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Glucose stimulation of insulin release in the absence of extracellular Ca^{2+} and in the absence of any increase in intracellular Ca^{2+} in rat pancreatic islets

(stimulus-secretion coupling/exocytosis/protein kinase A/protein kinase C)

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ABSTRACT **Insulin secretion has been studied in isolated rat pancreatic islets under stringent Ca^{2+}-depleted, Ca^{2+}-free conditions. Under these conditions, the effect of 16.7 mM glucose to stimulate insulin release was abolished. Forskolin, which activates adenylyl cyclase, also failed to stimulate release in the presence of either low or high glucose concentrations. A phorbol ester (phorbol 12-myristate 13-acetate; PMA) increased the release rate slightly and this was further increased by 16.7 mM glucose. Remarkably, in the presence of both forskolin and PMA, 16.7 mM glucose strongly augmented insulin release. The augmentation was concentration dependent and monophasic and had a temporal profile similar to the "second phase" of glucose-stimulated insulin release, which is seen under normal conditions when Ca^{2+} is present. Metabolism is required for the effect because mannoheptulose abolished the glucose response. Other nutrient secretagogues, α-ketoisocaproate, and the combination of leucine and glutamine augmented release under the same conditions. Norepinephrine, a physiological inhibitor of insulin secretion, totally blocked the stimulation of release by forskolin and PMA and the augmentation of release by glucose. Thus, under the stringent Ca^{2+}-free conditions imposed, the stimulation of insulin release by forskolin and PMA, as well as the augmentation of release by glucose, is under normal physiological control. As no increase in intracellular $[Ca^{2+}]$ was observed, the results demonstrate that glucose can increase the rate of exocytosis and insulin release by pancreatic islets in a Ca^{2+}-independent manner. This interesting pathway of stimulus-secretion coupling for glucose appears to exert its effect at a site beyond the usual elevation of intracellular $[Ca^{2+}]$ and is not due to an activation by glucose of protein kinase A or C.**

Glucose is one of the most important regulators of insulin secretion from pancreatic β cells with both short-term stimulatory and long-term modulatory effects. After an increase in the plasma glucose concentration, glucose metabolism in the pancreatic β cells results in two clearly defined, but still interdependent, pathways of stimulus-secretion coupling. In one, glucose metabolism leads to closure of the ATP-sensitive potassium (K_{ATP}) channels, with resultant depolarization of the plasma membrane, increased influx of Ca^{2+} via L-type voltage-dependent Ca^{2+} channels, and elevated intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) (1–4). The elevated $[Ca^{2+}]_i$ then triggers an increase in exocytosis by mechanisms that still are largely unknown (5). This sequence of events is known as the K_{ATP}-channel-dependent pathway for stimulation of insulin secretion by glucose. A second Ca^{2+}-dependent pathway by which glucose increases insulin secretion has been defined recently (6, 7) and is referred to as the K_{ATP}-channel-independent pathway (8–12). Under conditions in which glucose is unable to affect the K_{ATP} channel—for example, after closing the channel with sulfonylureas or after opening the channel with agents such as diazoxide—glucose has been shown to stimulate insulin secretion. When sulfonylureas are used, the β cell depolarizes and $[Ca^{2+}]_i$ is elevated. Thus, the glucose effect is to augment the insulin release stimulated by the elevated $[Ca^{2+}]_i$ (13). In the presence of diazoxide, the augmentation by glucose is not seen unless $[Ca^{2+}]_i$ is elevated—for example, by a depolarizing concentration of KCl (6, 7). Another important effect of glucose with an enhancing effect on insulin release is time-dependent potentiation. This pathway too is Ca^{2+} dependent (14).

In contrast to these Ca^{2+}-dependent effects, we report here the existence of an interesting Ca^{2+}-independent effect of glucose to augment insulin secretion when protein kinase C (PKC) is activated in the β cell. Furthermore, when protein kinase A (PKA) and PKC are activated simultaneously, this Ca^{2+}-independent augmentation of insulin release is of large magnitude.

