Original Article

Dynamics of Glucose-Induced Localization of PKC Isoenzymes in Pancreatic β-Cells

Diabetes-Related Changes in the GK Rat

Nasim Warwar,1 Suad Efendic,2 Claes-Göran Östenson,2 Esther P. Haber,1 Erol Cerasi,1 and Rafael Nesher1

Glucose metabolism affects most major signal pathways in pancreatic β-cells. Multiple protein kinases, including protein kinase C (PKC) isoenzymes, are involved in these effects; however, their role is poorly defined. Moreover, the dynamics of kinase isoenzyme activation in reference to the biphasic insulin secretion is unknown. In perfused pancreas of Wistar rats, PKCα staining was strongly associated with insulin staining, jointly accumulating in the vicinity of the plasma membrane during early first-phase insulin response. The signal declined before the onset of second phase and reappeared during second-phase insulin release as foci, only weakly associated with insulin staining; this signal persisted for at least 15 min after glucose stimulation. In the GK rat, glucose had minimal effect on β-cell PKCs. In control β-cells, PKCζ stained as granulated foci with partial association with insulin staining; however, no glucose-dependent translocation was observed. In the GK rat, only minimal staining for PKCζ was observed, increasing exclusively during early first-phase secretion. In Wistar β-cells, PKCε concentrated near the nucleus, strongly associated with insulin staining, with dynamics resembling that of biphasic insulin response, but persisting for 15 min after cessation of stimulation. In GK rats, PKCε staining corresponded to changes or association with insulin. PKCζ exhibited bimodal dynamics in control β-cells: during early first phase, accumulation near the cell membrane was observed, dispersing thereafter. This was followed by a gradual accumulation near the nucleus; 15 min after glucose stimulus, clear PKCζ staining was observed within the nucleus. In the GK rat, a similar response was only occasionally observed. In control β-cells, glucose stimulation led to a transient recruitment of PKCθ, associated with first-phase insulin release, not seen in GK β-cell. Data from this and related studies support a role for PKCε in glucose-induced insulin granule recruitment for exocytosis; a role for PKCζ in activation of insulin granules for exocytosis and/or in the glucose-generated time-dependent potentiation signal for insulin release; and a dual function for PKCζ in initiating insulin release and in a regulatory role in the transcriptional machinery. Furthermore, diminished levels and/or activation of PKCα, PKCε, PKCθ, and PKCζ could be part of the defective signals downstream to glucose metabolism responsible for the deranged insulin secretion in the GK rat. Diabetes 55: 590–599, 2006

Glucose metabolism is the primary initiator and regulator of most pancreatic β-cell functions: multiple β-cell genes are controlled by signals originating from glucose metabolism, as are all functions related to production, storage, and exocytosis of insulin. While the sequence of events involved in initiating glucose-induced insulin response have been well studied, those of extended release (second phase; see below) or insulin biosynthesis are considerably less clear. Multiple protein kinases are known to participate in relaying downstream signals of glucose metabolism; however, the precise role of most of them is poorly defined. When activated, most protein kinases, including the protein kinase C (PKC) family, translocate to the site of their effector and bind an anchoring protein, which keeps them in close proximity with their downstream effector (1–4). Signal termination usually results in dissociation of the kinase from its scaffold protein (1–4). Several isoforms of PKC were identified in pancreatic β-cells and many have been reported to undergo translocation subsequent to stimulation by glucose, acetylcholine, glucagon-like peptide 1, or fatty acids (5–10). We have shown that selective inhibition of the translocation of PKCε or PKCζ partially inhibits glucose-induced insulin release (5).

When subjected to extended (>20 min) glucose stimulation, the healthy pancreas responds with a typical biphasic insulin response that is believed to reflect the net sum of messages that initiate, amplify, and inhibit insulin release (11,12). Type 2 diabetes is characterized by a diminished first-phase insulin response to glucose and a progressively delayed and diminished second-phase insulin secretion. In an attempt to understand the role of PKC isoenzymes in the dynamic regulation of glucose-induced insulin release, we studied the activation and translocation of six PKC isoenzymes in the isolated perfused rat pancreas, a most sensitive isolated organ preparation, which permits a close approximation of the in vivo situation. Using digital confocal histochemistry, we determined the relationship between glucose-induced biphasic insulin re-
lease and the β-cell localization of specific PKC isoenzymes both in the normal pancreas and in the pancreas of the GK rat, an animal model for type 2 diabetes presenting diminished glucose-induced insulin secretion (13–15).

