Temporal Coding of Insulin Action through Multiplexing of the AKT Pathway

Hiroyuki Kubota,1,* Rei Noguchi,2 Yu Toyoshima,1 Yu-ichi Ozaki,1,5 Shinsuke Uda,1 Kanako Watanabe,1 Wataru Ogawa,3 and Shinya Kuroda1,2,4,*

1Department of Biophysics and Biochemistry, Graduate School of Science
2Department of Computational Biology, Graduate School of Frontier Sciences
University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan
3Department of Internal Medicine; Division of Diabetes and Endocrinology; Kobe University Graduate School of Medicine; Kusunoki-cho 7-5-1, Chuo-ku, Kobe 650-0017, Japan
4CREST, Japan Science and Technology Corporation, Bunkyo-ku, Tokyo 113-0033, Japan
5Present address: Laboratory for Cell Signaling Dynamics, Quantitative Biology Center, RIKEN, 6-2-3, Furuedai, Suita, Osaka 565-0874, Japan
*Correspondence: kubota@bi.s.u-tokyo.ac.jp (H.K.), skuroda@bi.s.u-tokyo.ac.jp (S.K.)
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SUMMARY

One of the unique characteristics of cellular signaling pathways is that a common signaling pathway can selectively regulate multiple cellular functions of a hormone; however, this selective downstream control through a common signaling pathway is poorly understood. Here we show that the insulin-dependent AKT pathway uses temporal patterns multiplexing for selective regulation of downstream molecules. Pulse and sustained insulin stimulations were simultaneously encoded into transient and sustained AKT phosphorylation, respectively. The downstream molecules, including ribosomal protein S6 kinase (S6K), glucose-6-phosphatase (G6Pase), and glycogen synthase kinase-3β (GSK3β) selectively decoded transient, sustained, and both transient and sustained AKT phosphorylation, respectively. Selective downstream decoding is mediated by the molecules’ network structures and kinetics. Our results demonstrate that the AKT pathway can multiplex distinct patterns of blood insulin, such as pulse-like additional and sustained-like basal secretions, and the downstream molecules selectively decode secretion patterns of insulin.

INTRODUCTION

Hormones and growth factors use a different combination of signaling pathways to exert specific functions. In addition, even a common signaling pathway selectively regulates multiple downstream molecules depending on the temporal patterns. However, how a hormone selectively regulates multiple downstream functions through a common signaling pathway remains to be clarified, and the physiological role of the selective regulation has yet to be demonstrated. Many hormones show distinct temporal patterns in vivo (Brabant et al., 1992). In particular, blood insulin, a hormone that regulates various metabolic processes, such as glycogenesis, gluconeogenesis, protein synthesis, and lipid synthesis (Whiteman et al., 2002), reportedly exhibits several temporal patterns, including additional secretion, which is observed in response to meals, basal secretion, which is characterized by persistently low circulating insulin concentrations (Lindsay et al., 2003; Polonsky et al., 1988), and small-amplitude oscillations recurring approximately every 10 to 15 min (O’Meara et al., 1993; O’Rahilly et al., 1988). The relevance of insulin secretion abnormalities, such as additional secretion and the 10 to 15 min oscillations in secretion, in the pathogenesis of type 2 diabetes mellitus has been recognized as important for optimizing insulin action on target tissues (Bruce et al., 1988; O’Rahilly et al., 1988; Pratley and Weyer, 2001; Del Prato, 2003). These studies indicate that insulin selectively regulates metabolic processes depending on its temporal patterns.

Liver, skeletal muscle, and adipose tissue are the three major organs targeted by insulin. Among them, the temporal patterns of insulin strongly affect the liver because the temporal patterns of insulin are most evident in the portal vein, which delivers blood from the pancreas to the liver. In the liver, the AKT pathway plays a pivotal role in the metabolic functions of insulin. AKT regulates protein synthesis through the phosphorylation of ribosomal protein S6 kinase (S6K), glycogen synthesis through the phosphorylation of glycogen synthase kinase-3β (GSK3β), and gluconeogenesis through glucose-6-phosphatase (G6Pase) transcription (Figure 1A) (Cheng et al., 2010; Jastrzebski et al., 2007; Whiteman et al., 2002; Yabaluri and Bashyam, 2010). G6Pase is one of the rate-limiting enzymes in gluconeogenesis, and its expression increases during fasting and decreases after meals based on insulin levels. However, how the temporal patterns of insulin selectively regulate specific downstream responses remains unknown.

RESULTS

The Insulin-Dependent AKT Pathway Model

We experimentally measured the time courses of phosphorylated AKT (pAKT), phosphorylated S6K (pS6K), and phosphorylated GSK3β (pGSK3β) and the G6Pase transcription level
Figure 1. Development of Insulin-Dependent AKT Pathway Model

(A) Schematic overview of the insulin-dependent AKT pathway model. A detailed description can be found in Figure S1 and Supplemental Experimental Procedures.
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(G6Pase) in an insulin dose-dependent manner in rat hepatoma Fao cells (Figures 1B and 1C). We used the step stimulation of insulin from 0.01 to 1 nM in consideration of the physiological ranges in blood (Jaspand et al., 1986; Polonsky et al., 1988; Song et al., 2000). The step stimulation of insulin induced strong transient and weak sustained responses from pAKT, a strong transient response from pS6K, weak transient and strong sustained responses from pGSK3β, and the strong sustained suppression of G6Pase without an obvious transient response. Importantly, S6K always returned to the basal level, regardless of the insulin concentration. The temporal patterns of the downstream molecules differed from one another and from that of the upstream molecule, pAKT, indicating that the three downstream molecules captured the distinct properties of pAKT.

