Glucose stimulates voltage- and calcium-dependent inositol trisphosphate production and intracellular calcium mobilization in insulin-secreting β TC3 cells

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The cellular processes leading to a rise in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) after glucose stimulation and K^+ depolarization were investigated in insulin-secreting β TC3 cells. Stimulation with 11.2 mM glucose causes inositol 1,4,5-trisphosphate production and release of Ca^{2+} from intracellular stores. A strong correlation was observed between the changes in $\text{Ins}(1,4,5)\text{P}_3$ concentration and the rise in $[\text{Ca}^{2+}]_i$, consistent with the former compound being responsible for release of Ca^{2+} from intracellular stores. The increase in $\text{Ins}(1,4,5)\text{P}_3$ production was reduced by $68 \pm 4\%$ when $[\text{Ca}^{2+}]_i$ was kept low on glucose stimulation by loading cells with the Ca^{2+} chelator 1,2-bis(2-aminophenoxy)ethane- $\text{NNN}'\text{N}'$ -tetra-acetic acid (BAPTA). The $\text{Ins}(1,4,5)\text{P}_3$ production was prevented in cells hyperpolarized with diazoxide, an opener of ATP-sensitive K^+ -channels, con-

sistent with the membrane potential controlling the rate of $\text{Ins}(1,4,5)\text{P}_3$ synthesis. Depolarizing K^+ concentrations evoked changes in $[\text{Ca}^{2+}]_i$ and $\text{Ins}(1,4,5)\text{P}_3$ production in both the presence and the absence of extracellular Ca^{2+} , and from the relation between the extracellular K^+ concentration and membrane potential we found a half-maximal $\text{Ins}(1,4,5)\text{P}_3$ production by a 28 mV depolarization from a resting potential of -56 mV and by a rise in $[\text{Ca}^{2+}]_i$ of 390 nM. We conclude that stimulation-induced changes in membrane potential and $[\text{Ca}^{2+}]_i$ are important in controlling $\text{Ins}(1,4,5)\text{P}_3$ production in β TC-3 cells and that glucose-stimulated Ca^{2+} mobilization from intracellular stores is due to voltage-dependent $\text{Ins}(1,4,5)\text{P}_3$ production and depends on the concurrent increase in $[\text{Ca}^{2+}]_i$.

INTRODUCTION

In the pancreatic β -cell there is clear evidence that $\text{Ins}(1,4,5)\text{P}_3$ is the cellular messenger inducing Ca^{2+} mobilization from intracellular stores by binding to specific receptors located on intracellular Ca^{2+} stores [1–4]. Calcium ions play a central role in stimulus–secretion coupling in pancreatic β -cells, and an increase in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is necessary for the fusion of the secretory granules with the plasma membrane causing insulin release [5,6]. The role for $\text{Ins}(1,4,5)\text{P}_3$ in glucose-induced changes in $[\text{Ca}^{2+}]_i$ remains unclear. It has been proposed that activation of phospholipase C (PLC) by Ca^{2+} itself accounts for most of the $\text{Ins}(1,4,5)\text{P}_3$ generated in response to glucose stimulation [3]. However, glucose may exert effects on inositol phosphate metabolism independently of Ca^{2+} , the nature of which remain unestablished [3,7]. Recent studies suggest that glucose stimulates release of intracellular Ca^{2+} [8] via a voltage-dependent intracellular Ca^{2+} release mechanism, possibly through increasing $\text{Ins}(1,4,5)\text{P}_3$ production [9].

In the pancreatic β -cell the cellular regulation of $\text{Ins}(1,4,5)\text{P}_3$ production is not well understood and it is the aim of this study to characterize the role of membrane potential (V_m) and $[\text{Ca}^{2+}]_i$ on the rate of $\text{Ins}(1,4,5)\text{P}_3$ production. To investigate these processes we used the insulin-secreting β -cell line β TC3 as a model for the pancreatic β -cell, to measure changes in cellular inositol phosphate content, $[\text{Ca}^{2+}]_i$ and V_m in response to elevated

glucose and extracellular K^+ concentrations ($[\text{K}^+]_o$). Here we demonstrate that glucose and K^+ depolarization-evoked $[\text{Ca}^{2+}]_i$ responses are complex processes involving voltage-dependent $\text{Ins}(1,4,5)\text{P}_3$ production and Ca^{2+} mobilization from internal stores, combined with Ca^{2+} influx through voltage-dependent Ca^{2+} channels.

