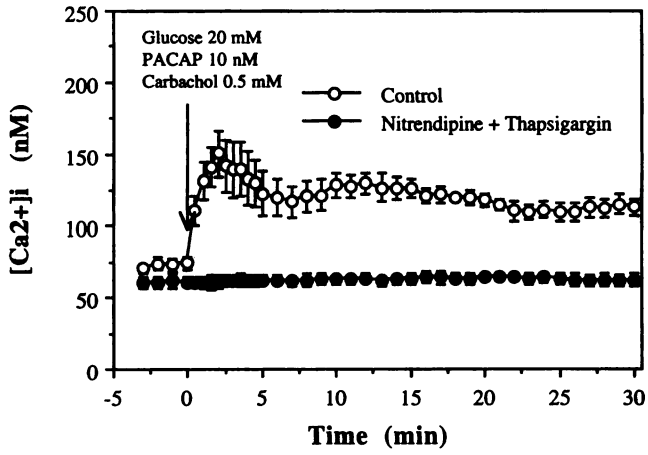

Fig. 10. Effects of nitrendipine and thapsigargin on changes in $[Ca^{2+}]_i$ in response to the combined stimulation with 20 mM glucose, 10 nM PACAP, and 0.5 mM carbachol in the HIT-T15 cells. $[Ca^{2+}]_i$ was monitored and calculated as described in Experimental Procedures. ○, Control responses. ●, Changes in $[Ca^{2+}]_i$ in the presence of 5 μM nitrendipine and 2 μM thapsigargin. Nitrendipine and thapsigargin were present throughout the experiments except for the periods of Fura-2 loading. Values represent the mean ± standard error of four determinations.

$[Ca^{2+}]_i$, nitrendipine and diazoxide also prevented the stimulation of insulin secretion. However, these data only confirm that elevation in $[Ca^{2+}]_i$ is necessary in glucose-induced insulin release when glucose is the sole secretagogue. It does not prove that glucose-stimulated insulin secretion requires elevated $[Ca^{2+}]_i$ in all circumstances. It may be that other stimuli and signal transduction pathways can provide a signal that substitutes for the rise in $[Ca^{2+}]_i$. It may be that the rise in $[Ca^{2+}]_i$ can be bypassed. Furthermore, we and others have reported that glucose can augment Ca^{2+}-induced insulin release from rat (11, 13, 15, 17, 18) and mouse (12, 14, 16) pancreatic β cells. In other words, glucose activates a signaling pathway that stimulates insulin secretion by a mechanism that does not increase $[Ca^{2+}]_i$ but does require increased $[Ca^{2+}]_i$ to be effective. This pathway in the action of glucose has been called the augmentation pathway as well as the K_{ATP} channel-independent pathway (11, 12). In studying this pathway, we found that the phenomenon of glucose-induced augmentation could be demonstrated under conditions in which the β cells were depleted of Ca^{2+} and in the absence of an increase in $[Ca^{2+}]_i$ (19). In this sense, it could be described as a Ca^{2+}-independent mechanism. We demonstrated the Ca^{2+}-independent augmentation by simultaneously exposing rat pancreatic islets to TPA, forskolin, and glucose under Ca^{2+}-free conditions. The purpose of the current study was to see whether the phenomenon occurred in β cells other than the rat pancreatic islet to characterize any such response in a cloned cell line to facilitate studies on the underlying mechanisms and to see whether the response could be induced by physiological stimulators that are thought to act, at least in part, via protein kinases A and C. From the data presented, it is clear that the HIT-T15 cell, like the rat pancreatic islet, can respond to glucose in the absence of extracellular Ca^{2+} and in the absence of a rise in intracellular Ca^{2+}. The lack of a rise in $[Ca^{2+}]_i$ was demonstrated in cell suspensions and by single-cell measurements, in both the current and the first report on this phenomenon (19). The single-cell determinations exclude the possibility