MATERIALS AND METHODS

Isolation of Pancreatic Islets and Insulin Secretion. Pancreatic islets were isolated from adult male Sprague–Dawley rats by collagenase dispersion as described (6, 15). Insulin release was measured under both static incubation and perifusion conditions. In static incubations, batches of five size-matched islets were incubated in 1 ml of Krebs–Ringer bicarbonate (KRB) buffer containing 129 mM NaCl, 5 mM $NaHCO_3$, 4.8 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2.8 mM glucose, 0.1% bovine serum albumin, and 10 mM Hepes (pH 7.4) for 60 min at 37°C (preincubation). Then the incubation medium was removed by aspiration and 1 ml of fresh KRB buffer containing test substances was introduced. Incubation under the test conditions was then continued for 30 or 60 min at 37°C. At the end of the incubations, the medium was aspirated and kept at −20°C until radioimmunoassayed for insulin. Rat insulin was used as a standard for radioimmunoassay. When Ca^{2+}-depleted conditions were needed, KRB buffer devoid of Ca^{2+} with different concentrations of EGTA was used throughout the experiments (for washing the islets and both the 60-min preincubation and the 60-min experimental incubation). When nitrendipine and thapsigargin were used, they were present throughout the preincubation and incubation periods. When norepinephrine was used, it was present during experimental incubation periods only. In perifusion experiments, 25 size-matched islets were placed in each 0.7-ml perifusion chamber (16, 17) and

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Abbreviations: $[Ca^{2+}]_i$, intracellular $[Ca^{2+}]$; PKC, protein kinase C; PKA, protein kinase A; PMA, phorbol 12-myristate 13-acetate.

perifused with KRB buffer devoid of Ca^{2+} with 1 mM EGTA at 37°C. The experiments were started after 60-min perifusion equilibration periods. Samples were collected every 1 or 2 min, and insulin in the perifusate was measured by radioimmunoassay.

Measurement of $[Ca^{2+}]_i$ in Single Rat β Cells. Isolated islets were placed in 100 μl of Ca^{2+}-free KRB buffer containing 1 mM EGTA and dispersed by gentle agitation. Dispersed islet cells were incubated on 35-mm glass coverslips in RPMI 1640 cell culture medium and maintained at 37°C for at least 12 hr prior to use in single cell studies. Cells were loaded with 1 μM indo-1 AM in KRB buffer containing 2.8 mM glucose for 30–45 min, washed, and placed in a 1-ml chamber. The chamber was placed in a Narishige microincubation system mounted on the stage of a Nikon Diaphot 200 inverted epifluorescence microscope. Excitation illumination from a xenon lamp was directed through a 360-nm bandpass filter. Emitted light was collected by a ×40 fluorescent objective and directed through the side port of the microscope to a dual-emission photometer (Photon Technology International, Princeton, NJ). A dichroic mirror with a longpass cutoff of 455 nm splits the emitted light. Reflected light is directed through a 405-nm bandpass filter. Light >455 nm passes through a 485-nm bandpass filter. Two photomultiplier tubes were used to detect light at 405 and 485 nm. Data from the photomultiplier tubes were relayed to a computer system for processing. The ratio of detected light (405 nm/485 nm) was calculated and displayed using OSCAR software (Photon Technology International) and a Dell Optiplex 433/L computer. Experimental details are given in the legend to Fig. 6.

Materials. Forskolin and phorbol 12-myristate 13-acetate (PMA), both from Sigma, were dissolved in dimethyl sulfoxide (final concentration, 0.02–0.2%). Nitrendipine and thapsigargin, which were also dissolved in dimethyl sulfoxide, were obtained from Research Biochemicals (Natick, MA). Control conditions had the same final concentrations of dimethyl sulfoxide as the test conditions. EGTA, dimethyl sulfoxide, mannoheptulose, α-ketoisocaproate, glutamine, and leucine were obtained from Sigma. Norepinephrine was from Fluka. Indo-1 AM was purchased from Molecular Probes.