Next, we developed a simple computational model of the insulin-dependent AKT pathway processes (Figures 1A, S1, and Supplemental Experimental Procedures). We confirmed experimentally that crosstalk between the AKT pathway and the ERK and JNK pathway was not observed in Fao cells (Figures 1E and 1G) (Boura-Halfon and Zick, 2009; Du et al., 2008; Ma et al., 2005). We assumed an mTOR-dependent negative regulation of pS6K in the model (Figures 1A and S1) because we could not reproduce the time courses of pS6K without delayed inhibition (Figure S4D; see also Discussion and Supplemental Experimental Procedures).

pAKT showed a partial adaptive response even in the presence of rapamycin in both experiments and the simulation. The reason remains unknown; however, in the simulation model, phosphorylation-dependent IRS degradation also contributed to the adaptive response in addition to negative feedback via mTOR, leading to the partial adaptive response in the absence of the negative feedback via mTOR. The partial adaptive response in the presence of rapamycin in the experiment may have occurred by the same mechanisms (see Supplemental Information). The model reproduced the characteristics of the experimental results in Figure 1C, except for the transient decrease of G6Pase at the intermediate insulin dose (0.03 nM) (Figure 1D, see also Discussion). The model also reproduced experimental results obtained using various inhibitors (Figure 1F), which were not used to estimate the parameters. This indicates that the model can capture the essential features of the transient and sustained responses of the AKT pathway. We previously found that transient and sustained responses in the ERK pathway depend on the increasing rates and final concentrations of growth factors, respectively (Sasagawa et al., 2005). This suggests that transient and sustained responses in the AKT pathway selectively encode the increasing rate and the final concentration of insulin, respectively.

**Transient Responses of pAKT, pS6K, and pGSK3β**

**Depend on the Increasing Rate of Insulin**

We examined whether transient peaks in the molecules of the AKT pathway depend on the increasing rate of insulin. We used the ramp stimulation of insulin with the same final concentrations in the simulation (Figure 2A, inset). As the increasing rate of insulin slowed, the transient peak in pAKT became smaller and delayed (Figure 2A). Among the downstream molecules, the peaks of pGSK3β and pS6K similarly became smaller and delayed, whereas a transient peak of decrease in G6Pase was not obvious. Eventually, pAKT, pGSK3β, pS6K, and G6Pase reached the same steady state regardless of the increasing rate of insulin, and only pS6K returned to the basal level (Figure S2A). This suggests that the sustained amplitudes of pAKT, pGSK3β, and G6Pase, but not that of pS6K, depend on the final concentration of insulin (see below). We examined experimentally whether transient peaks in the AKT pathway depend on the increasing rate of insulin. The step stimulation of insulin induced transient peaks in pAKT, pGSK3β, and pS6K, and as the increasing rate of insulin slowed, the transient peak in molecules became smaller and delayed (Figure 2B). In contrast, the sustained amplitudes of all the molecules at 600 min reached almost the same amplitude. This is consistent with the simulation results (Figure 2A).

To quantify the transient response, we defined the “Transient Peak Index (TPI),” which is the difference between the peak amplitude and the final amplitude normalized by the peak amplitude (Figure 2C inset and Experimental Procedures). A larger TPI indicates a larger transient peak amplitude relative to the final amplitude, and the TPI is 0 when the peak amplitude is equal to the final amplitude. As the duration of insulin increased (i.e., the increasing rate of insulin decreased), the TPIs of pAKT, pGSK3β, pS6K, and G6Pase decreased (Figure 2C). The TPI of pAKT decreased from 0.5 to 0, that of pS6K from 0.8 to 0.2, that of pGSK3β from 0.4 to 0, and that of G6Pase from 0.2 to 0. Because all the TPIs converged to 0, the response of the TPI to the step stimulation indicates the dynamic ranges versus the increasing rate of insulin. In both the simulation and experiments, the dynamic range of pS6K was the highest, whereas those of pAKT and pGSK3β were in the middle and those of G6Pase were the lowest (Figures 2D and 2E). Similar results were obtained when different final concentrations of insulin were used (Figure S2B). Thus, the transient peak in

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(B) Insulin-dependent time course of pAKT, pS6K, and pGSK3β were measured. The concentration of step stimulation of insulin is shown. This is a representative image of western blotting in Figure 1C.

(c) Insulin-induced time courses of pAKT, pS6K, pGSK3β, and G6Pase in Fao cells. The means and SEMs of six (pAKT, pS6K, pGSK3β) and three (G6Pase) independent experiments are shown. Insets show the dose and time courses of insulin.