EXPERIMENTAL

Cell culture

The β TC3 cells, derived from a transgenic mice insulinoma [10], were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 1000 mg/l D-glucose, 10% heat-inactivated fetal calf serum, 50 i.u./ml penicillin, 0.05 mg/ml streptomycin and 2 mM L-glutamine, and incubated in humidified air/ CO_2 , 19:1. Media were changed twice a week and cells were passaged once each week. All experiments were performed with cells from passage 30–45.

Measurements of $[\text{Ca}^{2+}]_i$

Images of $[\text{Ca}^{2+}]_i$ in single β TC3 cells were obtained with a fluorescence microscope (Zeiss Axiovert 135) and a digital image-processing system (Universal Imaging) as previously described [11]. Cells were loaded with $3 \mu\text{M}$ fura-2/AM for 40 min at 37°C

Abbreviations used: BAPTA/AM, 1,2-bis(2-aminophenoxy)ethane- $\text{NNN}'\text{N}'$ -tetra-acetic acid acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; $[\text{Ca}^{2+}]_o$, extracellular free Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium; $[\text{K}^+]_o$, extracellular K^+ concentration; PLC, phospholipase C; V_m , membrane potential.

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Figure 1 Effect of glucose on $[Ca^{2+}]_i$ in insulin-secreting β TC3 cells

Glucose-evoked changes in $[Ca^{2+}]_i$ in single β TC3 cells in (A) a Ca^{2+} -containing medium and (B) a low- Ca^{2+} medium ($[Ca^{2+}]_0$ approx. 100 nM). Effect of diazoxide (400 μ M) on the glucose-induced increase in $[Ca^{2+}]_i$ in (C) a low- Ca^{2+} medium and (D) a high- Ca^{2+} -medium. (D) No effect of 25 μ M nifedipine on glucose (11.2 mM) stimulated increase in $[Ca^{2+}]_i$ in a cell resuspended in a low- Ca^{2+} medium. Each trace is representative of 14–46 experiments from four to seven individual experiments.

and washed twice before the experiment with extracellular medium containing (in mM): 140 Na^+ , 4 K^+ , 2 Ca^{2+} , 1 Mg^{2+} , 150 Cl^- , 10 HEPES, pH 7.3. The Ca^{2+} -free medium contained no Ca^{2+} and had 100 μ M EGTA added unless otherwise stated. The recording chamber was continuously superfused with control or test solution at rates between 0.1 and 2 ml/min at 37 °C. Images were obtained by an oil-immersion UV objective (Zeiss Achrostat $\times 40$, 1.30 numerical aperture) and transferred to a videotape recorder (Umatic SP, VO 9600, Sony). The collected images were averages of eight frames and the sample rate was between 0.2 and 0.5 Hz. $[Ca^{2+}]_i$ was calculated by averaging pixel grey values in $4 \mu m \times 4 \mu m$ windows placed in the centre of the cell, and analysing pixel values as described below.

The intracellular free Ca^{2+} concentrations were calculated from the measurements of the ratio of fluorescence intensities according to the equation [12]:

$$[Ca^{2+}]_i = K_d[(R - R_{min.}) / (R_{max.} - R)](S_{f2}/S_{b2})$$

The grey pixel values obtained from the β TC3 cells for $R_{max.}$ amounted, on average, to 241. These were obtained by equilibrating Ca^{2+} across the plasma membrane with ionomycin (1 μ M). $R_{min.}$ and the proportionality constant (S_{f2}/S_{b2}) were obtained from a high- K^+ medium free of Ca^{2+} with 10 mM EGTA and fura 2 pentapotassium salt, and were 27 and 9.8 respectively. A value of 224 nM was used for the apparent K_d of Ca^{2+} binding to fura 2 [12].