Data Analysis. Data are presented as means ± SEM and statistical significance was evaluated by one-way analysis of variance with paired comparison by the Bonferroni method. Differences were considered significant at $P < 0.05$.

RESULTS

The effects of 16.7 mM glucose on insulin secretion were examined in the presence of the L-type Ca^{2+}-channel blocker nitrendipine and with forskolin and PMA still in the presence of nitrendipine. The results are shown in Fig. 1*A*. The stimulation of insulin release by 16.7 mM glucose, seen in the absence of nitrendipine (bar 7), was eliminated in the presence of nitrendipine (bar 2). Forskolin (bar 3), which activates adenylyl cyclase, had no effect on the secretion rate in the presence of a basal glucose concentration (2.8 mM). However, forskolin with 16.7 mM glucose increased insulin release to a level comparable with that of glucose in the absence of nitrendipine (cf. bars 4 and 7). PMA, which activates PKC, stimulated insulin secretion in the presence of 2.8 mM glucose (bar 5), and this was enhanced by 16.7 mM glucose (bar 6). These findings demonstrate that glucose is capable of stimulating insulin secretion even when the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels is blocked.

To determine the full extent of this potentially Ca^{2+}-independent response, the effects of glucose were examined under stringent Ca^{2+}-free conditions. The islets, after isolation, were depleted of Ca^{2+} by washing with Ca^{2+}-free KRB buffer containing 0.5 mM EGTA. Subsequently, they were incubated in the Ca^{2+}-free KRB/EGTA buffer for 60 min at

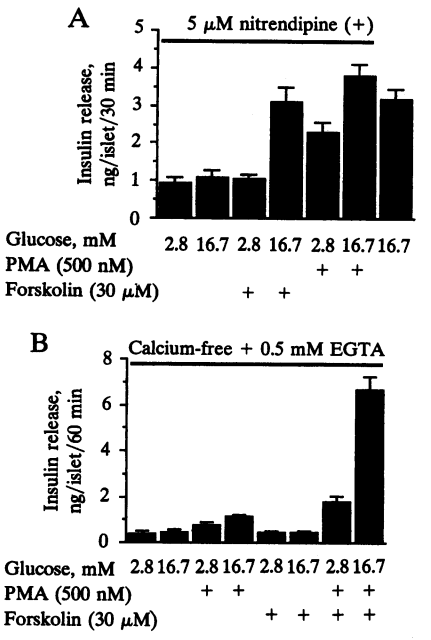

FIG. 1. (*A*) Effects of 2.8 and 16.7 mM glucose, PMA, and forskolin on insulin release from rat pancreatic islets in the presence of nitrendipine and the effect of 16.7 mM glucose in its absence. When nitrendipine was present, it was there throughout the preincubation and incubation periods. Solid line indicates the presence of 5 μM nitrendipine. The presence of 2.8 and 16.7 mM glucose, PMA, and forskolin is indicated at the bottom of the figure. (*B*) Glucose-induced augmentation of insulin release under Ca^{2+}-free conditions with 0.5 mM EGTA in the absence and presence of forskolin, PMA, or both forskolin and PMA. Values are means ± SEM (n = 5–6).

37°C to further deplete them of Ca^{2+}. After this 60-min preincubation, the islets were placed again in fresh Ca^{2+}-free KRB/EGTA buffer along with glucose and the test agents. Insulin release was measured over the next 60 min. As can be seen from the first two bars in Fig. 1*B*, 16.7 mM glucose was unable to stimulate insulin secretion under these Ca^{2+}-free conditions either alone or in the presence of forskolin (bar 6), which also failed to affect insulin secretion when added alone (bar 5). PMA caused a slight increase in insulin release (bar 3), and this was increased by 16.7 mM glucose (bar 4) (bar 3 vs. bar 4; $P < 0.05$). While these changes were slight, the effect of forskolin and PMA in combination stimulated insulin secretion (bar 7), and 16.7 mM glucose caused a remarkably large increase in secretion (see bar 8). Because of the potential importance of this Ca^{2+}-free stimulation, or augmentation, of insulin release by glucose, experiments were performed under even more severe conditions of Ca^{2+} depletion. The islets were studied in the presence of Ca^{2+}-free KRB containing 1 mM EGTA, 5 mM EGTA, and 1 mM EGTA plus 1 μM thapsigargin, an inhibitor of microsomal Ca^{2+}-ATPase that depletes intracellular Ca^{2+} stores (18). The results of these experiments are shown in Fig. 2. Under all three of these stringent Ca^{2+}-depleted conditions, the combination of forskolin and PMA stimulated insulin secretion, and this stimulation was always strongly augmented by 11.1 mM glucose.