(D) Insulin-induced time courses of pAKT, pS6K, pGSK3β, and G6Pase in the insulin-dependent AKT pathway model. Note that, to mimic the basal secretion, 0.01 nM of insulin was constantly added before and throughout the simulation and experiments unless specified.

(E) In the presence of the inhibitors rapamycin (200 nM), LY294002 (30 μM), or PD98059 (0.1 μM) (see also Supplemental Experimental Procedures), the step stimulation of insulin (1 nM) was performed and the amplitudes of pAKT, pS6K, pGSK3β, and G6Pase were measured in Fao cells.

(F) In the simulation, we fixed PNs 5, 7, 13, 19, or 21 at 0 to mimic the rapamycin and LY effects, and the amplitudes of pAKT, GSK3β, S6K, and G6Pase in response to the step stimulation of insulin (1 nM) were plotted. The colors indicate the same combination of insulin and inhibitors as in Figure 1E.

(G) Insulin stimulation did not induce the phosphorylation of JNK. The phosphorylation of JNK was measured by the indicated stimuli. A gel image of the immunoblot is shown. See also Figure S1.
pS6K was the most responsive to the increasing rate of insulin, and those of pAKT and pGSK3β were also responsive, whereas that of G6Pase was not responsive. Although a weak transient peak in G6Pase was observed in the simulation, a transient peak was not observed experimentally. Considering that AKT is an upstream molecule of S6K, GSK3β, and G6Pase, the information regarding the increasing rate of insulin was encoded into the transient peak in pAKT, which was, as a TPI, likely to be decoded by those of pS6K and pGSK3β, but not by that of G6Pase (see below).

**Figure 2. Increasing Rate of Insulin-Dependent pAKT, pS6K, pGSK3β and G6Pase**

(A) pAKT, pS6K, pGSK3β and G6Pase induced by the indicated ramp stimulations with insulin (inset) whose final concentration was 0.1 nM in the simulation. (B) Step (blue) and ramp (0.1 nM/240 min, green, and 0.1 nM/600 min, red) stimulations were performed (Figure 2A, blue, green and red lines), and the indicated molecules were measured during the experiment. Note that this step stimulation was performed by using the experimental setups for the ramp stimulation (see Experimental Procedures). This may cause the partial adaptive response of pS6K in response to step stimulation of insulin, which was different from the perfect adaptive response in Figure 1C. (C) Transient peak indexes (TPIs, inset) for the indicated molecules in the simulation (line) and experiments (dots). The colors of the dots are the same as those in Figure 2B. (D and E) Maximal TPIs for the molecules in simulation (D) and the experiments in Figure 2B (E). See also Figure S2.

Sustained Responses of pAKT, pGSK3β, and G6Pase Depend on the Final Concentration of Insulin

We next examined whether sustained amplitudes of the AKT pathway depend on the final concentration of insulin. We used the stepwise decrease stimulation with different final concentrations (Figure 3A, inset). We used this stimulation, rather than the step stimulation, in order to have the same increasing rate of insulin at the onset of stimulation to avoid the effect of different increasing rates of insulin and to quantify the sustained response in comparison with the maximal peak amplitude (see below). In response to the stepwise decrease stimulation with the different final concentrations, all the molecules had nearly reached a steady state at 480 min (Figure 3A). As the final concentration of insulin decreased, the sustained amplitude of pAKT also decreased (Figure 3A). Among the downstream molecules, the sustained amplitude of pGSK3β and G6Pase similarly decreased in the final concentration-dependent manner, whereas pS6K always returned to the basal level regardless of the final concentration of insulin (Figure 3A). We examined experimentally whether the sustained amplitudes of the AKT pathway depended on the final concentration of insulin. The step stimulation of insulin induced sustained responses of pAKT, pGSK3β, and G6Pase, whereas the stepwise decrease stimulation (0.1–0.01 nM) did
not (Figure 3B). In contrast, pS6K returned to the basal level by both step and stepwise decreases stimulation. The transient peaks in pAKT, pGSK3β, and pS6K reached nearly the same amplitudes with the step and stepwise decreases stimulation (0.1–0.01 nM), whereas a transient peak in G6Pase with a stepwise decrease stimulation reached half that of the step stimulation. This finding reflects the slow response of G6Pase compared with the other molecules (see below). The experimental results were consistent with the simulation results (Figure 3A).

