Systems analysis of GLP-1 receptor signaling in pancreatic β-cells

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Takeda Y., Amano A., Noma A., Nakamura Y., Fujimoto S., and Inagaki N. Systems analysis of GLP-1 receptor signaling in pancreatic β-cells. Am J Physiol Cell Physiol 301: C792–C803, 2011. First published July 6, 2011; doi:10.1152/ajpcell.00057.2011.—Glucagon-like peptide-1 (GLP-1) elevates intracellular concentration of cAMP ([cAMP]) and facilitates glucose-dependent insulin secretion in pancreatic β-cells. There has been much evidence to suggest that multiple key players such as the GLP-1 receptor, G protein, adenylate cyclase (AC), phosphodiesterase (PDE), and intracellular Ca2+ concentration ([Ca2+]i) are involved in the regulation of [cAMP]. However, because of complex interactions among these signaling factors, the kinetics of the reaction cascade as well as the activities of ACs and PDEs have not been determined in pancreatic β-cells. We have constructed a minimal mathematical model of GLP-1 receptor signal transduction based on experimental findings obtained mostly in β-cells and insulinoma cell lines. By fitting this theoretical reaction scheme to key experimental records of the GLP-1 response, the parameters determining individual reaction steps were estimated. The model reconstructed satisfactorily the dynamic changes in [cAMP] and predicted the activities of cAMP effectors, protein kinase A (PKA), and cAMP-regulated guanine nucleotide exchange factor [cAMP-GEF or exchange protein directly activated by cAMP (Epac)] during GLP-1 stimulation. The simulations also predicted the presence of two sequential desensitization steps of the GLP1 receptor that occur with fast and very slow reaction rates. The cross talk between glucose- and GLP-1-dependent signal cascades for cAMP synthesis was well reconstructed by integrating the direct regulation of AC and PDE by [Ca2+]i. To examine robustness of the signaling system in controlling [cAMP], magnitudes of AC and PDE activities were compared in the presence or absence of GLP-1 and/or the PDE inhibitor IBMX.

Adenyl cyclase; glucagon-like peptide-1; model stimulation; phosphodiesterase

Upon elevation of plasma glucose concentration ([glucose]), pancreatic β-cells generate bursts of action potentials to induce cyclic changes in [Ca2+]i (55) and regulate pulsatile insulin release (25). This glucose-dependent insulin secretion is synergistically enhanced by incretin hormones, which are released upon meal ingestion from endocrine cells distributed over the intestinal tract (16). The incretin hormones include glucagon-like peptide (GIP) and glucagon-like peptide-1 (GLP-1). GLP-1 is more effective than GIP to improve deteriorated incretin effect in diabetes and is widely used to treat patients with Type 2 diabetes (45). Elucidation of GLP-1 signaling system in β-cells, therefore, has been an extensive target of experimental studies. To date, it has been well established that GLP-1 activates adenylate cyclases (ACs) through binding to its G protein-coupled receptor and increases [cAMP], the key signal underlying the insulinotropic effects (17, 62).

The [cAMP] is determined primarily by the balance between cAMP production by ACs and degradation by phosphodiesterases (PDEs) (8). The activities of several isoforms of AC and PDE expressed in β-cells are controlled by [Ca2+]i (11, 28), which is regulated by Ca2+-permeable ion channels and transporters as well as Ca2+ release and uptake by the endoplasmic reticulum (ER). The increase in [cAMP] subsequently activates protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac), modulating the activities of multiple ion channels at the plasma membrane (26, 31, 35, 41, 42, 57) and ER (27, 36, 64), which in turn modify the pattern of Ca2+ transients. PKA and Epac also have direct effects on proteins that are involved in exocytosis of insulin vesicles (30), and thus the fine regulation of [cAMP] is critical for the adequate insulinotropic effects of GLP-1. However, since multiple signaling factors are involved in regulating [cAMP], the kinetic aspects of the reaction cascade during GLP-1 stimulation have not yet been determined in pancreatic β-cells.

To overcome this difficulty, we developed a mathematical model of GLP-1 receptor signal transduction. We adopted a strategy of estimating individual reaction rates and model parameters by fitting the theoretical reaction scheme to a variety of key experimental findings published to date (3, 11, 54, 66) in both β-cells and insulinoma cell lines. The model thus developed was validated by reconstructing the dynamic changes in [cAMP] during GLP-1 stimulation in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX) observed under various experimental conditions. The model well-simulated GLP-1-induced [cAMP] elevation and predicted the activities of cAMP effectors PKA and Epac as a function of IBMX. The simulation analysis revealed the presence of two transition steps of receptor desensitization that occur with fast and slow kinetics. The molecular basis for synergistic relationship between glucose and GLP-1 signaling in the cAMP synthesis were clarified by calculating the direct regulation of AC and PDE by [Ca2+]i. Finally, the robustness of the signaling system in controlling [cAMP] was examined by comparing the AC and PDE activities in the presence or absence of GLP-1 and/or the PDE inhibitor.