The concentration–response characteristics and time course of the augmentation of release by glucose under Ca^{2+}-free conditions are shown in Fig. 3. In contrast to the stimulation of insulin release by glucose under normal conditions (in the presence of Ca^{2+}), the augmentation of release by glucose in the absence of Ca^{2+} began at 2.8 mM glucose (Fig. 3*A*). The maximal response was seen at 11.1 mM glucose and the response subsequently declined as the concentration of glucose was increased further. The results of perifusion studies to illustrate the temporal profile of the response are shown in Fig.

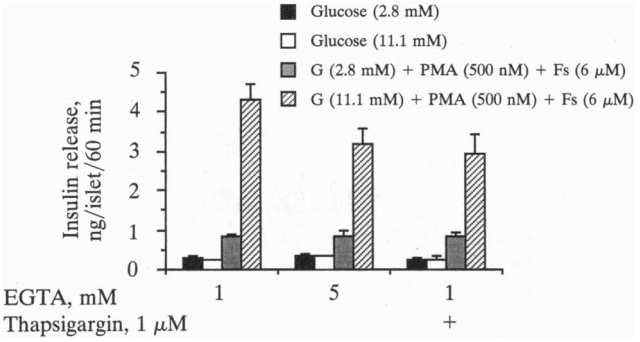

FIG. 2. Glucose-induced augmentation of insulin release under three different conditions of Ca^{2+} depletion. When thapsigargin was used, it was present throughout the experiments. Values are means ± SEM (n = 5). G, glucose; Fs, forskolin.

3*B*. The effect of glucose under these conditions was monophasic with increasing rates of insulin release over time. The response has a profile similar to that of the second phase of glucose-stimulated insulin release, which is seen under normal Ca^{2+}-containing conditions.

In preliminary studies on the mechanisms underlying this Ca^{2+}-independent augmentation of insulin release by glucose, metabolism of glucose was shown to be required. Mannoheptulose, which inhibits glucokinase and glucose metabolism, totally blocked the augmentation without affecting the response to forskolin and PMA (Fig. 4*A*). The augmentation was not specific for glucose, because other nutrient secretagogues also augmented release under the same conditions. As shown by the data in Fig. 4*B*, α-ketoisocaproate, and the combination of glutamine and leucine, strongly augmented the release of insulin. Finally, as a check on the nature of the mechanism of release, the effect of the physiological inhibitor norepinephrine was tested. As shown in Fig. 5, norepinephrine completely blocked the releasing effect of forskolin and PMA and the

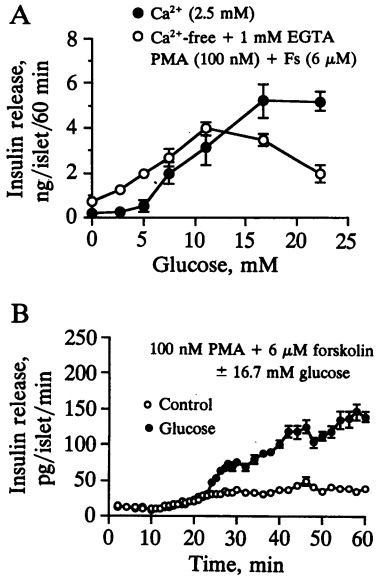

FIG. 3. (*A*) Concentration–response curves for glucose-induced insulin release in the presence of 2.5 mM Ca^{2+} (●) and glucose-induced augmentation of insulin release under Ca^{2+}-depleted conditions (○). Values are means ± SEM (n = 5). (*B*) Temporal profile of 16.7 mM glucose-induced augmentation of insulin release under Ca^{2+}-depleted conditions. Control islets were stimulated with 100 nM PMA and 6 μM forskolin from 10 min (○). Time course of glucose-induced augmentation of release is indicated (●). Glucose (16.7 mM) was introduced into the perifusates at 20 min. Values are means ± SEM (n = 4). Fs, forskolin.