We defined the “Sustained Amplitude Index (SAI)” as the normalized difference in the sustained amplitudes induced by a step and stepwise decrease stimulation (Figure 3C, inset). A larger SAI indicates a larger difference in the sustained amplitudes versus the difference in the final concentrations of insulin, and the SAI is 0 when the difference in the sustained amplitudes obtained by the step and stepwise decrease stimulation reaches 0. As the difference in the final concentrations increased (i.e., the final concentrations decreased), the SAIs of pAKT, pGSK3β, pS6K, and G6Pase increased. The SAI of pAKT increased from 0 to 0.4, that of pGSK3β from 0 to 0.5, that of pS6K from 0 to 0.2, and that of G6Pase from 0 to 0.8. Because all the SAIs converged to 0, the SAI obtained by the stepwise decrease stimulation (0.1–0.01 nM) indicates the dynamic ranges versus the final concentration of insulin. In both the simulation and experiments, the dynamic range of G6Pase was the highest, those of pAKT and pGSK3β were in the middle, and that of pS6K was the lowest (Figures 3D and 3E). Similar results were obtained when shorter (15 min) and longer (120 min) durations of insulin were used (Figure S3B). Thus, the sustained response of G6Pase was the most responsive and those of pAKT and pGSK3β were also responsive, whereas that of pS6K was hardly responsive. Considering that AKT is an upstream molecule of S6K, GSK3β, and G6Pase, the information regarding the final concentration of insulin was encoded into the sustained amplitude of pAKT, which was likely to be decoded by the sustained amplitudes of pGSK3β and G6Pase, but not by that of pS6K (see below). In particular, as an SAI, the information regarding the final concentration of insulin was enhanced and transmitted to G6Pase.

**Figure 3. Final Concentration of Insulin-Dependent pAKT, pS6K, pGSK3β, and G6Pase**

(A) pAKT, pS6K, pGSK3β, and G6Pase induced by the indicated stepwise decrease stimulation with the indicated final concentration for which the initial concentration (0.1 nM) and duration (30 min) were set at the same values (inset) in the simulation.

(B) Step (blue) and stepwise decrease (red, 0.1–0.01 nM) stimulations were performed (Figure 3A, blue and red lines), and the indicated molecules were measured during the experiment.

(C) Sustained amplitude indexes (SAIs, inset) for the indicated molecules in the simulation.

(D and E) Maximal SAIs for the molecules in the simulation (D) and the experiments in Figure 3B (E). See also Figure S3.
Selective Decoding of the Increasing Rate and Final Amplitude of pAKT by Downstream Molecules

The responses of pS6K, pGSK3β, and G6Pase to pAKT differed from one another. What kind of information on pAKT was selectively decoded by these molecules? To explore the decoding mechanisms, we used ramp stimulations of pAKT and examined the responses of pS6K, pGSK3β, and G6Pase in a simulation (Figures 4A and 4B). In response to ramp stimulations of pAKT, pGSK3β and G6Pase gradually increased and reached the same steady state, without an apparent transient peak, regardless of the increasing rate of pAKT (Figure 4A). In contrast, pS6K showed a transient peak in response to the ramp stimulation of pAKT. As the increasing rate of pAKT slowed, the transient peak in pS6K became smaller and was delayed (Figure 4B). Eventually, pS6K returned to the basal level, regardless of the increasing rate of pAKT. Thus, the response of pS6K to ramp stimulations differed from those of pGSK3β and G6Pase, while those of pGSK3β and G6Pase were similar. This finding indicates that, in response to the step stimulation of insulin, the transient response of pS6K depends on the increasing rate of pAKT, whereas that of pGSK3β depends on the transient response of pAKT. Furthermore, the transient responses of pS6K and pGSK3β, although they appear similar, decode the distinct features of pAKT.

The network structure of the AKT to S6K module was different from those of the AKT to GSK3β and the AKT to G6Pase modules (Figure S1), which may account for the different response of S6K compared with those of GSK3β and G6Pase. The network structure of the AKT to S6K module in the model was an incoherent feed-forward loop (IFFL): pAKT-dependent rapid activation and delayed inactivation of S6K (Figures 1, S1, and S4A). In contrast, those of the AKT to GSK3β and AKT to G6Pase modules in the model had a feed-forward structure (FF): pAKT-dependent activation and pAKT-independent constant inactivation (Figures S1 and S4A).
An IFFL can generate two remarkable features: an increasing rate of stimulation-dependent transient response and perfect adaptation (Ma et al., 2009; Muzzey et al., 2009; Sasagawa et al., 2005; Tyson et al., 2003), the latter of which is a property of the maintenance of the same steady state against different final concentrations of stimulation. Because of these features of the IFFL, the transient response of pS6K depended on the increasing rate of pAKT (Figures 4A, S4B, and S4C) and pS6K always returned to the basal level regardless of the increasing rate (Figure 4A) or the final amplitude (Figures 1C, 2A, 2B, 3A, 3B, 4A, 4C, S2A, S3A, and S4B). Similar features can also be seen in other network structures such as a Negative FeedBack Loop (NFB) (Ma et al., 2009; Ozaki et al., 2005) (see below). The structural difference between the IFFL and FF is the mode of inactivation. The conversion of the delayed inactivation of the AKT to S6K module to a constant inactivation converted the structure and the response of pS6K from IFFL to FF (Figure S4C), and an FF structure of the AKT to S6K module could not reproduce the adaptation of pS6K in response to step stimulation of insulin (Figure S4D).