Measurements of inositol phosphate levels

Inositol phosphate levels were measured in cells grown in Nunc 6-well multidishes (35 mm) for 3 days. The cells were loaded for 24 h with 10 μ Ci/ml $[^3H]$ myo-inositol in modified DMEM without glucose and inositol. After incubation the cells were washed

three times in modified DMEM without glucose and with 2 mM myo-inositol. After stimulation the reaction was terminated at appropriate times by the addition of ice-cold trichloroacetic acid to a final concentration of 0.7 M. After the samples had been transferred to test tubes (1.5 ml) and centrifuged at 12000 g for 1 min, the supernatants were decanted to 10 ml conical glass tubes and trichloroacetic acid was extracted with diethyl ether.

Inositol phosphates were separated by HPLC on an anion-exchange column (Vydac 303NT405) as described elsewhere [13]. The data are presented as a percentage of the total inositol phosphate release. All experiments were carried out at 37 °C.

Electrophysiology

Membrane-potential recordings were made by using the perforated-patch whole cell configuration [14] (amphotericin B technique) with an EPC-9 patch-clamp amplifier (Heka Elektronik G.m.b.H., Lambrecht, Germany). The data were stored on videotape and displayed directly on a chart recorder (DASH IV, Astro Med, West Warwick, RI, U.S.A.). The pipette solution consisted of (in mM): 76 K_2SO_4 , 10 $NaCl$, 10 KCl , 1 $MgCl_2$, 5 HEPES, pH 7.35 (KOH) and 0.24 mg/ml amphotericin B.

Chemicals

Fura 2 acetoxymethyl ester, fura 2 pentapotassium salt and 1,2-bis(2-aminophenoxy)ethane- NNN' -tetra-acetic acid acetoxymethyl ester (BAPTA/AM) were obtained from Molecular Probes (Eugene, OR, U.S.A.); U73122 and U73343 from Biomol (Plymouth Meeting, PA, U.S.A.); EGTA from Fluka (Buchs, Switzerland); ionomycin from Calbiochem (San Diego, CA,

U.S.A.); DMEM, trypsin, penicillin, streptomycin, and L-glutamine from Gibco (Roskilde, Denmark); fetal calf serum from A. H. Diagnostic (Aarhus, Denmark); and [³H]myo-inositol from NEN Products (Boston, MA, U.S.A.). All other chemicals were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

Statistical analysis

All data are expressed as mean values $\pm$ S.E.M. for n tested cells.

RESULTS

Effect of glucose on [Ca²⁺]_i

Stimulation of β TC3 cells with 11.2 mM glucose causes an increase in [Ca²⁺]_i, which is typically 200–400 nM in amplitude from a resting level of 187 ± 6 nM ($n = 176$) and has a latency of 30–90 s. A glucose-induced rise in [Ca²⁺]_i was, however, only observed in a fraction (18%) of the tested cells, as described previously [11]. Short-duration Ca²⁺ spikes were frequently superimposed on the sustained increase in [Ca²⁺]_i (Figure 1A). When β TC3 cells were resuspended in a low-Ca²⁺ medium ([Ca²⁺]_o approx. 100 nM), raising external glucose to 11.2 mM surprisingly caused an increase in [Ca²⁺]_i, although with a reduced amplitude (Figure 1B). Addition of diazoxide (400 μ M), an opener of ATP-sensitive K⁺-channels, suppressed the increase in [Ca²⁺]_i in cells resuspended in a low-Ca²⁺ medium (Figure 1B) as

well as in a high-Ca²⁺ medium (Figure 1C). We found that diazoxide (400 μ M) hyperpolarized the β TC3 cell membrane potential from -56 ± 5 to -74 ± 4 mV ($n = 5$). This indicates that the release of Ca²⁺ from internal stores and Ca²⁺ influx from the extracellular medium after glucose stimulation is dependent on V_m . In β TC3 cells pretreated with 25 μ M nifedipine, an L-type Ca²⁺ channel blocker, and resuspended in a low-Ca²⁺ medium we observed an increase in [Ca²⁺]_i of the same magnitude as that found in non-pretreated cells after stimulation with 11.2 mM glucose (Figure 1D), consistent with glucose causing Ca²⁺ mobilization from intracellular stores.

