

# $\beta$ -Cell Protein Kinases and the Dynamics of the Insulin Response to Glucose

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**A full biphasic insulin response is the most sensitive index for well-coupled  $\beta$ -cell signal transduction. While first-phase insulin response is extremely sensitive to potentiating and inhibiting modulations, full expression of second-phase response requires near maximally activated  $\beta$ -cell fuel metabolism. In the isolated rat pancreas, accelerated calcium entry or activation of protein kinase (PK)-A or PKC result in no insulin response in the absence of fuel metabolism. At submaximal levels of  $\beta$ -cell fuel secretagogue, arginine (which promotes calcium entry) or glucagon (which activates PKA) produces a small first-phase insulin response but minimal or no second-phase response; carbachol (which activates PKC and promotes calcium entry) generates biphasic insulin response in the presence of minimal fuel (3.3 mmol/l glucose). Glucagon produces full biphasic response in the presence of 10.0 mmol/l glucose, whereas arginine requires near-maximal stimulatory glucose (16.7 mmol) to produce full biphasic insulin response. Thus, PKA and PKC signal pathways potentiate primary signals generated by fuel secretagogues to induce full biphasic insulin response, while calcium recruitment alone is insufficient to potentiate primary signals generated at low levels of fuel secretagogue. We suggest that three families of PKs (calmodulin-dependent PK [CaMK], PKA, and PKC) function as distal amplifiers for stimulus-secretion coupling signals originating from fuel metabolism, as well as from incretins acting through membrane receptors, adenylate cyclase, and phospholipase C. Several isoenzymes of PKA and PKC are present in pancreatic  $\beta$ -cells, but the specific function of most is still undefined. Each PK isoenzyme is activated and subsequently phosphorylates its specific effector protein by binding to a highly specific anchoring protein. Some diabetes-related  $\beta$ -cell derangements may be linked to abnormal function of one or more PK isoenzymes. Identification and characterization of the specific function of the individual PK isoenzymes may provide the tool to improve the insulin response of the diabetic patient. *Diabetes* 51 (Suppl. 1):S68–S73, 2002**

**B**iphasic insulin response is the ultimate indication of a well-coupled healthy  $\beta$ -cell and can be accurately estimated only when the pancreas, islet, or  $\beta$ -cell is subjected to a square-wave protocol of glucose stimulus. The physiological benefit of this aspect of the dynamics of insulin secretion has not yet been fully resolved (see article by Alan D. Cherrington in this issue); nevertheless, loss or decline of first-phase insulin response to glucose is one of the most sensitive indicators of decline in  $\beta$ -cell function. The exceptional sensitivity of first-phase insulin response to potentiating, synergistic, and inhibiting modulation justifies its selection as a gauge for pancreatic function. However, we still lack comprehensive information on the nature of the initiating and amplifying signals involved in the production of a full, sustained insulin response. In this review, we will focus on the role of  $\beta$ -cell PKs in potentiating the insulin response to metabolic and nonmetabolic stimuli, as well as in the expression of both first- and second-phase insulin responses.

## METABOLIC REQUIREMENTS FOR BIPHASIC INSULIN RESPONSE

A full biphasic insulin response requires accelerated  $\beta$ -cell metabolism and the involvement of numerous proximal intermediates, as well as a distal coupling intermediate (Fig. 1), whereas this is not true for nonmetabolizable secretagogues, which elicit only first-phase insulin response. Thus, when the isolated perfused rat pancreas is used as a sensitive preparation to study the dynamics of insulin response to secretagogue, low-level metabolic stimulus (6.9 mmol/l glucose) elicits almost only first-phase insulin response (see Fig. 6 in the article by Cerasi and Neshet in this issue). Stimulation by higher concentration of glucose (8.3 mmol/l) produces a stronger first-phase insulin response, as well as a moderate second-phase response. It is only with stronger glucose stimuli (16.7 mmol/l) that a full second-phase insulin response is observed. Furthermore, a linear relationship exists between the expression of time-dependent potentiation (TDP) (see article by Cerasi and Neshet in this issue) and the slope of second-phase insulin response (1). In the absence of ambient glucose, no insulin release is observed in response to nonmetabolic stimuli (e.g., 5 mmol/l arginine, which is a membrane-depolarizing agent that promotes calcium influx); applied together with 5.0 or 7.0 mmol/l glucose, arginine elicits first-phase insulin response with minimal or no second-phase response (Fig. 2). An identical stimulus applied 20 min later displays the expression of a strong time-dependent inhibitory sig-