In contrast to pS6K, pGSK3β and G6Pase shared the same network structure and showed similar responses to step and ramp stimulation of pAKT. However, their responses to the step stimulation of insulin were different: pGSK3β showed both a transient and a sustained response, whereas pGSK3β showed only a sustained response (Figures 1C and 1D). The time constant of the FF indicates how rapidly the downstream molecule can follow changes in the upstream molecules. We measured the apparent time constants of pGSK3β and G6Pase, which are defined as the time to reach 63.2% of the steady state by the step stimulation of pAKT. The apparent time constants of pGSK3β and G6Pase were 1.6 and 18.4 min, respectively. By decreasing the time constant of pGSK3β, the transient response of pGSK3β began to disappear and the shape of the time course of pGSK3β became similar to that of G6Pase (Figure S4E). Thus, the difference in the time constants of pGSK3β and G6Pase accounted for their distinct responses, such that pGSK3β can decode both transient and sustained responses of pAKT, whereas G6Pase can specifically decode a sustained response of pAKT, rather than a transient one.

We next examined the responses of pS6K, pGSK3β, and G6Pase at steady state using the step stimulation of pAKT (Figure 4C). pS6K, pGSK3β, and G6Pase had nearly reached a steady state at 480 min. The dose response curve of pS6K versus pAKT at 480 min revealed the adaptation of pS6K at the steady state; regardless of the final amplitude of pAKT, pS6K returned to the basal level (Figure 4D), consistent with the above results. The steady state of pGSK3β monotonically increased as that of pAKT increased. The change in pGSK3β from 25% to 75% of the maximal response required a change in pAKT from 0.1 to 0.9, and the half-maximal concentration (EC50) of pAKT, which gives a 50% of the maximal response, was 0.31 (Figure 4D). The steady state of G6Pase also monotonically increased as that of pAKT increased. However, the change in G6Pase from 25% to 75% of the maximal response required a change in pAKT from only 0.03 to 0.14, and the EC50 of pAKT was 0.06 (Figure 4D). These results indicate a wider dynamic range of pGSK3β than G6Pase against pAKT and that pAKT can transmit large amounts of information to pGSK3β (Yu et al., 2008). These results also indicate the higher sensitivity of G6Pase than pGSK3β against changes at a low amplitude of pAKT and that G6Pase has a small EC50, enabling it to show a maximal response even at the low amplitude of pAKT. This also means that GSK3β and G6Pase selectively use different concentration ranges of pAKT and insulin. The steady states of pS6K, pGSK3β, and G6Pase versus that of pAKT in the experiments shown in Figure 1C produced similar results (Figure 4D, red dots). Thus, the downstream molecules pS6K, pGSK3β, and G6Pase show distinct steady state responses, indicating that they selectively decode different information on pAKT.

Encoding and Decoding In Vivo-like Temporal Patterns of Insulin in the AKT Pathway

We next examined how in vivo-like temporal patterns of insulin are encoded into pAKT and decoded by the downstream molecules. Insulin exhibits additional and basal secretions (Polonsky et al., 1988). The additional secretion corresponds to postprandial secretion and is characterized by a pulse- like pattern, the duration of which is a few hours (Polonsky et al., 1988; Simon et al., 1987). Basal secretion is the low and constant secretion that occurs during fasting. Both secretions in obese subjects are reportedly higher than those in normal subjects, but the ratio between the peak concentration of the additional secretion and the basal secretion remains the same (Polonsky et al., 1988). In addition, insulin is regularly secreted every 15 min, independent of meals (O’Meara et al., 1993; O’Rahilly et al., 1988). Therefore, we examined the selective roles of the additional, basal, and 15 min pulsatile insulin secretions in the regulation of the AKT pathway. Because it is experimentally difficult to perform continuous pulsatile stimulation, we used the single pulse stimulation to examine the effect of 15 min pulse stimulation on the transient responses. Therefore, we used three types of constant and pulse stimulations in both the simulation and experiment: a 120 min pulse (0.1 nM of insulin) with basal stimulation (0.01 nM) (Figures 5A and 5C, red lines), which resembles the additional and basal secretions in normal subjects; a 120 min pulse (1 nM of insulin) with basal stimulation (0.1 nM) (Figure 5A, blue lines), which resembles the additional and basal secretion in obese subjects; and a 15 min pulse (0.1 nM of insulin) with basal stimulation (0.01 nM) (Figure 5C, green lines), which resembles the 15 min pulsatile insulin secretion.

In response to the basal-like stimulation, the basal levels of pAKT and pGSK3β increased and that of G6Pase decreased as the basal levels of the stimulation increased (Figures 5A and 5B). In contrast, the basal level of pS6K remained the same, regardless of the basal level of the stimulation (Figures 5A and 5B). This change is consistent with the experimental results if the steady state at 480 min in Figure 1C is regarded as the basal level (Figure 5B, red dots). These results indicate that pAKT, pGSK3β, and G6Pase can respond to the basal secretion of insulin, and G6Pase is highly sensitive to changes at low concentrations of insulin, whereas pS6K perfectly adapts to the basal secretion of insulin.