RESEARCH DESIGN AND METHODS

Male Wistar and GK rats (Karolinska Institute Colony) weighing 250–300 g were anesthetized by intraperitoneal injection of 100 mg/kg thiopentone sodium. Pancreata were isolated and perfused as previously described (16,17). Mean (nonfasting) morning blood glucose in GK rats was 7.2 ± 0.4 mmol/l as compared with 4.7 ± 0.1 mmol/l in age-matched control Wistar rats. Animal use was with full adherence to Institutional Ethical Committee guidelines. At specific perfusion time points, shown in Fig. 1, the pancreas was fixed with 4% formaldehyde in PBS by perfusion (18) and stored at 4°C in PBS.

Immunohistochemical staining. Pancreatic sections were deparaffinized by three immersions, 5 min each in xylene, twice for 2 min in 100% ethanol, and 2 min in 95, 90, 80, and 70% ethanol and water, once each. Sections were next washed three times in PBS for 5 min, blocked in 10% nonimmune goat serum for 1 h, and treated overnight with the specific PKC isoenzyme antibody. Following three washes in PBS for 5 min, the second antibody was added for 1.5 h at 37°C, with all subsequent procedures being performed in the dark. For counterstaining, the sections were washed three times in PBS for 5 min, incubated at 37°C for 1 h with anti-insulin, and followed by second antibody for 1.5 h. Following three washes of 5 min in PBS, the slides were mounted in 90% glycerol in PBS. All stainings were performed in pairs of sections of the isolated perfused pancreata from control Wistar rats and squares from GK rats. Arrows indicate time points selected for histochemical analysis of distribution of PKC isoenzymes in pancreatic β-cells. 0 time: basal release, before onset of glucose stimulus; 2 min: during accelerated release rate, rise toward peak first phase; 5 min: decline in release rate toward nadir between first- and second-phase insulin response; 15 min: accelerating release rate toward peak second-phase insulin response; 75 min: near basal, poststimulatory release rate. Bottom panel: Insulin release rate relative to basal (3.3 mmol/l glucose) release rate. Each point represents mean ± SE of 4–20 isolated perfused pancreata.

Digital confocal microscopy imaging. A Zeiss LSM 410 confocal laser scanning system attached to the Zeiss Axiovert 135 M inverted microscope was utilized. The system was equipped with a 25-mW air-cooled argon laser (488-nm excitation line with 51-mW long-pass barrier filter) for the excitation of green fluorescence. Red fluorescence was excited with helium-neon lasers at 543-nm line with 570-nm long-pass filter or 633-nm line with 665-nm emission filter. Double- or triple-labeled specimens were excited with laser lines of 488 and 543, 488 and 633, or 543 and 633 nm and monitored simultaneously using double or triple detectors and the Zeiss-supplied filter block combinations with dichroic beam splitters and emission filters specific for each fluorescence combination. In the same experiments, images were collected with Nomarski differential interference contrast optics using transmitted light detectors. The excitation powers (output voltage of laser beam) and emission filters were tuned to keep the overlapping from each channel at a minimum. The laser illumination intensity was attenuated by an interfering variable neutral density filter placed in the illumination path. The pinhole aperture was set to give the best signal-to-noise ratio and best image resolution, thus optimizing optical sectioning. Specimens’ autofluorescence was set to background level. To reduce the visual noise, each confocal optical section was performed in the fast line-scan acquisition mode (512 pixels/line) by averaging 8 or 16 images before the final image was produced on the monitor. In each experiment, exciting light intensity, background level, photomultiplier gain, imaging filters, aperture, contrast, and electronic zoom were adjusted by averaging 8 or 16 images before the final image was produced on the monitor. In each experiment, exciting light intensity, background level, photomultiplier gain, imaging filters, aperture, contrast, and electronic zoom were adjusted.
TABLE 1
Association between PKC isoenzymes and insulin granules during glucose stimulus