Effect of glucose on inositol phosphate production

Figure 2(A) shows typical HPLC profiles of [³H]inositol phosphates prepared from resting β TC3 cells and after stimulation with 11.2 mM glucose for 3 min. Inositol phosphate production was measured in cells cultured without glucose for 24 h (see the Experimental section). However, such prolonged glucose deprivation did not affect their subsequent response to glucose, because no differences in their Ca²⁺ responses to glucose stimulation (11.2 mM) and K⁺ depolarization (45 mM) were observed compared with control cells. Figure 2(B–D) shows the time dependencies of changes in all the different inositol phosphates separable with this HPLC procedure after stimulation with

Figure 2 Changes in inositol phosphate contents after glucose stimulation

(A) HPLC elution profile of the different ³H-labelled inositol phosphates prepared from β TC3 cells at rest (solid line) and after stimulation with 11.2 mM glucose for 3 min (●). Inositol phosphates were separated by HPLC with an anion-exchange column and eluted with a 0–100% gradient of NaH₂PO₄ (broken line) at a flow rate of 3 ml/min; 55 fractions each containing 1 ml were collected. To determine the retention time of each inositol phosphate, the column was calibrated with various [³H]inositol phosphate standards. (B, C and D) Time dependencies of the changes in cellular inositol phosphate contents after glucose stimulation (11.2 mM). The ordinate shows how much (as a percentage) a given inositol phosphate contributes to the sum of all the inositol phosphates produced when measured 3 min after stimulation. Points are means $\pm$ S.E.M. for three experiments. Where error bars are not visible they are masked by the data points.

Figure 3 Correlation between changes in Ins(1,4,5) P_3 and $[Ca^{2+}]_i$ in β TC3 cells after stimulation with 11.2 mM glucose

(A) Average changes in $[Ca^{2+}]_i$ in 46 glucose-responsive β TC3 cells in a Ca^{2+} -containing medium. (B) Changes in the cellular Ins(1,4,5) P_3 content after stimulation with 11.2 mM glucose. Points are means $\pm$ S.E.M. for three experiments.

11.2 mM glucose. We found considerable amounts of inositol phosphates in the unstimulated state and observed glucose stimulation (11.2 mM) to cause a rapid rise in the Ins(1,4,5) P_3 content, followed by a decline towards the prestimulatory level reaching approx. 50 % of its maximal value within 10 min. The Ins(1,3,4,5) P_4 content showed a large increase (from approx. 3 % to 30 % of the total inositol phosphate content within 3 min) but with a slower time course than Ins(1,4,5) P_3 . The Ins(1,3,4) P_3 content increased with a time dependence similar to that of Ins(1,4,5) P_3 although no subsequent decrease was observed after stimulation. By contrast, the Ins(1,4) P_2 content displayed a slow increase (from 10 % to 20 % of total inositol phosphate release) reaching a constant value less than 5 min after stimulation. It is also evident from Figure 2(B) that a large fraction of the inositol phosphates formed during stimulation accumulated in the cells as Ins4P.

We have investigated the correlation between glucose-induced changes in $[Ca^{2+}]_i$ and the Ins(1,4,5) P_3 content in greater detail. Figure 3(A) shows the average changes in $[Ca^{2+}]_i$ in all tested glucose-responsive β TC3 cells: it is evident that $[Ca^{2+}]_i$ starts to increase after 0.5 min and reaches a maximum level 1 min after glucose stimulation (11.2 mM), which remains essentially the same for up to 3 min. The question arises whether or not the cellular Ins(1,4,5) P_3 content rises rapidly enough to account for the Ca^{2+} release processes. We therefore measured the Ins(1,4,5) P_3 content shortly after stimulation with 11.2 mM glucose and it seems that the rate of increase closely follows the rate of increase in $[Ca^{2+}]_i$ (Figure 3B). The rise in cellular Ins(1,4,5) P_3 content starts after 0.5 min and a full response is observed 1 min after

Figure 4 Effect of $[Ca^{2+}]_i$ and diazoxide on glucose-stimulated Ins(1,4,5) P_3 production in β TC3 cells

Cells were stimulated in a Ca^{2+} -containing medium (●), after incubation with BAPTA/AM (50 μ M for 20 min) and resuspension in a low- Ca^{2+} medium (○), or after pretreatment with 400 μ M diazoxide (▽). Points are means $\pm$ S.E.M. for three experiments.

stimulation. The Ins(1,4,5) P_3 content does not level off within the measured time interval (3 min) as shown in Figure 3(B).