The pulse stimulations increased pAKT, pGSK3β, and pS6K and decreased G6Pase (Figure 5A). pAKT, pGSK3β, and pS6K showed similar peak amplitudes in response to both 120 and
15 min pulse stimulations, whereas G6Pase showed a lower peak amplitude in response to the 15 min pulse stimulations than to the 120 min pulse stimulations (Figures 5C–G). G6Pase did not exhibit a maximal amplitude in response to the 15 min pulse stimulation because of the large time constant of G6Pase, as described in the previous section. To quantify response to the 15 min pulse stimulation, we defined the “Peak Ratio Index (PRI),” which is the ratio of the peak amplitudes of the 15 min pulse to that of the 120 min pulse (Figure 5E and Experimental Procedures). A larger PRI indicates that a molecule is more responsive to the 15 min pulse stimulation. In both the simulation and experiments the PRI of pAKT was almost 1, indicating that pAKT similarly responded to the 15 min and 120 min pulse stimulations (Figures 5F and 5G). The PRIs of pGSK3β and pS6K were almost 1, whereas the PRI of G6Pase was 0.6. Thus, pAKT, pGSK3β, and pS6K can respond to the 15 and 120 min pulse insulin stimulation. These results clearly demonstrate the different responses to insulin secretion.
patterns: pAKT and pGSK3β can respond to the additional, basal, and 15 min pulse secretions; pS6K can respond to the additional and 15 min pulse secretion but not the basal secretion; and G6Pase can respond to the additional and basal secretions but not the 15 min pulse secretion.

We also examined the steady state responses of pAKT, pGSK3β, pS6K, and G6Pase in response to the 15 min pulsatile insulin secretion in simulation. We found that pAKT and pGSK3β similarly respond to the 15 min and the 120 min pulsatile insulin secretion, whereas pS6K and G6Pase preferentially respond to the 120 min pulsatile insulin stimulation (Figure S5). Further experimental study is necessary to address the steady state responses to the pulsatile stimulation.

**Similar Temporal Coding of the AKT Pathway in Primary Hepatocytes**

We next examined whether the responses of pAKT, pS6K, pGSK3β, and G6Pase are similar in primary hepatocytes, which better represent a physiological response. The responses of all molecules in primary hepatocytes appeared similar to those in Fao cells except that the peak time of pAKT, pS6K, and pGSK3β in primary hepatocytes were delayed compared to those in Fao cells (Figures 5D and 6). In principle, this suggests that primary hepatocytes and Fao cells share some similar temporal coding properties. Similar responses of pAKT, pS6K, pGSK3β, and G6Pase were also observed in response to the 120 min (Figure 6A, red lines) and 15 min pulse stimulations (Figure 6A, green lines), except that the peak amplitudes of pAKT and pS6K in response to the 15 min pulse stimulation were smaller than those in response to the 120 min pulse stimulation.

We further quantified the responses of pAKT, pS6K, pGSK3β, and G6Pase in primary hepatocytes by the indexes TPI, SAI, and PRI. The TPI of pS6K was the highest, that of pAKT and pGSK3β were in the middle, and that of G6Pase was the lowest (Figure 6B), indicating that the transient peak in pS6K was the most responsive to the step stimulation of insulin, those of pAKT and pGSK3β were also responsive, but that of G6Pase was not responsive. This is consistent with the results in Fao cells (Figures 2D, 2E, and 6B inset). In contrast, the SAI of G6Pase was the highest, those of pAKT and pGSK3β were in the middle, and that of pS6K was the lowest (Figure 6C), indicating that G6Pase had the greatest sustained response, pAKT and pGSK3β were also responsive, but pS6K was hardly responsive. This is in principle consistent with the results in Fao cells (Figures 3D, 3E, and 6C inset), although the SAI of pAKT and pGSK3β were lower than those in Fao cells, because their peak amplitudes compared to the sustained amplitudes were larger in primary hepatocytes. The PRI of pGSK3β was the highest, that of pAKT was in the middle, and those of pS6K and G6Pase were the lowest (Figure 6D), indicating that the peak amplitude of pGSK3β responded similarly to the additional-like 120 and 15 min pulse stimulations of insulin, whereas pAKT was partially responsive to the 15 min pulse stimulation, and pS6K and G6Pase were not efficiently responsive to the 15 min pulse stimulation. The response of pGSK3β is consistent with that in Fao cells, whereas the responses of pAKT and pS6K were weaker than those in Fao cells (Figures 5F, 5G, and 6D inset). The weaker responses of pAKT and pS6K may be due to the slower response of pAKT in primary hepatocytes as seen by the delayed peak time of pAKT in response to 120 minute pulse stimulation (see Discussion). Despite the difference in the PRIs of pAKT and pS6K between primary hepatocytes and Fao cells, these results clearly demonstrate that the temporal coding of the AKT pathway is conserved in primary hepatocytes.

**Figure 6. Temporal Patterns of pAKT, pS6K, pGSK3β, and G6Pase in Primary Hepatocytes**

(A) pAKT, pS6K, pGSK3β, and G6Pase in response to the step (cyan) and 15 min pulse (green) and 120 min pulse (red) stimulations in primary hepatocytes with 1 nM insulin (inset). The means and SEMs of three independent experiments are shown.

(B–D) The TPIs (B), SAIs (C), and the PRIs (D) of each molecule were plotted from data in Figure 6A. Insets indicate each index of Fao cells with the same scales and orders from Figure 2E (B), Figure 3E (C), and Figure 5G (D), respectively.