<table>
<thead>
<tr>
<th>Time</th>
<th>0 min</th>
<th>2 min</th>
<th>5 min</th>
<th>15 min</th>
<th>75 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKCα</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar</td>
<td>72</td>
<td>92†‡</td>
<td>52†‡</td>
<td>68</td>
<td>64</td>
</tr>
<tr>
<td>GK</td>
<td>22‡</td>
<td>45†‡</td>
<td>45†‡</td>
<td>43‡‡</td>
<td>32‡‡</td>
</tr>
<tr>
<td>PKCδ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar</td>
<td>44</td>
<td>54</td>
<td>56</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>GK</td>
<td>26.5</td>
<td>28‡</td>
<td>11‡</td>
<td>33‡‡</td>
<td>17.5</td>
</tr>
<tr>
<td>PKCε</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar</td>
<td>49</td>
<td>82†‡</td>
<td>51*</td>
<td>66</td>
<td>82†‡</td>
</tr>
<tr>
<td>GK</td>
<td>23‡</td>
<td>21‡</td>
<td>20‡</td>
<td>24‡‡</td>
<td>30‡‡</td>
</tr>
<tr>
<td>PKCθ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>23</td>
<td>35</td>
<td>23</td>
<td>26</td>
<td>17</td>
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<tr>
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<td>11‡‡</td>
<td>10‡</td>
<td>9†‡</td>
<td>9‡</td>
</tr>
<tr>
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</tr>
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<td>Wistar</td>
<td>41</td>
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<tr>
<td>GK</td>
<td>30</td>
<td>28</td>
<td>43</td>
<td>25</td>
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</table>

Sections of pancreata obtained from perfused Wistar or GK rats were fixed and double-stained for insulin and PKC isoenzymes at the indicated times, prior to (0 min), during (2, 5, 15 min), or after (75 min) a 1-h glucose (16.7 mmol/l) stimulus. Histochemical analysis was performed using confocal laser microscopy. Data are presented as percent of the fields scanned (positive fields/total recorded fields).

RESULTS

Insulin response to glucose stimulus. The GK rat pancreas showed a high basal insulin secretion rate compared with control Wistar rats when perfused with 3.3 mmol/l glucose (495 ± 83 vs. 30 ± 5 μU/min; P < 0.0001). The brisk response to 16.7 mmol/l glucose in the GK was misleading, as only 485 ± 105 μU were secreted during the first phase (initial 10 min) as compared with 2,717 ± 46 μU released during that period from the Wistar rat pancreas (P < 0.0001). During second phase, insulin release in the Wistar rat was 32,900 ± 147 vs. 16,512 ± 162 μU/50 min in the GK rat (P < 0.02) (Fig. 1, top panel). Thus, due to the high basal insulin release rate, the relative insulin response to glucose (fold of basal) was minimal during both first- and second phases of secretion in pancreata isolated from GK rats (Fig. 1, bottom panel), indicative of poor insulin responsiveness to glucose in this model of type 2 diabetes. In addition, in the GK rat, there was a transient off response in the release rate upon cessation of the glucose stimulus.

Dynamics of β-cell PKCoa distribution in response to glucose stimulus. PKCoa has been shown by us (5) and by others (19,20) to participate in the regulation of the insulin response to glucose. Figure 2A clearly shows lower levels of the isoenzyme in β-cells of GK rats. In Western blot, total islet extract from Wistar rat also indicated considerable higher levels of PKCoa as compared with GK rat (83.6-fold; Fig. 3). In Wistar rat β-cells, 2 min after initiating the glucose stimulus, at the onset of first-phase insulin response, pronounced accumulation of PKCoa was observed in the vicinity of the cell membrane (Fig. 2A). Membrane-associated staining faded off at 5 min during the decline toward the nadir between the first- and second phases of insulin release, while clear granulated staining reappeared during early second-phase (15-min) secretion. PKCoa staining was now dispersed throughout the cell, which persisted throughout the second phase (not shown) and at least for 15 min after the glucose stimulus was withdrawn (Fig. 2A). During second-phase insulin release some membrane localization of PKCoa signal was observed, albeit considerably weaker than during first phase. During the poststimulatory period of diminishing insulin release, membrane localization of PKCoa had diminished as well. In β-cells of GK rats, 16.7 mmol/l glucose initially induced only minimal membrane-associated PKCoa staining; during and subsequent to second-phase insulin response, aggregation of PKCoa similar to that in control Wistar rat β-cells was observed, but at much lower intensity. While variation in staining intensity was observed between islets in any given pancreatic section, the images of Fig. 2 represent the typical staining: analyzing over 60 fields, we observed a typical membrane accumulation of PKCoa at 2 min of stimulation in 73% of the fields of islets from Wistar rats, whereas only 12.5% of GK islets showed similar distribution at that time point. The pronounced decline seen at 5 min in Wistar islets was evident in 72% of 56 fields, and the persisting granulated structures 15 min after the cessation of glucose stimulus (75 min in Fig. 2) was observed in 70% of 42 fields.