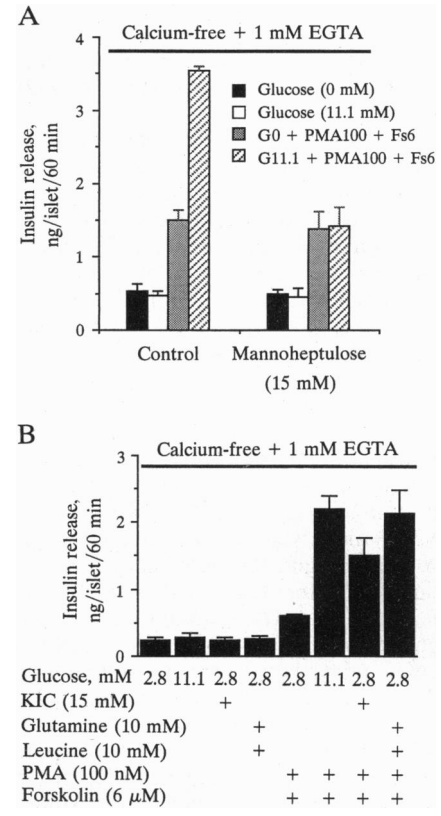

FIG. 4. (*A*) Effects of mannoheptulose on glucose-induced augmentation of insulin release under Ca^{2+}-depleted conditions. Mannoheptulose (15 mM) was present during the experimental incubation periods only. (*B*) Nutrient-induced augmentation of insulin release also under Ca^{2+}-depleted conditions. Values are means ± SEM (n = 5). G0, glucose (0 mM); G11.1, glucose (11.1 mM); PMA100, PMA (100 nM); Fs6, forskolin (6 μM); KIC, α-ketoisocaproate.

augmentation of release by glucose. We presume, therefore, that normal physiological control mechanisms are still operative.

Despite the severity of the Ca^{2+}-free conditions imposed, measurements of $[Ca^{2+}]_i$ were made in order to be quite certain that there was no increase in $[Ca^{2+}]_i$ during the stimulation with glucose, PMA, and forskolin and that the effect, therefore, was Ca^{2+} independent. Experiments were performed on single rat β cells, which were loaded with the Ca^{2+} indicator indo-1. Two types of experiments were performed. In one, the cells were exposed to the combined stimulus of glucose, PMA, and forskolin in KRB with 2.5 mM

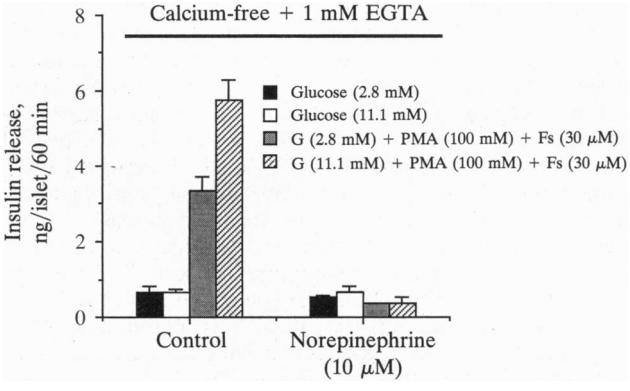

FIG. 5. Effect of norepinephrine on insulin release stimulated by forskolin and PMA and on glucose-augmented release. Values are means ± SEM (n = 5). G, glucose; Fs, forskolin.