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The transient response of pGSK3β was induced by the transient response of pAKT because the network structure of the AKT to GSK3β module is an FF with a small time constant. Thus, pGSK3β responds to both 120 and 15 min pulse insulin stimulations and the sustained stimulation. Therefore, it is likely that in vivo pGSK3β efficiently responds to all stimulation patterns of insulin, including the additional, the 15 min pulse, and basal secretion of insulin (Figure 7, pink, orange, and blue). Considering that GSK3β regulates glycogenesis, the rapid response of pGSK3β is physiologically reasonable because glycogenesis should be induced immediately after meals when the blood glucose level is high. We confirmed that glycogen is synthesized within 30 min after the addition of insulin in Fao cells (data not shown). The sustained response of pGSK3β was induced by the sustained response of pAKT, and pGSK3β showed an almost linear response against a wide dynamic range of pAKT (Figure 4D). This suggests that glycogenesis is tightly and precisely controlled over a wide range of insulin concentrations.

The sustained response of G6Pase responds to the sustained response of pAKT, and G6Pase shows a high sensitivity to pAKT because the network structure of the AKT to G6Pase module is an FF with a small EC50. Because of the large time constant of the AKT to G6Pase module, G6Pase can respond to a transient response of pAKT with longer duration (120 min) but not with shorter duration (15 min) (Figures 5C, 5D, and 6A). Thus, it is likely that in vivo G6Pase responds to the 120 min pulse and sustained insulin stimulation but not to the 15 min pulse stimulation. Therefore, G6Pase might decode information on basal insulin secretion in vivo, particularly in the presence of a low insulin concentration, and additional secretion, but not 15 min pulse secretion (Figure 7, blue and pink). A discrepancy existed between the experiments and simulation for the transient and sustained response of G6Pase at the intermediate doses of insulin. The reason for the discrepancy of the transient response of G6Pase remains unknown; however, a transient decrease of G6Pase should accompany delayed inhibition of G6Pase degradation or delayed activation of G6Pase synthesis. The reason for the discrepancy of the sustained response of G6Pase remains unknown, but it may be because of cooperativity of the expression of G6Pase through multiple sites (Yabaluri and Bashyam, 2010), which was not modeled by the current framework of mass action. Further studies are necessary to clarify these
discrepancies. However, these discrepancies do not affect our conclusions regarding the G6Pase response in our study (slow and highly sensitive to insulin). Considering that G6Pase regulates gluconeogenesis, the highly sensitive but slow time constant characteristics of G6Pase allow gluconeogenesis to be induced only when the blood glucose and insulin levels are low for long periods, such as during fasting and overnight, and to be tightly inhibited by the additional secretion that occurs when insulin is triggered by postprandial high glucose levels. The slow response of G6Pase may also prevent a response to the 15 min pulse insulin secretion that occurs during fasting, thus avoiding the unnecessary consumption of energy. PEPCK (phosphoenolpyruvate carboxykinase), a gene encoding another rate-limiting enzyme of gluconeogenesis, showed characteristics similar to those of G6Pase (data not shown). Therefore, these characteristics might guarantee robust control of gluconeogenesis only in response to slow and low insulin secretion, such as basal secretion.

DISCUSSION

Temporal Patterns Multiplexing as a General Property of Signaling Pathways

One of the general characteristics of signaling pathways is the ability to code multiple cellular information through the common signaling pathway (Kholodenko et al., 2010). Multiple cellular information coded in specific temporal patterns of growth factors are encoded into specific temporal patterns in signaling pathways, which are selectively decoded by distinct downstream molecules by taking advantage of the combination of differences in the network structures, time constants, and ECso values (Behar and Hoffmann, 2010). The concept of temporal pattern multiplexing, known as kinetic insulation, has been proposed on a theoretical basis (Behar et al., 2007). Thus, we propose that temporal pattern multiplexing may be a general coding mechanism used by signaling pathways. In addition, cells can change the coding mechanisms of the common signaling pathway by controlling the expression of regulatory molecules, allowing constant and delayed inactivation. For example, we found that the AKT pathway, including S6K, serves as a low-pass filter, one of the FF structures with a constant inactivation, in PC12 cells (Fujita et al., 2010). Our modeling study revealed that the FF structure of the AKT to S6K module could not reproduce the adaptation of pS6K in Fao cells (Figure S4D), indicating the existence of unidentified pathway(s) for regulation of pS6K, such as an IFFL or NFBL. These findings suggest that defects in the temporal coding mechanism of the same signaling pathway by controlling the expression of the regulatory molecules for constant and delayed inactivation.