In double imaging, PKCoa was observed to associate with staining for insulin: the isoenzyme’s signal was associated with insulin during the resting (nonstimulated) period in 64–72% of the fields, peaking at 92% of the fields toward maximal first-phase insulin release (2 min) and declining to 52% during the interphasic nadir (Table 1). In GK rats, PKCoa association with insulin staining was minimal: only 22–32% of the fields showed clear association between insulin and PKCoa during the resting period, rising to only 43–45% during the stimulatory period (Table 1). Dynamics of β-cell PKCoa distribution in response to glucose. PKCoa staining in Wistar rat β-cells revealed distinct granulated structures, but no glucose-dependent alterations (Fig. 2B). In diabetic GK rats, isoenzyme staining seemed weaker than that of Wistar β-cells. Western blot analysis of total islet extract (Fig. 3) revealed that the difference was minimal (1.6-fold). Moreover, 74% of the fields examined in sections from GK rats revealed some signal increase at 2 min of glucose stimulus. Association between insulin and PKCoa was observed in 56–60% of the fields during glucose stimulation in control rat pancreas, with somewhat lower association during resting period (30–40%) (Table 1). In sections of the GK rat pancreas no association between insulin and PKCoa was evident.
Dynamics of β-cell PKCε distribution in response to glucose. Figure 2C shows the typical changes in β-cell staining for PKCε as function of glucose stimulation. While in paired staining, GK rat β-cells normally displayed strong expression of PKCε, mostly concentrated in polar region close to the nucleus, no glucose-dependent change could be observed. In control Wistar β-cells, dynamic changes were evident: some polar concentration of PKCε in the vicinity of the nucleus could be seen at 2 min, clearly dissipating at 5 min during the decline of insulin release. Unlike the histochemical images, analysis of PKCε levels by Western blot using total islet extract revealed considerably higher levels of the isoenzyme in islets of Wistar rat as compared with GK rat, implying that most of that isoenzyme signal was derived from non-β-cells of the islets (Fig. 3). In Wistar rat β-cells, localized staining of PKCε reappeared during second-phase (15-min) insulin response (Fig. 2C), and persisted after cessation of the glucose stimulus (75 min). As compared with 12.5% of the fields of the GK rat β-cells showing decline in PKCε staining at 5 min of stimulation, 67% exhibited clear decline in the Wistar rat pancreas. In addition, staining for PKCε was associated with insulin in Wistar β-cells (Table 1): at baseline, approximately half of the fields exhibited clear PKCε association with insulin, rising to 82% during early insulin response, declining back to basal levels (51%) at 5 min, and increasing again during second-phase insulin response and remaining high 15 min after termination of the glucose stimulus. In contrast, in β-cells of the diabetic GK rats, association between PKCε and insulin was minimal: only 20–30% of the fields displayed some association, and glucose-induced changes were not observed (Table 1).

The discrepancy between paired histochemical staining of pancreatic sections and Western blot analysis of total (isolated) islet (Fig. 3) can only indicate that the majority of the PKCε signal of total islet extract came from non-β-cell sources.

Bimodal translocation of PKCζ. In β-cells of the control Wistar rat, a bimodal glucose-dependent translocation of PKCζ was observed (Fig. 2D). Closely associated with the onset of glucose stimulus (2 min), staining for PKCζ disappeared from most areas of the cell (observed in 76% of the scanned fields), while clear translocation to the cell membrane was evident in 69% of the scanned fields. Staining for PKCζ increased again during the decline in first-phase insulin response (5 min), with clear concentration in the vicinity of the nucleus by 15 min of glucose stimulation. One hour later (15 min after secession of glucose stimulation), strong concentration of PKCζ near the nucleus was observed in 76% of the fields and clear evidence of intranuclear staining was observed in 66% of the fields. On the other hand, sections of the corresponding time points of the GK rat showed no significant changes: only 15% of the fields showed some decline in β-cell PKCζ signal at 2 min, and none resulted in membrane translocation. Only 27% of the scanned fields showed some translocation and entry into the nucleus by 75 min (Fig. 2D).