Glossary

| [L] | GLP-1 (ligand) |
| [R] | free GLP-1 receptor |
| [R1] | total GLP-1 receptor |
| [R2] | active GLP-1 receptor |
| [RD1] | desensitized GLP-1 receptor in state 1 |
| [RD2] | desensitized GLP-1 receptor in state 2 |
| [LR] | GLP-1 receptor bound with ligand |

1 This article is the topic of an Editorial Focus by Harvey (29a).

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A minimal model of the GLP-1 receptor signaling transduction in pancreatic β-cells was constructed. Parameters to define the model, including concentrations, binding constants (K), of signaling factors, maximum activity (Vmax) and half-maximal effective concentration (Km) of substances for activation of enzymes, rate constants, and various magnitude factors of kinetic equations are listed in APPENDIX I. The time-dependent integration of six differential equations (Eqs. 3–8) were performed using the Euler method with a time step of ≤2 ms on the Microsoft Visual Studio platform. The units of time and substrate concentrations are seconds and millimolar (indicated otherwise), respectively.

**Activation of GLP-1 receptor.** Figure 1 shows the reaction scheme of the minimal model of the GLP-1 receptor cascade. Active receptors may form three different conformations: free receptor (R), ligand (L)-bound receptor (LR), and the Gs-bound LR complex (LRG). Binding reactions indicated by black arrows were assumed to be much faster than the rest of reactions associated with conformational changes (22), and thus an instantaneous equilibrium was assumed for the reactions enclosed within the red rectangle. The dissociation constant (Kd) for GLP-1 binding to the receptor has been determined in expression systems (45, 65), whereas Kd for LR and G was estimated by fitting the [GLP-1]-dependent [cAMP] accumulation (66). In calculating the reaction cascade, total amounts [R], [G], and [Gβγ] were conserved by applying Eqs. 1 and 2, respectively.

\[
[R] = [R]_{0} + [R_{D2}] + [R_{D1}]
\]

Where \( [R]_{0} = [R] + [LR] + [LRG] \).

\[
[G] = [G_{αβγ}] + [G_{βγ}]
\]

Where \( [G_{αβγ}] = [G] + [LR] \).

Upon ligand binding, the GLP-1 receptor undergoes desensitization through phosphorylation by unknown mechanisms (67). Although the molecular mechanism has not been elucidated, the computer simulation of the spontaneous decay in [cAMP] during continuous stimulation with GLP-1 (see Fig. 3) as well as desensitized [cAMP] production after preconditioning of GLP-1 receptors (see Fig. 4) suggested the presence of two sequential desensitization states R_D1 and R_D2. We assume that the first desensitization step to R_D1 occurs from the ligand-bound form of the receptor, (LR + LRG), followed by the second transition to R_D2 with a recovery steps to R, (see Fig. 1). The rate constants for the time-dependent desensitization were determined by fitting the kinetic scheme to experimental recordings, and the time-dependent changes in [R_D1] and [R_D2] are defined by Eqs. 3 and 4, respectively.

\[
d[R_{D1}] = k_{1} \cdot ([LR] + [LRG]) - k_{2} \cdot [R_{D1}] - k_{3} \cdot [R_{D1}] + k_{4} \cdot [R_{D2}]
\]

\[
d[R_{D2}] = k_{3} \cdot [R_{D1}] - k_{4} \cdot [R_{D2}]
\]

The kinetics for the activation and deactivation of G protein are calculated by rate constants of G dissociation into GαGTP and Gβγ subunits and subsequent hydrolysis of GTP (APPENDIX I), which have been biochemically investigated (5, 56). The time-dependent changes in [GαGTP], [Gβγ], and [Gαβγ] are described by the following differential equations:

\[
d[G_{αGTP}] = k_{5} \cdot [LRG] - k_{6} \cdot [G_{αGTP}]
\]

\[
d[G_{βγ}] = k_{6} \cdot [G_{αGTP}] - k_{7} \cdot [G_{αβγ}]
\]

\[
d[G_{αβγ}] = k_{5} \cdot [LRG] - k_{7} \cdot [G_{αGTP}] \cdot [G_{βγ}]
\]

**AC and PDE activities.** The level of [cAMP] is determined by the balance between production rate (V_{Ac,GTP}) and degradation rate (V_{PDE}) by ACs and PDEs, respectively (Eq. 8).

\[
\frac{dcAMP}{dt} = V_{Ac,GTP} - V_{PDE}
\]