Possible Physiological and Pathological Roles of the Temporal Coding of Insulin Functions

In vivo administration of pulsatile insulin stimulation has been shown to be more effective than that of continuous insulin stimulation on hepatic glucose production (Bratusch-Marrain et al., 1986; Koopmans et al., 1996) and on the stimulation of glucose uptake in peripheral tissue (Grubert et al., 2005; Koopmans et al., 1996). Such effective responses to the pulsatile insulin stimulation suggest that the network structures for these insulin actions are likely to be an IFFL or NFBL, which effectively respond to pulsatile stimulation rather than constant stimulation of insulin. Indeed, pS6K preferentially responded to pulsatile insulin stimulation in the simulation (Figure S5). Hepatic glucose production is thought to be dependent on glycogen metabolism and gluconeogenesis. However, the network structures of GSK3β and G6Pase were likely to be FF, not IFFL or NFBL, at least in Fao cells. This suggests that the network structures of the downstream GSK3β and G6Pase or the other pathways might be IFFL or NFBL, so that pulsatile insulin stimulation is more effective for the inhibition of hepatic glucose production. The pathophysiology of type 2 diabetes is characterized by defects in insulin action and insulin secretion. Evidence suggests that the 15 min pulsatile insulin secretion does not occur in subjects with type 2 diabetes (O’Rahilly et al., 1988). Moreover, on intravenous glucose challenge, the first phase of insulin secretion (rapid pulse-like insulin secretion) is preferentially impaired, whereas the second phase (slow and sustained insulin secretion) is maintained, at least in an early phase of the disease (Chaillous et al., 1996). Although Fao cells may not perfectly reflect the effects of insulin on metabolism in the liver, our findings suggest that defects in both the amplitude as well as the temporal pattern of insulin secretion affect the development of type 2 diabetes through conceptually similar mechanisms. Here we have shown that members of the insulin signaling network respond differentially to the temporal pattern of insulin stimulation. It is thus possible that alterations in the temporal pattern of insulin action lead to selective impairment of a certain insulin action. Signaling for both gluconeogenesis and lipogenesis is exaggerated in the liver of insulin-resistant type 2 diabetes, whereas the former is inhibited and the latter is stimulated by insulin in normal conditions (Brown and Goldstein, 2008). It is possible that such a paradoxical phenomenon (i.e., selective resistance in a certain branch of insulin signaling in one organ) is attributable to the different responses of each insulin action to the temporal pattern of insulin stimulation. Further study is necessary to address the role of temporal coding of insulin functions in the pathogenesis and treatment of type 2 diabetes. Moreover, insulin actions depend on not only by the temporal patterns of insulin, but also on the crosstalk of other counteracting hormones, such as glucagon. Thus, a systems biological approach will be needed to understand insulin actions with complex crosstalk.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials

The Fao cells were cultured and used accordingly (Supplemental Experimental Procedures). Unless specified, 0.01 nM of insulin was constantly added before the simulation and throughout the simulations and experiments. Ramp stimulation was performed by adding 59 nl of medium containing the insulin 340 times evenly for the indicated duration using a noncontact microdispenser robot (Mr. MJ; MECT Corporation). Because the ramp stimulation requires the continuous addition of insulin, we used the same protocol for the step stimulation; we added insulin onto the media and left the plate open in a CO2 incubator. Hepatocytes were isolated as previously described (Supplemental Experimental Procedures). Antibodies against pAKT [Ser473, #9271], pS6K [Thr389, #9205], and pGSK3β [Ser21, #9336] were purchased from Cell Signaling Technology and used for immunoblotting. Procedures of
RT-PCRs for G6Pase and PEPCK were described in Supplemental Experimental Procedures.

**Simulation and Parameter Estimation**

We developed the insulin-dependent AKT pathway model based on the law of mass action and performed simulation and parameter estimations using Matlab (version R2008b, Math Works). The methods of parameter estimations were described in Supplemental Experimental Procedures. The parameters and equations used in this study are shown in Figure S1. Unless specified, 0.01 nM of insulin was constantly added before the stimulation and throughout the simulation and experiments.

**Definition of TPI, SAI, and PRI**

TPI is defined as

$$TPI = \frac{\text{Peak}_\text{final} - \text{Peak}_\text{peak}}{\text{Peak}_\text{peak}}$$

where peak and final are the peak and final amplitudes of the time course, respectively. TPI indicates the transient peak amplitude relative to the peak amplitude.

SAI is defined as

$$SAI = \frac{\text{Step}_\text{final} - \text{Pulse}_\text{final}}{\text{Maxres} - \text{Pulse}_\text{final}}$$

where Step_final, Pulse_final, and Maxres are the final amplitudes of step and pulse stimulation and the maximal amplitude of the time courses, respectively. SAI indicates the normalized difference in the sustained amplitudes induced by the step and stepwise decrease stimulation.

PRI is defined as

$$PRI = \frac{\text{15min}_\text{Pulse}_\text{peak} - \text{120min}_\text{Pulse}_\text{peak}}{\text{120min}_\text{Pulse}_\text{peak}}$$

where 15 min_Pulse_peak and 120 min_Pulse_peak are the peak amplitudes of 15 and 120 min pulse stimulation, respectively. PRI indicates the ratio of the peak amplitudes of 15 and 120 min pulse stimulation.

Note that in this study all the indexes of G6Pase were determined based on the absolute difference from the basal level.

**ACCESSION NUMBERS**

The BioModels Database accession number for the data referred to this paper is MODEL1204060000.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.molcel.2012.04.018.

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