Western blot analysis of total islet extract revealed a 6.7-fold–stronger signal of PKCζ of the Wistar rat as compared with that of GK islets (Fig. 3). In extract of Wistar islets, two bands were observed: a major band at ~80 kDa and a minor band at ~78 kDa. GK islets exhibited only a single band corresponding to the smaller 78-kDa band. The 6.7-fold difference in PKCζ signal in Wistar islets could only be associated with histochemical images seen in β-cells at 75 min, 15 min following glucose stimulus (Fig. 2D).

No change in β-cell PKCα distribution during glucose stimulation. The β-cells of Wistar rats exhibited a very weak signal for PKCα with no significant glucose-induced redistribution within 1 h of stimulation. In β-cells of diabetic GK rats, no histochemical PKCα signal was registered (Fig. 2E). Western blot of total islet protein indicated the presence of PKCα in both lines (Fig. 3). However, it is possible that the majority of the PKCα signal originates from non-β-cells, while the relative contribution of β-cell is too low to show in histochemical studies, more so in the GK rat.

Dynamics of β-cell PKC9 distribution in response to glucose. In Wistar β-cells, a pronounced PKC9 signal was observed after 2 min of glucose stimulation (Fig. 2F), displaying granulated foci, distributed throughout the extranuclear compartment of the β-cell. By 15 min, most of the granulated foci faded off, suggesting a prompt and transient glucose-induced activation at 2 min; 71.4% of the fields exhibited increased β-cell localization of PKC9 at 2 min, while 77% of the fields displayed the decline and dispersion of the enzyme by 15 min. In sections of pancreata of GK rats, some 80% fields displayed some increase in β-cell PKC9 signal; 64% of sections revealed persisting subsequent lower signals as compared with the 2-min point of glucose stimulation. Western blots of total islet extract also showed stronger PKC9 signal in Wistar rat islets (fivefold) as compared with GK islet extract (Fig. 3). The association between insulin staining and PKC9 was minimal in both Wistar and GK β-cells; it was detected in only 17–35% of the scanned fields in Wistar rats and unaltered by glucose stimulus (Table 1); practically none was seen in GK rat β-cells.

DISCUSSION

Protein kinases, including the PKC family of lipid-dependent kinases, play multiple regulatory functions in signal transduction of most cells, including growth, differentiation, apoptosis, exocytosis, and cell movement (21). Up to nine isoenzymes of protein kinase C have been identified in pancreatic β-cells (18,22); however, the function of most of them is poorly defined and often controversial. Following activation, most PKC isoenzymes unfold, translocate, and bind an anchoring protein at the site of their physiological action (2,23–26). It has been repeatedly shown that inhibition of binding of protein kinases to their anchoring site diminishes their biological action (5,27–29).

We have shown that glucose, the primary, multifunctional regulatory stimulus of the pancreatic β-cell, induces the translocation of several isoenzymes of PKC to new cellular compartments (5). While some of these events have been linked with exocytosis (9,10,30–32), β-cell proliferation (8), or apoptosis (33), neither PKC-isoform functional redundancy nor performance of multiple tasks by single isoenzyme can be ruled out. We have also shown that in Psamommys obesus, a gerbil model of nutrition-induced type 2 diabetes, the expression of PKCo, PKCζ, PKCε, and PKC9 was altered (18). We were able to link changes in PKC isoenzyme expression with the prevailing hyperglycemia (PKCo) or with the diabetes trait (PKC9, PKCε, and PKCζ) in this model. In the present study we employed the GK rat, a model for nonobese type 2 diabetes, and its genetic ancestor the Wistar rat as normoglycemic control in order to describe the effect of glucose stimulation on the
FIG. 2. Localization of PKCα, δ, ε, ζ, λ, and θ in β-cells of perfused Wistar and GK diabetic rat pancreas. Perfusions were terminated at the time points indicated in Fig. 1. Pancreata fixed in formaldehyde and sections prepared for digital confocal histochemical analysis. Signal intensity is presented as color scale: blue-background, green, red, and bright orange represent increased signal intensity, respectively (for details see RESEARCH DESIGN AND METHODS). Representative pictures of 12–115 scanned fields are presented. β-Cells were identified by insulin counterstaining. Each experiment was performed by staining the full time scale; sections of Wistar and GK pancreata were stained in pairs (magnification ×100; digital zoom ×3).
expression and translocation of six β-cell PKC isoenzymes. We opted to use the isolated perfused pancreas preparation, despite its considerable technical difficulty and manpower demand, because it is the only in vitro system that replicates the time resolution of physiological in vivo glucose-induced biphasic insulin release and the cellular events that may be related to it. Since we have postulated that the biphasic dynamics of insulin response to glucose is the net effect of the interaction of multiple regulatory signals (12,34), we compared here the dynamics