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AKAP, A kinase anchoring protein; C, catalytic; CaMK, calmodulin-dependent protein kinase; PK, protein kinase; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; R, regulatory; RACK, receptors for active C kinase; RICK, receptors for inactive C kinase; TDI, time-dependent inhibitory signal; TDP, time-dependent potentiation; TPA, tissue plasminogen activator.

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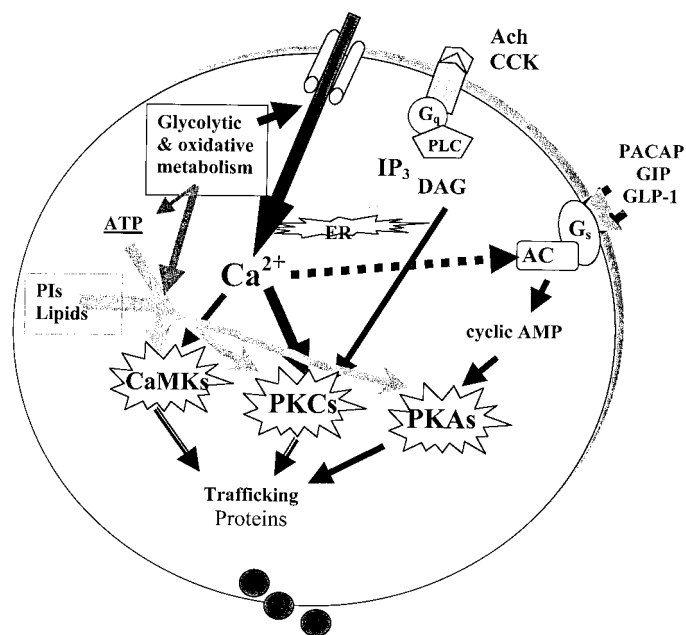


FIG. 1. A simplified scheme for  $\beta$ -cell distal signal transduction pathway. Primary coupling signals originating from fuel metabolism mobilize cytosolic calcium and activate three major families of PKs. The kinases in concert activate distal trafficking proteins, which move insulin granules and activate exocytosis. Hormones and neurotransmitters act as signal amplifiers by converging on a number of isoforms of the PK families. PI, phosphoinositide; CCK, cholecystokinin; Ach, acetylcholine; ER, endoplasmic reticulum; PACAP, pituitary adenylate cyclase activating polypeptide; AC, adenylate cyclase;  $G_s$ , heterotrimeric GTP (guanosine triphosphate)-binding protein, s type class;  $G_q$ , heterotrimeric GTP-binding protein, q type class; IP<sub>3</sub>, inositol trisphosphate; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1.

nal (TDI) (see article by Cerasi and Neshar in this issue) (Fig. 2 and references 1 and 2). Given in combination with arginine, a high concentration of glucose (10.0 mmol/l, which produces a brisk second-phase insulin response when applied alone) is required to display moderate second-phase response, as well as to overcome the expression of TDI, as gauged by a second, identical stimulus (Fig. 2).

Activation of cAMP-dependent protein kinase (PK)-A is regarded as an important pathway that amplifies stimulus-secretion signals in pancreatic  $\beta$ -cells. The occasionally resurfacing debate addressing whether PKA is directly activated by glucose metabolism or by incretins has only recently been settled by the elegant studies of Takahashi et al. (3,4), demonstrating a new important role for ATP in promoting distal exocytotic steps via activation of PKA. Thus, stimulation of the isolated perfused pancreas with glucagon (5.0  $\mu$ g/ml, which activates adenylate cyclase and PKA pathways) without glucose or any other fuel produces no insulin response. A minimal first-phase insulin response may be observed when glucagon is applied together with 5.0 mmol/l glucose, while the combination of glucagon and 7.0 mmol/l glucose results in the appearance of moderate first- as well as second-phase insulin responses; moderate expression of TDI is also evident upon the application of a second similar stimulus (Fig. 3).