Effect of $[Ca^{2+}]_i$ and V_m on glucose-evoked inositol phosphate production

To investigate whether a change in $[Ca^{2+}]_i$ by itself plays a role in the generation of Ins(1,4,5) P_3 after glucose stimulation we incubated β TC3 cells with BAPTA/AM (50 μ M for 20 min), which completely prevented a rise in $[Ca^{2+}]_i$, and resuspended the cells in a Ca^{2+} -free medium with EGTA. Glucose was still capable of stimulating Ins(1,4,5) P_3 release with a time dependence similar to that observed in experiments where $[Ca^{2+}]_i$ is rising (Figure 4). However, the overall amount of Ins(1,4,5) P_3 released to the cytosol was decreased by 68 ± 4 % ($n = 3$).

To characterize the role of V_m in the cellular regulation of the Ins(1,4,5) P_3 synthesis seen in the absence of a rise in $[Ca^{2+}]_i$, we pretreated cells with diazoxide (400 μ M). Under this condition the rise in Ins(1,4,5) P_3 content after stimulation with 11.2 mM glucose was abolished (Figure 4). Thus the data reveal that glucose stimulation causes Ins(1,4,5) P_3 production that to a large extent depends on the concurrent increase in $[Ca^{2+}]_i$ but is controlled by changes in V_m .

Effect of depolarizing V_m on Ca^{2+} signalling and inositol phosphate production

Because glucose stimulates Ins(1,4,5) P_3 production and because the subsequent rise in $[Ca^{2+}]_i$ depends on V_m , we have investigated the possibility that membrane depolarization with elevated $[K^+]_o$ can cause Ins(1,4,5) P_3 production and Ca^{2+} release from intracellular stores. Figure 5(A) depicts a typical time course of the rise in $[Ca^{2+}]_i$ in a cell exposed to 45 mM K^+ . The $[Ca^{2+}]_i$ increased in all cells tested, reaching an average peak amplitude of 703 ± 58 nM ($n = 35$). In a Ca^{2+} -free medium with 100 μ M EGTA, we still observed a small transient increase in $[Ca^{2+}]_i$ in response to depolarization with 45 mM K^+ (Figure 5B). The changes in $[Ca^{2+}]_i$ showed considerable cell-to-cell variability and

Figure 5 Effects of KCl on $[Ca^{2+}]_i$ and Ins(1,4,5) P_3 synthesis

Typical time courses of changes in cellular Ins(1,4,5) P_3 content and $[Ca^{2+}]_i$ in cells exposed to 45 mM K^+ . β TC3 cells were resuspended in either a Ca^{2+} -containing (A) or a Ca^{2+} -free medium with 100 μ M EGTA (B) before elevation of the extracellular K^+ concentration from 4 to 45 mM. Each trace is representative of 35 or 36 cells from seven different experiments. (C) Ins(1,4,5) P_3 production in response to depolarization with 45 mM K^+ . The cells were stimulated in a Ca^{2+} -containing medium (●) or when $[Ca^{2+}]_i$ was kept low with BAPTA/AM (50 μ M for 20 min) and after resuspension in a Ca^{2+} -free medium with 100 μ M EGTA (○). The points are means $\pm$ S.E.M. for three experiments.

the responses were between 80 and 130 nM in amplitude ($n = 36$). In cells pretreated for 10 min with 5 μ M U73122, an inhibitor of PLC-coupled processes [15,16], the Ca^{2+} transients in response to depolarizing V_m with 45 mM K^+ were abolished. U73343, a close structural analogue of U73122, had no effect on the increase in $[Ca^{2+}]_i$ after K^+ (45 mM) depolarization (results not shown). These data are consistent with a depolarization of V_m causing activation of the PLC reaction, leading to production of inositol phosphates, which subsequently causes release of Ca^{2+} from intracellular stores.