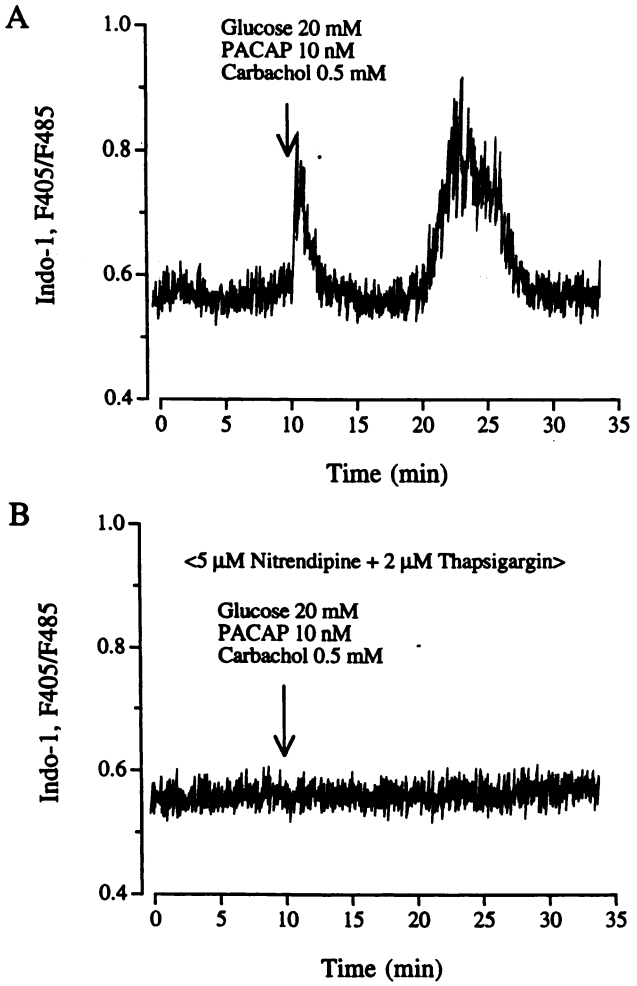

Fig. 11. Effect of 20 mM glucose, 10 mM PACAP, and 0.5 mM carbachol in the absence (A) and presence (B) of 5 μM nitrendipine and 2 μM thapsigargin on $[Ca^{2+}]_i$ in single HIT cells. $[Ca^{2+}]_i$ was monitored by Indo-1. *Traces*, representative examples of the 24 experiments performed.

that a small subpopulation of Ca^{2+}-responding β cells could be responsible for the stimulation. Furthermore, the results demonstrate that the response is not limited to the rat islet and does not require the presence of other islet cells. An additional finding was that activation of protein kinases A and C, neither of which alone in the absence of extracellular Ca^{2+} caused a significant stimulation of insulin release with glucose, was able to act together to cause secretion. This implies that some point of convergence of the effects of protein kinases A and C allows the stimulus/secretion pathway to proceed to the stimulation of exocytosis. This may be viewed as substituting for a rise in $[Ca^{2+}]_i$ or bypassing the point in stimulus/secretion coupling at which raised $[Ca^{2+}]_i$ is required. Finally, the simultaneous addition of PACAP and carbachol has a permissive effect that is similar to that of TPA and forskolin with respect to glucose-augmentation. This suggests that *in vivo*, where the pancreatic β cell is exposed to multiple secretagogues and incretins, such as PACAP and acetylcholine, this Ca^{2+}-independent mechanism could be operating yet masked by the presence of the normal Ca^{2+}-dependent mechanism.

Several questions arise from these data. Glucose signaling has been conveniently divided into the K_{ATP} channel-dependent pathway, by which $[Ca^{2+}]_i$ is elevated, and the K_{ATP} channel-independent pathway, by which the Ca^{2+}-stimulated insulin secretion is augmented (11–18). With the demonstration of an insulinotropic action of glucose separate from its dependence on elevated $[Ca^{2+}]_i$ (19), the possibility exists that a third pathway of glucose signaling is present in the β cell. This would be a K_{ATP} channel-independent and Ca^{2+}-independent augmentation pathway of glucose signaling. Alternatively, there may be only one augmentation pathway for glucose, which requires either raised $[Ca^{2+}]_i$ or simultaneous activation of protein kinases A and C for activation and stimulation of insulin release. It is interesting to speculate that this glucose action may be the "permissive" action of glucose for the potentiating effects of protein kinases A and C and that it might be correct to describe the effects of protein kinases A and C as "permissive" for the augmentation of insulin release by glucose.

In conclusion, protein kinases A and C have a synergistic effect to stimulate exocytosis in the presence of glucose, which has a novel Ca^{2+}-independent insulinotropic action. Both of these effects are exerted at a site distal to the elevation of $[Ca^{2+}]_i$ in stimulus/secretion coupling in the pancreatic β cell. Thus, the following components are involved in the distal steps of stimulus/secretion coupling: 1) elevation of $[Ca^{2+}]_i$, 2) activation of protein kinase A, 3) activation of protein kinase C, 4) synergy of the protein kinase A and C activations, and 5) Ca^{2+}-dependent and -independent augmentation by glucose. Despite our lack of knowledge about the mechanisms underlying the glucose-induced augmentation, it is obviously powerful and quantitatively important in the control of insulin release.

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