Stimulation with glucagon together with 10.0 mmol/l glucose produces fully amplified first- and second-phase insulin response as well as two- to threefold TDP in subsequent stimulation.

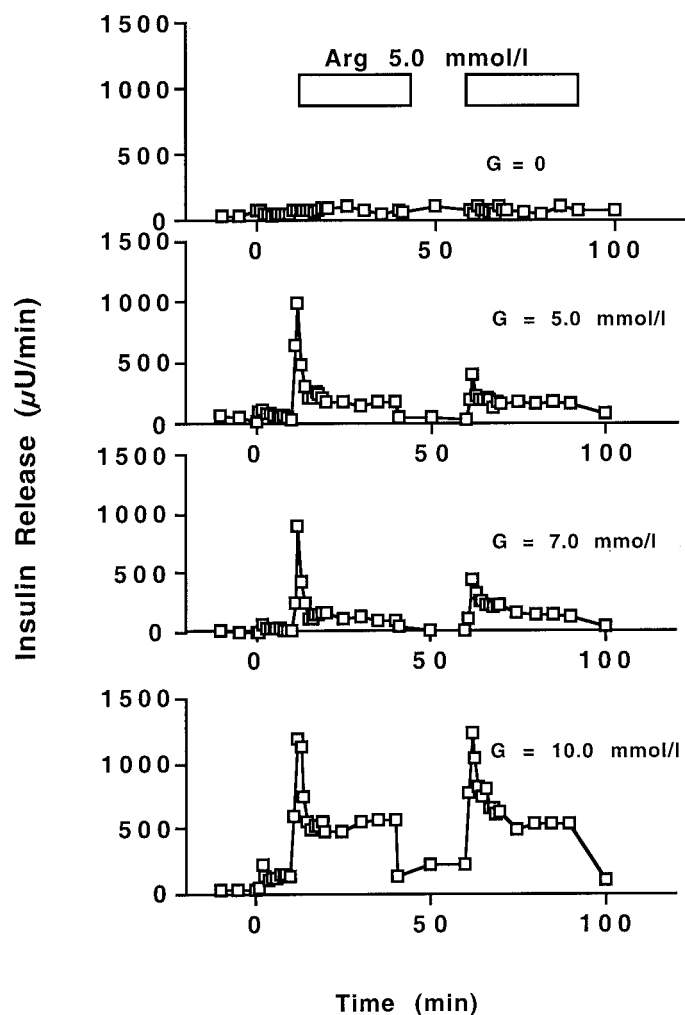


FIG. 2. Glucose-dependence of the insulin response to arginine (Arg) in the isolated perfused rat pancreas. The isolated pancreas was subjected to 40-min stimulation with arginine (5.0 mmol/l, a membrane-depolarizing agent that facilitates calcium entry) at various concentrations of glucose (G). In the absence of ambient glucose, no insulin release was observed. At low or moderate concentrations of glucose (5.0 or 7.0 mmol/l), only first-phase insulin response was observed. The expression of TDI of insulin response (see article by Cerasi and Neshar in this issue) could be demonstrated during a second similar stimulus applied 20 min later. Higher concentrations of glucose (10.0 mmol/l) lead to higher first-phase insulin response as well as the appearance of second-phase insulin response concomitant with the abolishment of the expression of TDI during the second stimulus. Each figure is the mean data from five or more isolated pancreata  $\pm$  SE.