To investigate further the involvement of PLC in the cellular response to membrane depolarization we measured Ins(1,4,5) P_3 production in response to depolarization with 45 mM K^+ in the presence of extracellular Ca^{2+} and under conditions where $[Ca^{2+}]_i$ is kept low with BAPTA and chelation of extracellular Ca^{2+} with EGTA (Figure 5C). The magnitudes of the increases in the

Figure 6 Relationship between changes in $[Ca^{2+}]_i$, membrane potential and Ins(1,4,5) P_3 production after depolarization with high- K^+

(A) Changes in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$). (B) Changes in membrane potential (ΔV_m) 5 s after high- K^+ depolarization (●) and the predicted changes in β -cell V_m from the Nernst equation (broken line). Each trace is representative of 31 and 39 cells from seven and nine experiments respectively. (C) Ins(1,4,5) P_3 responses when measured 5 s after stimulation in a Ca^{2+} -containing medium (●) and when Ca^{2+} was kept low during stimulation (○). The broken line represents the difference between the measured values under Ca^{2+} -containing and Ca^{2+} -free conditions. Points are means $\pm$ S.E.M. for three experiments.

individual inositol phosphate levels are similar to those observed after glucose stimulation but with a much faster time course. The Ins(1,4,5) P_3 content was maximal after 5 s and then started to decline towards the prestimulatory level (Figure 5C). The magnitude and time course of Ins(1,4,5) P_3 production in response to depolarization of V_m (45 mM K^+) in cells pretreated with 400 μ M diazoxide were unaffected and increased to the same extent as cells not pretreated with diazoxide (results not shown). This excludes the possibility that the lack of Ins(1,4,5) P_3 synthesis in diazoxide-treated cells after glucose stimulation (compare Figure 4) was due to some adverse effect of diazoxide on the PLC reaction.

Figure 6 shows the relationship between changes in $[Ca^{2+}]_i$, V_m and Ins(1,4,5) P_3 production during depolarization with different extracellular K^+ concentrations measured 5 s after stimulation. Figure 6(C) shows the Ins(1,4,5) P_3 responses in a Ca^{2+} -containing medium and when $[Ca^{2+}]_i$ was kept low with BAPTA and chelation of extracellular Ca^{2+} with EGTA. Under the latter condition, which shows the effect of changes in V_m on Ins(1,4,5) P_3 pro-

duction, we found a half-maximal effect for the activation of the PLC reaction of approx. 28 mV depolarization (approx. 17 mM K^+) from a resting potential of -56 mV (Figures 6B and 6C). By subtracting the measured values at the different K^+ concentrations under Ca^{2+} -containing and Ca^{2+} -free conditions, we determined the Ca^{2+} dependency of the PLC reaction. From these data it can be seen that a half-maximal effect for Ca^{2+} -induced $Ins(1,4,5)P_3$ production is an approx. 390 nM rise in $[Ca^{2+}]_i$ (or approx. 16 mM K^+) (Figures 6A and 6C).

DISCUSSION

In this study we found that addition of glucose to a final concentration of 11.2 mM causes $Ins(1,4,5)P_3$ production and release of Ca^{2+} from intracellular stores in pancreatic β TC3 cells. The increase in $Ins(1,4,5)P_3$ production results from membrane depolarization and is sensitive to changes in $[Ca^{2+}]_i$. An essential finding in the present study is that PLC activation, and consequently $Ins(1,4,5)P_3$ production, is initiated by small changes in V_m and $[Ca^{2+}]_i$. In response to elevated $[K^+]_o$ the cellular $Ins(1,4,5)P_3$ production was half-maximal for an approx. 28 mV membrane depolarization and by an approx. 390 nM rise in $[Ca^{2+}]_i$.

The present study supports earlier work with islets, showing that Ca^{2+} -mediated $Ins(1,4,5)P_3$ production is important after glucose stimulation [3]. However, this effect seems to be secondary to $Ins(1,4,5)P_3$ production due to glucose-induced changes in V_m . Our data therefore confirm and extend the results of Roe et al. [9] on mouse β -cells that a component of the glucose-induced rise in $[Ca^{2+}]_i$ represents voltage-dependent intracellular Ca^{2+} release.