Ca^{2+} to document the increase in $[Ca^{2+}]_i$. Then the cells were washed in Ca^{2+}-free medium containing 1 mM EGTA (these conditions were less stringent than some that were applied in the secretion studies) and rechallenged with glucose, PMA, and forskolin while monitoring $[Ca^{2+}]_i$. In the second type of experiment, the cells were challenged first in the Ca^{2+}-free medium, subsequently washed with normal Ca^{2+}-containing KRB, and then rechallenged to demonstrate that cells that had no response in Ca^{2+}-free medium did respond in normal Ca^{2+}-containing medium. In no case was there an increase in $[Ca^{2+}]_i$ under Ca^{2+}-free conditions. An example of the first type of experiment is provided in Fig. 6*A*, which shows that stimulation with glucose, PMA, and forskolin caused a large increase in $[Ca^{2+}]_i$ and initiated oscillations in $[Ca^{2+}]_i$. After washing in Ca^{2+}-free medium containing 1 mM EGTA, $[Ca^{2+}]_i$ was reduced and no response was seen when the cell was rechallenged with glucose, PMA, and forskolin. Similar results were obtained when the cells were challenged first in Ca^{2+}-free medium, in which no increase in $[Ca^{2+}]_i$ occurred,

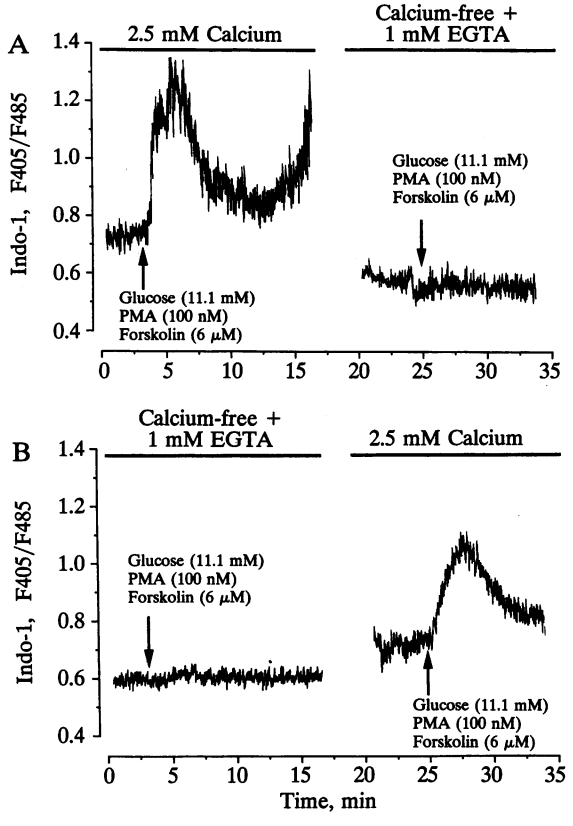

FIG. 6. (*A*) Effect of 11.1 mM glucose, 100 nM PMA, and 6 μM forskolin to increase $[Ca^{2+}]_i$ in a rat pancreatic β cell in Ca^{2+}-containing KRB (*Left*) and the lack of effect of this stimulatory mixture in Ca^{2+}-free KRB (*Right*). Cells were maintained at 37°C throughout these experiments. In one type of experiment, as shown, cells were incubated in Ca^{2+}-containing KRB buffer with 2.8 mM glucose for 15–30 min before adding PMA, forskolin, and glucose to the chamber to final chamber concentrations of 100 nM, 6 μM, and 11.1 mM, respectively (*Left*). Fluorescence was recorded at 405 and 485 nm for ≈15 min. Then, the incubation chamber was washed twice with Ca^{2+}-free KRB buffer containing 2.8 mM glucose and 1 mM EGTA and the chamber was filled with 1 ml of Ca^{2+}-free KRB buffer containing 1 mM EGTA. After 2–5 min, the test compounds were reintroduced into the chamber and fluorescence was monitored for an additional 10–15 min (*Right*). (*B*) In a second type of experiment, the same procedure was repeated except that the order of the buffer solutions was reversed. Cells were challenged first under Ca^{2+}-free conditions and then retested in Ca^{2+}-containing KRB buffer. Shown is the ratio of fluorescence detected at wavelengths of 405 and 485 nm. An increase in the ratio indicates increased $[Ca^{2+}]_i$.