PKC signal pathways play a clear role in potentiating the insulin response to glucose and incretins (5–7). Carbamyl choline (carbachol 5.0  $\mu$ mol/l, which activates  $\beta$ -cell phospholipase C [PLC] and PKC and facilitates calcium mobilization) produces no insulin response in the absence of added fuel (not shown), but a moderate biphasic insulin response can be seen when applied in the presence of minimal fuel secretagogue (3.3 mmol/l glucose) (Fig. 4). Moderate expression of TDP can also be observed upon subsequent, similar stimulus.

These observations may lead to the following important conclusions:

- The production of a biphasic insulin response requires the presence of adequate fuel secretagogue.
- A full biphasic insulin response may be produced by

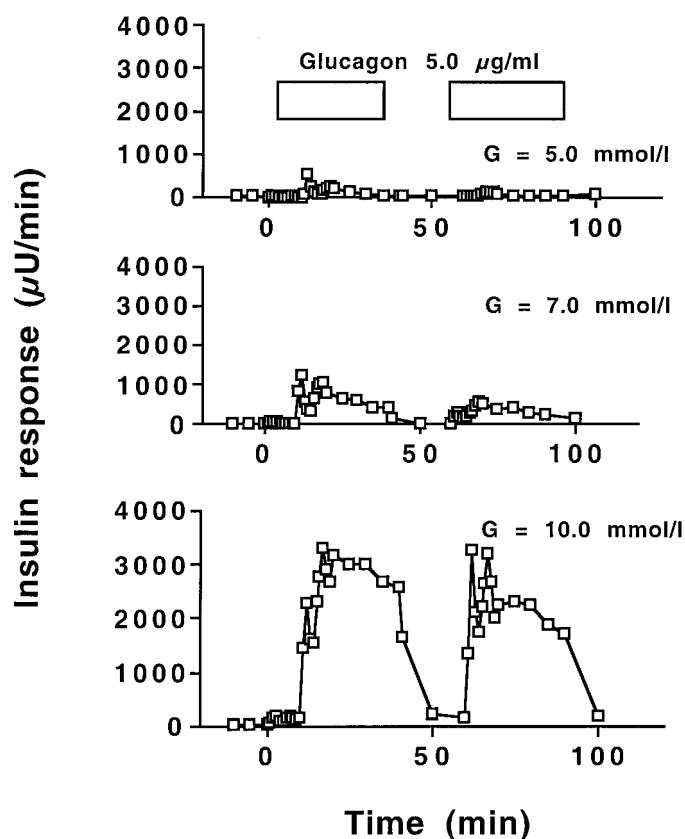


FIG. 3. Glucose-dependence of the insulin response to glucagon in the isolated perfused rat pancreas. The isolated pancreas was subjected to 40-min stimulation with glucagon (5.0  $\mu\text{g/ml}$ , an activator of PKA) at various concentrations of glucose (G). In the absence of ambient glucose, no insulin release was observed (not shown). At low concentrations of glucose (5.0 mmol/l), minimal first-phase insulin response was observed. The disappearance of first-phase response during the subsequent similar stimulus is evidence for the expression of TDI of insulin response (see article by Cerasi and Nesher in this issue). Higher concentrations of glucose (7.0 mmol/l) lead to higher first-phase insulin response as well as the appearance of a moderate second-phase insulin response. Full biphasic response was observed at 10.0 mmol/l glucose. Each figure is the mean data from five or more isolated pancreata  $\pm$  SE.

either fully activated metabolic signal pathways within the pancreatic  $\beta$ -cell or submaximal metabolic signals amplified by the activation of nonmetabolic potentiating stimuli.

- Activation of either PKA or PKC pathways potentiates the insulin response to a metabolic stimulus.
- Activation of either of the PKA- or PKC-amplifying pathways produces stronger insulin responses than those strictly causing calcium mobilization.