It is important to emphasize that in the present and previous study on β TC3 cells [11] it was only a subpopulation (18%) of the tested cells that responded to glucose stimulation with a rise in $[Ca^{2+}]_i$. Inositol phosphate production as measured in the present study represents the average of a whole cell population constituting both glucose responsive and non-responsive cells. Because the non-responsive β TC3 cells are defined by their lack of Ca^{2+} response to glucose stimulation, these cells do not contribute to the ' Ca^{2+} -dependent' component of the observed inositol phosphate production after glucose stimulation. Furthermore measurements of the membrane potential in non-responsive β TC3 cells (with the perforated patch-clamp technique) revealed only a minor membrane depolarization after stimulation with 11.2 mM glucose. This small membrane depolarization in the non-responsive cells affects the 'membrane potential-dependent' component of the glucose-induced inositol phosphate production to only a small extent. Thus the measured inositol phosphate production after glucose stimulation results from the glucose-responsive cells, which is also the subpopulation of cells described in the Ca^{2+} measurements. The large population of non-responding cells should thus not contribute to synthesis of inositol phosphates.

In the β -cell there seem to be different mechanisms available for stimulating $Ins(1,4,5)P_3$ production: (1) a direct activation involving coupling between a plasma membrane receptor and a PLC enzyme, (2) through changes in V_m and (3) through modulation of PLC activity by changes in $[Ca^{2+}]_i$, possibly affecting different PLC isoenzymes. With regard to the mechanism of PLC activation it is interesting to note that all major PLC isoforms (β , ϵ and γ) are present in β TC3 cells [17]. It is generally accepted that PLC- β couples to G-proteins whereas PLC- γ interacts with tyrosine kinase-linked receptors [18,19]. The mode of activation of PLC- ϵ is unknown [20,21]. It might represent a calcium-sensitive form as it contains one EF-hand motif [22]. With regard to changes in V_m affecting the PLC

reaction it is noteworthy that none of the known PLC isoforms contains a membrane-spanning sequence. However, the phospholipid substrates for these enzymes are all in membranes and reversible membrane binding of PLC is thought to explain changes in phosphatidylinositol turnover on stimulation of cells [20,23]. Exactly how V_m controls $Ins(1,4,5)P_3$ production remains to be elucidated. Both direct and indirect effects of V_m could be envisaged. However, there is evidence for direct regulation of enzyme activity by changes in V_m : the adenylate cyclase of the monocellular organism *Paramecium*, which functions as a K^+ -channel, is stimulated by hyperpolarization [24]. Furthermore, in skeletal muscle $Ins(1,4,5)P_3$ may be produced in response to membrane depolarization [25] and in smooth muscle cells hyperpolarizing V_m reduces agonist-induced accumulation of $Ins(1,4,5)P_3$ and consequently Ca^{2+} mobilization from intracellular stores [26].

A hypothesis for explaining voltage-dependent inositol phosphate production in β TC3 cells is that membrane depolarization causes a rearrangement of $PtdIns(4,5)P_2$ in the plasma membrane, resulting in better availability of this phospholipid to PLC. In addition, one could imagine that depolarization of V_m enhances the association between PLC and plasma membrane constituents, leading to an increased translocation of PLC to the plasma membrane. Thus the combination of better availability of substrate to PLC with increased PLC translocation to the plasma membrane could explain the increased $Ins(1,4,5)P_3$ production observed in the present study after membrane depolarization. In contrast, hyperpolarization would diminish $PtdIns(4,5)P_2$ availability and abolish PLC translocation to the plasma membrane, thereby preventing stimulation of the PLC reaction and consequently $Ins(1,4,5)P_3$ production.

The actions of $Ins(1,4,5)P_3$ are mediated by specific receptors identified in different cellular locations [18,27–31]. However, the recent demonstration of $Ins(1,4,5)P_3$ receptors on insulin secretory granules [27] implies a novel and very important mechanism of action for $Ins(1,4,5)P_3$ in regulating insulin secretion from the β -cell [32]. Release of Ca^{2+} from the secretory granules would cause a localized Ca^{2+} transient necessary for exocytosis of granules immediately adjacent to the plasma membrane. A more pronounced $Ins(1,4,5)P_3$ -induced increase in $[Ca^{2+}]_i$ would augment the secretory response by facilitating the movement of secretory granules into a position ready for exocytosis. Such a mechanism assigns further significance to $Ins(1,4,5)P_3$ in glucose-stimulated insulin secretion.

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