FIG. 2—Continued.
of localization and the relative level of the PKC isoenzymes during stimulation with the sugar.

**PKCα.** The classical PKCα is highly ubiquitous and strongly expressed in most cells. Link between the activity of PKCα and insulin response to glucose was first reported by Rasmussen’s laboratory (19,20) and repeatedly confirmed thereafter (5,6,9,35). We have previously shown that PKCα translocates to the cell membrane following glucose stimulation in isolated rat islets and that selective inhibition of the isoenzyme’s translocation reduces the glucose-induced insulin release. In the present study we have shown that in the control Wistar rat, PKCα translocated to the vicinity of the β-cell membrane during the accelerated rate of insulin release of the early first-phase response to glucose. In addition, strong colocalization of PKCα with insulin staining was observed during this stage. It should be noted that, while insulin granules cannot be observed by light microscopy, over 98% of β-cell insulin is stored in granules and less than 2% represents newly synthesized hormone, mostly en route in the endoplasmic reticulum or Golgi systems. Therefore, we refer in this work to positive association of PKC and insulin staining as PKC localized in or near insulin granules. No such localization of PKCα was observed at 5 min, when insulin release declined to a nadir. Relocalization with insulin granules was observed during the rise toward peak second-phase insulin response to glucose. Data obtained from most of the fields screened suggest that at both instances (first- and second-phase insulin response), PKCα localization followed the dynamics of insulin granule recruitment and shuttling.

GK rat β-cells showed a different profile of PKCα localization, closely correlating with the overall diminished insulin response to glucose: as insulin release rates increased only minimally above basal rates, PKCα exhibited little localization at the vicinity of the membrane at any time during the glucose perfusion. It should be noted that GK β-cells were not depleted of insulin as compared with β-cells of the control Wistar rat (data not shown); indeed, other studies also revealed no deficiency in insulin synthesis or storage in the GK rat (36). Furthermore, the degree of expression of PKCα was reduced in the GK β-cell, confirmed both histochemically and by Western blot analysis. A similarly reduced PKCα expression was also observed in β-cells of *P. obesus*, but only in hyperglycemic animals; expression was near normal in islets of normoglycemic prediabetic *P. obesus* (18). Furthermore, incubation of islets from hyperglycemic *P. obesus* for 18 h in low concentrations of glucose completely restored the islets’ insulin response to glucose (18). Taken together, the data suggests that PKCα plays a role in insulin granule shuttling and movement toward secretory sites on the cell membrane and that its expression can be modulated by hyperglycemia.

**PKCε.** Studies by our group and by others implicated PKCε in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCε staining during glucose stimulation: the increased staining associated with the early insulin response faded off during the nadir in insulin release between the first- and second phases, reappearing during late insulin response. Interestingly, strong PKCε staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCε is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCε staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCζ.** This study and studies done elsewhere support the concept that PKCζ is involved in a dual regulatory pathway in pancreatic β-cells: using pseudosubstrate or kinase-dead PKCζ for isoenzyme-selective inhibition, Buteau et al. (8) showed that PKCζ participates in the proliferative effect of glucagon-like peptide-1 via the phosphatidylinositol-3 kinase (PI3K) pathway, which leads to Akt phosphorylation and the development of insulin resistance in pancreatic β-cells. Furthermore, the expression of PKCζ in GK rat β-cells was reduced as compared with control Wistar rat β-cells, confirming the observations made elsewhere (5,6,35). In our study, PKCζ was expressed in GK rat β-cells, but with a reduced level compared with control Wistar rat β-cells. This reduced expression was also observed in GK rat β-cells of the GK/P. obesus diabetic model, and in the normal *P. obesus* (18). Furthermore, the expression of PKCζ was restored in GK/P. obesus diabetic model, but not in normal *P. obesus* (18).