#### PKs AS AMPLIFIERS OF THE B-CELL INSULIN RESPONSE

The concept according to which most if not all PKC isoenzymes following their activation are translocated to the site of their action is now widely adopted (8,9). Mochly-Rosen et al. (10) developed and refined the model by characterizing specific receptors for inactive C kinase (RICK)-anchoring proteins that bind the kinase in its folded form, thereby protecting it from degradation. Following its activation, each PKC isoenzyme translocates to a new site in order to colocalize in close proximity with its substrate by binding to a second highly specific anchoring

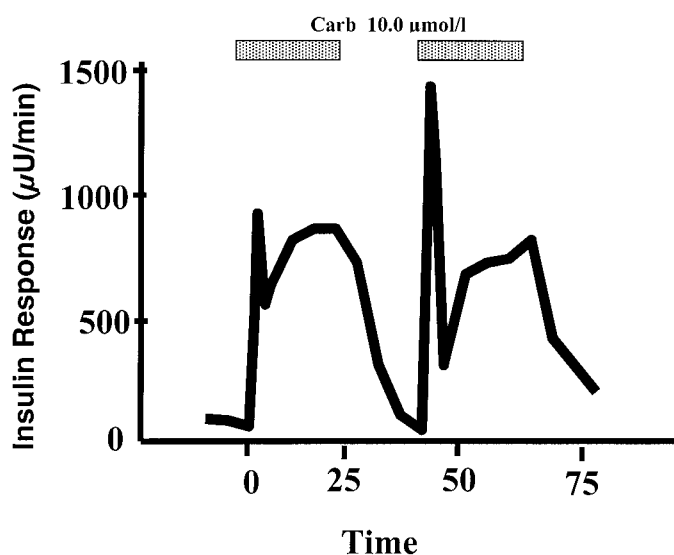


FIG. 4. Biphasic insulin response of the isolated rat pancreas to carbamyl choline. Carbamyl choline (carbachol [Carb]) 10.0  $\mu\text{mol/l}$ , activates PLC and PKC, and recruits  $\text{Ca}^{2+}$  produces biphasic insulin response in the isolated pancreas in the presence of substimulatory (3.3 mmol/l) glucose. Note the expression of TDP during a second stimulus applied after a 20-min period of rest at basal glucose. The mean data from five isolated pancreata are shown.

protein termed RACK (receptor for activated C kinase). Thus, according to the RICK-RACK concept, compartmentation and substrate specificity are achieved by translocation and binding of the PKC to a highly selective RACK protein that maintains the kinase juxtaposed with its effector substrate. Sim and Scott (11) have developed a slightly modified model for isoforms of PKA, in which the inactive holoenzyme is bound to a scaffold protein termed AKAP (A kinase anchoring protein) via its regulatory (R) subunit, maintaining it in close proximity to the specific effector substrate. Activation by cAMP releases the catalytic (C) subunits to phosphorylate the adjacent substrate protein. Since there are only four R isoforms and three C isoforms in the PKA family, compartmental and functional diversity is achieved by the very large number of AKAPs (over 30 have been described thus far), while in the case of PKC 12 isoforms have already been cloned (eight have been identified in pancreatic  $\beta$ -cells [7,12]), and multiple isoforms are known to be present in any cell type.

Since stimulus-induced translocation of a given PKC isoenzyme is a sensitive indicator for its activation, we used quantitative confocal histochemical imaging to study the PKC dynamics during glucose-induced insulin release. We observed glucose-induced translocation of six PKC isoforms to new sites within the  $\beta$ -cells of the isolated perfused rat pancreas ( $\beta$ -cells were identified by counterstaining for insulin). By selecting specific time points during the resting state (3.3 mmol/l glucose), 2 min after onset of stimulation (16.7 mmol/l), 15 or 30 min into the stimulatory period, and 20 min after the cessation of stimulation, we could register the site and concentration of any of the eight PKC isoenzymes identified in pancreatic  $\beta$ -cells and correlate their activity with the dynamics of the insulin response to glucose. Our preliminary data indicate that  $\alpha$ PKC, in a fully dispersed form during perfusion with basal glucose, migrates to the nucleus within 2 min of glucose stimulus, promptly exiting this compartment upon