At least nine different isoforms of membrane-bound AC have been identified (29). In pancreatic β-cells, ACVIII was suggested to play a predominant role in synthesis of cAMP during GLP-1 stimulation of β-cells (53). The modulation of ACVIII by both Ca2⁺-bound calmodulin (Ca_CaM) and GαGTP would provide the molecular basis for synergistic relationship between glucose and GLP-1 stimulation in the cAMP synthesis (11). In addition to this adaptable component...
The G<sub>a</sub>GTP-dependent activation of AC was calculated with a $K_{1/2}$ determined by Sunahara and colleagues (58). An [ATP] of 3 mM was used in the present study, and the $K_{1/2}$ of [ATP] defining the substrate dependency of V<sub>AC</sub> and V<sub>AC,G</sub> were adopted from Dessauer et al. (13). The term for Ca<sup>2+</sup>-dependent regulation of V<sub>AC,G</sub> in Eq. 11 was originally developed in Aplysia neurons (69) and was modified to fit the ACVIII activity (21) in the β-cell model by Fridlyand et al. (23). Eq. 11 contains both Ca<sub>2+</sub>CaM ([Ca<sub>2+</sub>CaM]+[Ca<sub>2+</sub>CaM]) and Ca<sup>2+</sup>-dependent activation and Ca<sup>2+</sup>-dependent inhibition. We additionally introduced f<sub>CL,AC</sub>, the fraction of Ca<sub>2+</sub>CaM-dependent V<sub>AC,G</sub>. A [Ca<sup>2+</sup>] of 500 nM was assumed under a high-glucose condition and a resting [Ca<sup>2+</sup>] of 100 nM for a lower glucose concentration used in experiments (18, 33, 60). An instantaneous equilibrium was assumed for the binding of Ca<sup>2+</sup> to CaM using the association and dissociation rate constants given by Yu and colleagues (69). The V<sub>AC,i</sub> was determined at 1.8 μM/s from the initial rate of rise (dashed line in Fig. 3A) of [cAMP] evoked by GLP-1 in the presence of high [IBMX] > 250 μM and [glucose] > 20 mM. Based on this estimation, V<sub>max</sub> of AC activities (V<sub>max,AC</sub> and V<sub>max,AC,G</sub>) and fractions (f) of the Ca<sup>2+</sup>-dependent component of V<sub>AC,G</sub>(f<sub>CL,AC</sub>) were optimized (see APPENDIX I) by reconstructing experimental findings with the whole reaction scheme.

In β-cells, it has been suggested that several PDE isoforms (1C, 3B, 4, 5B, and 10A) are involved in regulation of insulin secretion (15, 51). However, the fractional contribution of each isofrom to cAMP degradation in intact cells has not yet been determined. Sams and Montague (54) observed over 70% of total PDE activity in the supernatant fraction of an homogenate of islets of Langerhans. Their kinetic analysis of the soluble PDEs suggested the presence of at least two fractions with different activities, as indicated by two linear components (dashed and solid black lines) in the Lineweaver-Burk plot (Fig. 2). We reevaluated the experimental results by fitting the data with a sum of two Michaelis-Menten functions (Eq. 12) in the present study.

The half-maximal value of Ca<sub>2+</sub>CaM for the stimulation of PDE (68) was adopted from the PDE model developed for β-cells (23), and f<sub>CL,PDE</sub> reflects the fraction of the Ca<sub>2+</sub>CaM-dependent component of the enzyme. Given the [cAMP]s determined under various experimental conditions (Table 1), V<sub>max,PDE</sub> and f<sub>CL,PDE</sub> (APPENDIX I) were finely adjusted by reconstructing these experimental findings using 100 or 500 nM [Ca<sup>2+</sup>] according to the [glucose] used in experiments. In experimental studies, the rise in [cAMP] evoked by GLP-1 saturates even in the presence of a maximal inhibitory concentration of IBMX, indicating that some fraction of PDE activity still remained, controlling [cAMP]. Ahmad and colleagues (1) showed that ~80% of the soluble PDE activity in a β-cell line (BRIN-BD11 cells) was blocked by [IBMX] > 200 μM, and thus we assumed that the

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Table 1. Comparison of [cAMP] between experimental measurements in rat primary pancreatic β-cells and model simulations under control conditions and after 15 min stimulation with GLP-1 with or without IBMX

<table>
<thead>
<tr>
<th>Experimental Data</th>
<th>Simulation Result</th>
<th>Experimental Data</th>
<th>Simulation Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP levels, μM</td>
<td>1.4 mM Glucose</td>
<td>100 nM Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>20 mM Glucose</td>
</tr>
<tr>
<td>Resting (control)</td>
<td>3.4</td>
<td>1.6</td>
<td>3.2</td>
</tr>
<tr>
<td>10 nM GLP-1</td>
<td>10.5</td>
<td>12.0</td>
<td>11.7</td>
</tr>
<tr>
<td>10 nM GLP-1/1w IBMX</td>
<td>38.2</td>
<td>37.0</td>
<td>55.2</td>
</tr>
</tbody>
</table>