**PKCθ.** This study and studies done elsewhere support the concept that PKCθ is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCθ staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCθ staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCθ is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCθ staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCl.** This study and studies done elsewhere support the concept that PKCι is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCι staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCι staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCι is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCι staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCδ.** This study and studies done elsewhere support the concept that PKCδ is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCδ staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCδ staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCδ is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCδ staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCγ.** This study and studies done elsewhere support the concept that PKCγ is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCγ staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCγ staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCγ is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCγ staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCε.** This study and studies done elsewhere support the concept that PKCε is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCε staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCε staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCε is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCε staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCζ.** This study and studies done elsewhere support the concept that PKCζ is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCζ staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCζ staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCζ is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCζ staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCθ.** This study and studies done elsewhere support the concept that PKCθ is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCθ staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCθ staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCθ is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCθ staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.

**PKCδ.** This study and studies done elsewhere support the concept that PKCδ is involved in the stimulus-secretion coupling of the β-cell (5,31,37). In the present study, we observed dynamic changes in PKCδ staining during glucose stimulation: the increased staining associated with the early insulin response, reappearing during late insulin response. Interestingly, strong PKCδ staining was observed after cessation of the glucose stimulus and the return of insulin release to basal rates. It remains to be investigated whether this poststimulation expression of PKCδ is involved in the priming, time-dependent insulin release–potentiating effect of glucose (11,12,34). In the GK diabetic model, clear staining was evident but no glucose-dependent dynamic changes were seen. Furthermore, while glucose-dependent association between insulin and PKCδ staining was observed in control β-cells, minimal or no such association could be demonstrated in GK rat β-cells.
sitol-3 kinase pathway. Tang and Sharp (6) used the inhibitory pseudosubstrate to show that PKCζ is important for calcium-independent insulin release triggered by carbachol in RINm5f cells. Both studies reported that the proliferative or secretory effects of PKCζ were glucose independent. In contrast, while our study may lend support to both functions, it clearly indicates glucose dependency for activation and translocation of this isoform: shortly after the onset of glucose stimulation (2 min), a major fraction of the previously dispersed isoenzyme was seen localized at the vicinity of the β-cell membrane, dispersing shortly later. Longer glucose stimuli lead to the migration of the isoenzyme toward the nuclear membrane and to detection of PKCζ within the nucleus. While membrane association may support a role for PKCζ in signals for first-phase insulin response, the late intranuclear accumulation may point to a regulatory role in the transcriptional machinery. The fact that both effects in the present study were induced by the glucose stimulus, whereas in the two previous studies no glucose effect was observed, may reflect the higher degree of physiological sensitivity of the isolated perfused pancreas as compared with isolated islets or cell lines. In contrast to the diabetic GK rat, β-cells of isolated islets of the diabetes-prone line of P. Obesus showed a significant increase in expression of PKCζ. In addition to species difference, it should be noted that the diabetic P. Obesus exhibits severe hyperinsulinism and >80% depletion of insulin stores. PKCζ is known to be phosphorylated upon activation (38), often displaying two bands of 76–78 kDa and 80 kDa. While the prominent band of the Wistar islets was of the larger size, the major band observed in GK islets corresponds to the smaller 78-kDa band, possibly the nonphosphorylated form. This observation should prompt further exploration of the role PKCζ in any aberrant β-cell function in the GK rat.

**PKCδ.** The novel PKC, PKCδ, plays a central role in T-cell proliferation, and in production of interleukin-2 (39–42). Of special interest is its proposed role in insulin resistance in muscle cells (43,44) and adipocytes (45), and hence its tentative role in insulin resistance in type 2 diabetes (46). We observed a marked, glucose-induced increase in PKCδ signal at 2 min during the onset of first-phase insulin response, which clearly faded off thereafter without significant late recurrence. At the present level of resolution, PKCδ distribution was diffused over the cell’s extranuclear compartment, without specific cellular localization or association with insulin granules. Of interest is the observation that in β-cells of the diabetic GK rat, the isoenzyme’s signal was either nonexistent or extremely faint. Western blot analysis of isolated islets confirmed marked difference in PKCδ expression between Wistar and GK rats. Low levels of PKCδ were also seen in β-cells of P. obesus; however, in that model, PKCδ levels increased considerably by high-energy diet (18). Together, these observations may implicate PKCδ in early glucose-triggered events in β-cells, events that may be important for initiating rather than sustaining the insulin response.