further stimulation and concentrating at the cell membrane by 30 min of ongoing glucose stimulus (6).  $\gamma$ PKC, also in a dispersed form during the resting state, also displays migration into the nucleus by 2 min and concentrates in a perinuclear region during early second-phase insulin response. The nonclassical  $\delta$ PKC appears in a granulated form during perfusion with basal glucose and shows some transient localization to the nucleus at the onset of glucose stimulus (2 min), with strong evidence for dispersion thereafter.  $\epsilon$ PKC slowly (15 min and longer) migrates to the cell periphery upon stimulation with glucose, quickly redispersing upon cessation of the glucose stimulus. Glucose did not cause new compartmental concentration of  $\eta$ PKC; however, a clear uniform and lasting decrease in antibody binding was seen, suggesting that  $\eta$ PKC underwent glucose-induced structural changes.  $\theta$ PKC, an additional nonclassical PKC isoenzyme, appears in a granulated form uniformly distributed in the cytoplasm, promptly moving into the nucleus (2 min) upon stimulation with glucose. The atypical  $\zeta$ PKC slowly (15 min) concentrated in a well-defined perinuclear region of the  $\beta$ -cell following glucose stimulation and revealed a clear migration into the nucleus. Cessation of glucose stimulus leads to its prompt exit from the nucleus and redispersion in the cytosol. Finally,  $\lambda$ PKC exhibited strong uniform distribution in the cytosol in a granulated form in the nonstimulated state, appeared fully dispersed within 2 min of glucose stimulus, and gradually regranulated in the cytosol upon on-going glucose stimulus.

Can a causal relationship between the dynamic redistribution of activated PKC isoenzymes and the biphasic kinetics of the insulin response to glucose be established? Certainly, the kinetics of the translocation-activation of several isoenzymes may fit that of TDI ( $\delta$ PKC,  $\eta$ PKC,  $\zeta$ PKC, and  $\lambda$ PKC) or TDP ( $\alpha$ PKC,  $\epsilon$ PKC, and  $\gamma$ PKC) in relation to the biphasic insulin response (see article by Cerasi and Neshar in this issue). Future attention should be given to the observation that glucose-induced concentration of  $\zeta$ PKC in the perinuclear region persists long after the glucose stimulus has subsided, and a relationship between the activity of this isoenzyme and TDP should be explored. Glucose-induced translocation of activated kinases ( $\alpha$ PKC,  $\gamma$ PKC,  $\theta$ PKC, and  $\zeta$ PKC) into the  $\beta$ -cell nucleus may imply that these specific isoenzymes are involved in glucose-dependent gene regulation. However, these speculative deductions obviously require additional comprehensive studies before causal relationships can be established. Clearly, to establish such a link, specific inhibitory and stimulatory studies must be performed in conjunction with highly sensitive preparations designed to monitor the stimulus-dependent kinetics of insulin release or glucose-dependent gene activities. The widely used broad stimulators (e.g., phorbol myristoyl acetate [PMA]) or inhibitors (e.g., downregulation by prolonged exposure to PMA, staurosporin, calphostin, etc.) are inappropriate for providing conclusive evidence. Overexpression of a specific isoenzyme or of an activity-deficient mutant isoenzyme may provide useful data (work in progress). However, the use of translocation inhibitory peptides (6,10) has one clear advantage; because they are highly isoenzyme-specific in nature, their introduction into  $\beta$ -cells permits acute studies with little time for the induction of compen-