and subsequently in Ca^{2+}-containing medium, in which an increase in $[Ca^{2+}]_i$ was observed (Fig. 6*B*). In additional experiments, the combination of glucose, PMA, and forskolin has been shown to stimulate insulin secretion in the absence of Ca^{2+} in the HIT-T15 β-cell line. When studied under fura-2-loaded conditions, no increase in $[Ca^{2+}]_i$ could be detected in these cells either (data not shown). We conclude that no increase in $[Ca^{2+}]_i$ takes place when insulin secretion is stimulated under Ca^{2+}-free conditions by the combination of glucose, PMA, and forskolin.

DISCUSSION

The results presented here demonstrate that glucose has an insulinotropic action in Ca^{2+}-depleted pancreatic islets in Ca^{2+}-free conditions under which no increase in $[Ca^{2+}]_i$ was detectable. The Ca^{2+}-depleted conditions were severe and included washing in a Ca^{2+}-free buffer containing EGTA, prolonged preincubation in this buffer, and then measuring insulin release during further incubation in Ca^{2+}-free buffer. Concentrations of EGTA up to 5 mM were used in the buffers in addition to thapsigargin to more rapidly deplete the intracellular Ca^{2+} stores. It is well known that glucose fails to raise $[Ca^{2+}]_i$ or to stimulate insulin secretion under these conditions or, in fact, under conditions of Ca^{2+} deprivation much less severe than these (19–22). The large Ca^{2+}-independent augmentation of insulin release by glucose demonstrated here requires stimulation of PKC and PKA as well as metabolism of glucose. It occurs over a physiological range of glucose concentration, although the response is maximal between 10 and 15 mM and decreased at 22.2 mM. The reason for the decrease at high glucose concentrations is not apparent, but it may be related to the stringent Ca^{2+}-free conditions imposed and resultant changed glucose metabolism. The augmentation is not specific for glucose and can be caused by other nutrient secretagogues. This glucose-activated pathway exerts its effect in a K_{ATP}-channel-independent manner, because neither sulfonylureas (glyburide and tolbutamide) nor diazoxide affected the glucose-induced augmentation in the Ca^{2+}-depleted conditions (data not shown). The mechanisms underlying the augmentation are not known. However, physiological control can be exerted over the release and total inhibition was imposed by norepinephrine. The release does not appear to be due to an activation of PKA or PKC—i.e., an activation further than that achieved by forskolin and PMA—because even with very high concentrations of these two agents (e.g., the combination of 60 μM forskolin and 1 μM PMA), augmentation by glucose was still large (data not shown). One possible exception to this conclusion, of course, would be the presence in the islet of a hypothetical glucose-sensitive PMA-insensitive PKC.

In stimulus-secretion coupling in the pancreatic β cell, it has been assumed that the elevation of $[Ca^{2+}]_i$ is an essential requirement for glucose to exert an effect. However, PKC activation can stimulate secretion under Ca^{2+}-free conditions (23) and in permeabilized cells even after they have lost their responsiveness to $[Ca^{2+}]_i$ (24). Similarly, the data presented here show that a glucose-activated pathway exists that delivers a stimulatory signal to a point beyond the elevation of $[Ca^{2+}]_i$ in normal stimulus-secretion coupling and that does not require elevated $[Ca^{2+}]_i$ to be effective. In fact, $[Ca^{2+}]_i$ under the stringent conditions imposed was lower than normal basal levels. The question that arises therefore is whether this interesting pathway is distinct from the K_{ATP}-channel-independent pathway, which has been described (6, 8, 10, 12), and is therefore a third branch of glucose stimulus-secretion coupling that exerts its effect only in the presence of activated PKC or more strongly in the presence of activated PKC and PKA. If this is the case, then it would explain why glucose is required for the potentiating effects of activators of PKA and