**PKCε and PKCα.** These two PKC isoenzymes showed no glucose-dependent changes in cellular localization, and their level of expression seemed lower in β-cells of diabetic GK rats. In contrast, Western blot analysis failed to reveal differences in Wistar and GK islets, suggesting that non-β-cells contributed significantly to the abolition of that difference. Evidence from other cells as well as from pancreatic β-cells implicated PKCδ as playing a role in apoptotic pathways (26,33). Using β-cell–targeted PKCδ knockout mice, Hashimoto et al. (32) linked that isoenzyme with glucose-regulated insulin release by direct regulation of the GLUT2 and HNF3β genes and of downstream control of Sur1, Kir6.2, hexokinase 1, and hexoki-

nase 2. It should be noted that the defective insulin response in the GK rat has not been linked to reduced insulin stores and changes in β-cell mass or apoptotic activity have, with few exceptions, been absent (14,36,47). In previous studies using cultured rat islets we have seen glucose-induced localization of PKCδ in perinuclear regions (5). This was not observed in the intact pancreas here, lending support to the general observation that cultured islets display much higher levels of apoptotic activity than in the intact pancreas.

The tightly glucose-coupled biphasic dynamics of insulin release is considered the most sensitive indicator of a healthy β-cell. We have proposed that this unique hormonal response to its physiological stimulus is the net result of the interaction of multiple signals, which both potentiate and inhibit the exocytosis (34). Multiple protein kinases are known to play a role in mediating distal β-cell signals generated by glucose metabolism; here we have provided information on the dynamic changes induced by glucose in the PKC family under as nearly physiological as possible conditions. The mechanism of impaired insulin release in GK β-cells is unclear. Nagamatsu et al. (15) as well as Gaisano et al. (48) demonstrated reduced levels of key components of the SNARE system, an important part of the distal exocytotic complex. While Guest et al. (36) reported an intact insulin biosynthetic machinery and unaltered insulin content in GK β-cells, Nagamatsu’s studies (15) pointed toward increased rate of insulin degradation and diminished insulin stores. Thus, diminished translocation of PKCζ into GK β-cell nuclei could be related to the impaired activation of one or more genes involved in production of the SNARE proteins, while the failure of PKCε to relocate may reflect a disturbed step in trafficking of insulin granules toward the exocytotic site. Direct intervention with the activation/translocation of PKCε and PKCζ in normal β-cells under glucose stimulation may help verify this hypothesis.

Figure 4 summarizes the timing of the major changes in PKC isoenzymes in relation to the dynamics of the insulin response in the perfused rat pancreas; it also indicates the major changes observed in the GK rat model of type 2 diabetes. PKCo, -ε, -ζ, and -θ appear to be activated by glucose immediately at the onset of the stimulation and are thus associated with the accelerated rate of first-phase insulin response. During these changes, PKCo and PKCe were mostly colocalized with the insulin granules. These two isoforms showed a second burst of activation/localization at 15 min of glucose stimulus, during second-phase release, and were still strongly associated with insulin granules. β-cells of the GK rat lacked this major activation of PKCo, -ε, and -ζ, and showed week association with insulin granules, raising the possibility that diminished activity of one or more of these isoenzymes plays a role in the loss of first-phase insulin response in type 2 diabetes. Of special interest is the observation that reactivation of PKCe and PKCζ was observed at 75 min, 15 min into the poststimulatory resting period, only PKCe being associated with insulin granules. Thus, it is possible that these PKC isoforms may be associated with the time-dependent potentiating signal (12,17) for insulin response. These two isoenzymes were not affected by glucose in β-cells of the
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that diminished levels and/or activation of PKCα, PKCe, PKCθ, and/or PKCζ in the β-cells of the GK rat. Although the GK rat is an inbred offspring of the Wistar rat, genetic contribution to these differences cannot be ruled out either. However, a direct link between diminished translocation/activity of PKCα and PKCe, and reduced insulin response in rat islets was previously shown (5). In summary, our results suggest differences cannot be ruled out either. However, a direct offspring of the Wistar rat, genetic contribution to these

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