satory pathways. We employed  $\beta$ -C2-4, a short peptide analogue of RACK1 binding site on  $\alpha$ PKC and  $\beta$ PKC, to inhibit both the translocation and function of  $\alpha$ PKC (6). Since  $\beta$ PKC has not been detected in pancreatic  $\beta$ -cells ([12] and our unpublished data), the peptide specifically blocked translocation of activated  $\alpha$ PKC and diminished the insulin response to glucose by  $\sim 40\%$  (6). The fact that the addition of mannoheptulose (an inhibitor of glycolysis) or omission of calcium also diminished the translocation of  $\alpha$ PKC and its localization (in the activated form) in the vicinity of the cell membrane could suggest that the isoenzyme plays a role in the activation of the insulin granule SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex and its membrane fusion. Introduction of  $\epsilon$ V1-2, a short peptide analogue of  $\epsilon$ PKC's binding region to RACK2, has been used by us as a specific inhibitor of translocation of the activated form of that isoenzyme. While the inhibitory peptide diminished insulin response to glucose by  $\sim 40\%$  (6),  $\epsilon$ PKC's localization to the membrane was slower than that of  $\alpha$ PKC (see above). RACK2, the anchoring protein for  $\epsilon$ PKC, has been identified as  $\beta$ COP1, a member of COP1 trafficking protein family involved in shuttling newly synthesized proteins between the endoplasmic reticulum and subsequent secretory compartments. Therefore, we are presently exploring the possibility that  $\epsilon$ PKC plays a role in proinsulin processing or in movement of newly synthesized insulin granules in  $\beta$ -cells. Thus, introduction of isoenzyme-specific inhibitory peptides (analogues of RACK binding sites) or stimulatory peptides (analogues of RICK binding sites), in conjunction with islet perfusion studies, should provide an extremely valuable tool for the identification of the role of individual kinases in phasic insulin release. Similar methodology would be useful (and has already been applied [13]) for resolution of the role of PKA isoenzymes; however, these studies are presently restricted by lack of comprehensive data on the full spectrum of AKAPs present in pancreatic  $\beta$ -cells.

## PK FUNCTION IN AN ANIMAL MODEL FOR TYPE 2 DIABETES

Additional support for the role of PKC isoenzymes in the dynamics of the insulin response to glucose may be obtained from studies using islets from spontaneously diabetic animals. We have used the islets of *Psammomys obesus*, an animal model for nutrition-dependent type 2 diabetes. When isolated *P. obesus* islets are subjected to two consecutive 50-min stimulations with glucose (Fig. 5), islets of the hyperglycemic animal display reduced and diminishing insulin response compared with islets obtained from normoglycemic prediabetic *P. obesus*. Tested in vitro in batch incubations, the islets of diabetic animals showed a diminished response to glucose but an adequate response to activators of adenylate cyclase-PKA pathway (7). On the other hand, islets obtained from hyperglycemic *P. obesus* exhibited diminished response to PMA (a non-selective activator of PKC isoenzymes) as compared with the response seen in normoglycemic islets (7). Eighteen-hour conditioning of islets obtained from hyperglycemic *P. obesus* in low ambient glucose dramatically improved their glucose responsiveness as well as their response to PMA (Fig. 6), suggesting hyperglycemia-dependent diminished

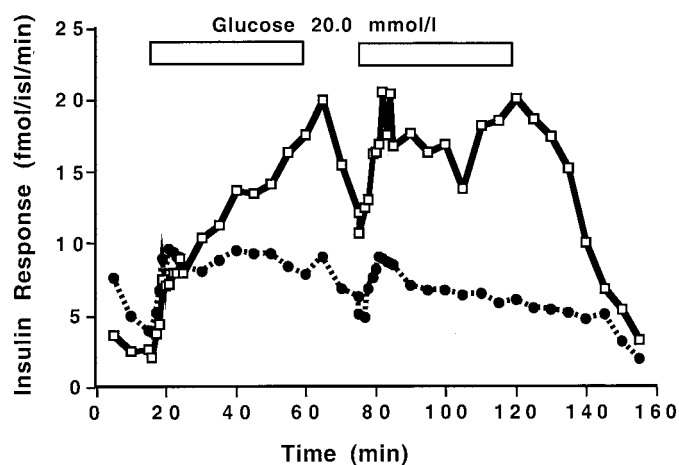


FIG. 5. The dynamics of insulin response to glucose stimulus in an animal model for nutrition-induced type 2 diabetes. Islets isolated from normoglycemic *Psammomys obesus* ( $\square$ , bold line) or from hyperglycemic *P. obesus* ( $\bullet$ , dotted line) were subjected to two consecutive stimuli with 20 mmol/l glucose, 20 min apart. Note the diminishing insulin response in islets of hyperglycemic *P. obesus*. Each line is the mean of five to seven experiments; 20 islets are perfused in each experiment.

activity of one or more PKC isoenzyme. Quantitative confocal histochemical studies corroborated these assumptions:  $\beta$ -cells of hyperglycemic animals displayed diminished levels of  $\alpha$ PKC,  $\theta$ PKC, and  $\lambda$ PKC as compared with those isoforms in the diabetes-resistant line of *P. obesus* (7). These observations await on-going studies to establish a direct link between a specific isoenzyme and the aberrant dynamics of insulin response to glucose in *P. obesus*.