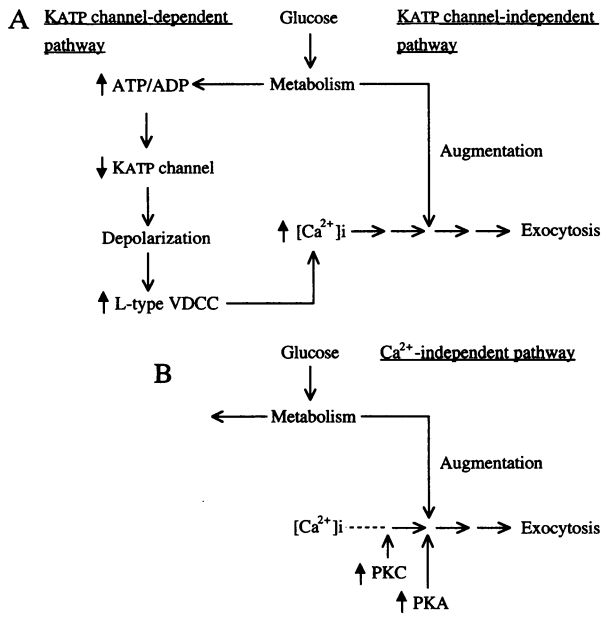

FIG. 7. Pathways of stimulus-secretion coupling for effects of glucose on insulin secretion. (*A*) K_{ATP}-channel-dependent pathway (*Left*) and K_{ATP}-channel-independent pathway (*Right*). The K_{ATP}-dependent pathway leads, via closure of the K_{ATP} channel, to increased Ca^{2+} influx, increased $[Ca^{2+}]_i$, and increased exocytosis. The K_{ATP}-channel-independent pathway augments the effect of increased $[Ca^{2+}]_i$ on exocytosis at a distal site in stimulus-secretion coupling. The effect of both pathways to increase insulin release is Ca^{2+} dependent. (*B*) Ca^{2+}-independent, K_{ATP}-channel-independent pathway for effect of glucose on insulin secretion. In the presence of activated PKC, and even more so in the presence of simultaneously activated PKA and PKC, glucose exerts a large stimulatory effect on insulin secretion. This occurs even after severe Ca^{2+} deprivation under Ca^{2+}-free conditions.

PKC on insulin secretion. On the other hand, it is possible that the strong activation of PKA and PKC that has been imposed in this study is mimicking, and thereby replacing, the effect of elevated $[Ca^{2+}]_i$. In this case, the augmentation of release demonstrated here could be due to the same mechanism that underlies the K_{ATP}-channel-independent effect of glucose, whatever that mechanism might be.

In summary, the mechanisms by which glucose stimulates insulin secretion can be divided into two main pathways. These are described as K_{ATP}-channel dependent and K_{ATP}-channel independent. The former has a fairly well-described mechanism involving closure of the K_{ATP} channel, depolarization of the cell membrane, increased Ca^{2+} entry, elevated $[Ca^{2+}]_i$, and consequent stimulation of secretion. The K_{ATP}-channel-independent pathway is less well defined and its mode of action is unknown. Until now it was thought that the pathway required an elevated $[Ca^{2+}]_i$ for it to be expressed. With the demonstration in this paper of an effect of glucose to augment insulin secretion even under severely Ca^{2+}-depleted conditions and in the absence of an increase in $[Ca^{2+}]_i$, two possibilities arise. The first is that only one K_{ATP}-channel-independent pathway exists and that elevated $[Ca^{2+}]_i$ and activated PKA/PKC both act on, or lead to, a common site in the pathway. It is on this site, or a more distal site in the pathway, that glucose exerts its K_{ATP}-channel-independent effect. The second possibility is that two K_{ATP}-channel-independent pathways exist in glucose stimulus-secretion coupling. One of these would be Ca^{2+} dependent and one would be Ca^{2+} independent. If this proves to be the case, then the actions of PKC and PKA appear to be permissive for the augmentation effect of glucose. These possibilities are illustrated in Fig. 7.

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