In summary, the complex  $\beta$ -cell signals responsible for production of a full, sustained insulin response to glucose may be simplified and modeled as follows:

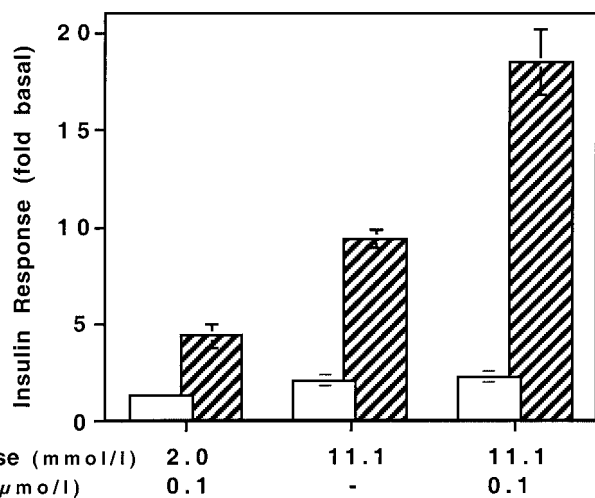


FIG. 6. PMA effect in an animal model for nutrition-induced type 2 diabetes. Islets isolated from hyperglycemic *P. obesus* failed to respond to PMA (activator of PKC), to glucose, or to the combination of the two ( $\square$ ). After overnight conditioning of islets of hyperglycemic *P. obesus* in low glucose ( $\boxtimes$ ), a brisk insulin response to PMA, glucose, and the combination of the two was observed. Islets were incubated in batch for 60 min, in groups of 5, in quadruplicates; each bar is the mean of four to six experiments  $\pm$  SE.

- Extracellular glucose concentrations promptly equilibrate across the  $\beta$ -cell plasma membrane, leading to accelerated glycolytic and oxidative metabolism of the sugar. The newly generated ATP shifts the steady-state ATP-to-ADP ratio, causing closure of  $K^+$ ATP channels, membrane depolarization, and accelerated calcium entry via voltage-dependent calcium channels.
- Step one results in the generation of a number of intermediates required for recruitment and movement of granules and for transmission of the primary and potentiating signals. Among them ATP, guanosine triphosphate, and lipid intermediates.
- Intermediates generated in step one activate both primary, inhibitory, and potentiating signal pathways including adenylate cyclase-PKA (via ATP,  $Ca^{2+}$ , and others), PLC-PKC (via ATP, diacylglycerol, and  $Ca^{2+}$ ), and Ca calmodulin-dependent PK (CaMK) (via calmodulin and  $Ca^{2+}$ ). At present, we have no data permitting to specifically distinguish between the primary versus the inhibitory and potentiating signal pathways; however, these may differentially activate or inhibit specific isoenzymes of the PK families. Multiple trafficking proteins are downstream effectors of the different kinase isoenzymes.
- Neurotransmitters and hormonal secretagogues activate membrane effectors; the signals generated converge on the three families of kinases to amplify or inhibit the signal(s) generated metabolically within the cell.

Primary  $\beta$ -cell signals generated by fuel metabolism converge on amplifying signals generated by potentiating stimuli, by activating three major PK families to produce the entire spectrum of glucose-regulated functions, including insulin production, storage, and release. Activation or inhibition of specific isoenzymes may be responsible for the phasic dynamics of the insulin response. Deranged activity of one or more isoenzyme may be linked to abnormal dynamics of insulin response in animal models for type 2 diabetes; future studies will indicate the relevance of these findings to diabetes in man.

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