The cAMP levels were indicated in units of fmol-10<sup>10</sup> cells<sup>-1</sup> in the experimental work (11), and we converted these units to μM by assuming the cytoplasmic volume of a single β-cell [764 fl. (10)]. Administration of IBMX (250 μM) was simulated by decreasing PDE activity by 80%. See text for definitions of abbreviations.
RESULTS

[cAMP] in pancreatic β-cells under resting conditions and GLP-1 stimulation. The basal level of [cAMP] was 1.6 μM at 100 nM [Ca^{2+}] and 1.4 μM at 500 nM [Ca^{2+}] in our model simulation (Table 1). These values of [cAMP] are comparable to 3.4 and 3.2 μM measured in rat primary β-cells at 1.4 and 20 mM [glucose], respectively (11). Upon stimulation with 10 nM GLP-1 for 15 min, [cAMP] increased to 4.2 μM at 100 nM [Ca^{2+}] and to 5.6 μM at 500 nM [Ca^{2+}] in our model. If PDE was inhibited by 80% (corresponding to 250 μM IBMX) in the absence of GLP-1, [cAMP] increased to ~11 μM independently of [Ca^{2+}] levels. These results also agreed well with the experimental observations. When stimulated with 10 nM GLP-1 in the presence of IBMX, [cAMP] elevated to 37.0 μM at 100 nM [Ca^{2+}] and further to 57.6 μM at 500 nM [Ca^{2+}], similarly to the experimental records of 38.2 and 55.2 μM obtained at the low (1.4 mM) and high (20 mM) [glucose], respectively.

The experimental time course of [cAMP] accumulation induced by GLP-1 (66) was also examined (Fig. 3). Upon stimulation with GLP-1 at 25 mM [glucose] in the presence of IBMX, [cAMP] increased rapidly and slowly declined after reaching peaks within 4 min (filled circles, Fig. 3A). The time course of [cAMP] was well reconstructed by simulation at 20% PDE activity and 500 nM [Ca^{2+}] (black curve, Fig. 3A). The velocity of cAMP degradation by PDE gradually increases with increasing [cAMP], and the peak is attained when the production rate of cAMP (V_{AC,t}) matched the degradation rate by PDE (V_{PDE} in Eq. 8). Simulations revealed that the time to peak as well as the subsequent slow decline in [cAMP] were also influenced by desensitization of the GLP-1 receptor, predominantly due to the state transition to R_{D1} (Fig. 1). In the absence of IBMX, the balance between AC and PDE activities are attained at a much lower [cAMP], thereby giving a time to peak of <1 min (gray curve, Fig. 3A). The simulation result was also in good agreement with experimental data (open circles, Fig. 3A).

The experimental dose-response relationship obtained by Widmann and colleagues (66) was reconstructed by calculating the [cAMP] accumulation attained over 10 min application of different concentrations of GLP-1 at 20% PDE activity and 500 nM [Ca^{2+}] (Fig. 3, B and C). At 0.001 and 0.01 nM [GLP-1], [cAMP] increased to a stable saturation level within 2 min (Fig. 3B), whereas at higher [GLP-1], the time to peak was delayed with increasing [GLP-1] and the desensitization became more pronounced. As [GLP-1] increases, the activation of V_{AC,G} (see Eqs. 9−11) became significant when [GLP-1] > 0.1 nM and nearly saturated at [GLP-1] slightly larger than 10 nM. In Fig. 3C, the dose-dependent accumulation of cAMP at the end of 10 min application of different concentrations of GLP-1 are compared between the simulation and experimental
results in INS-1 cells. The half-maximal [GLP-1] is 0.56 nM in the simulation, which is only slightly larger than that obtained in INS-1 cells (0.50 nM).

Ultra-slow desensitization of the GLP-1 receptor. The simulation analysis suggested that the gradual decay of [cAMP] after the peak (Fig. 3, A and B) during GLP-1 stimulation largely reflects desensitization of the ligand-bound receptor (LR and LRG in Fig. 1) to RD1. However, the RD1 kinetics alone failed to reconstruct the very slow inactivation remaining 1 h after washing out agonist as observed by Baggio and colleagues (3). The model including an RD2 state in series with RD1 reconstructed well the desensitization phenomenon in response to the experimental protocol (Fig. 4 A). When the 2-h pretreatment protocol was applied (Fig. 4 B), the RD1 fraction (blue curve) increased to a maximum of 0.28 at the expense of the active fraction (Ra, red curve) within the initial 10 min, and then both Ra and RD1 slowly declined thereafter due to a continuous transition to RD2 (green curve). During the washout period, the RD1 fraction quickly became insignificant, whereas 85% of RD2 remained even after 1 h washout. The result suggests that the fraction of Ra available for the second application of agonist, the [GLP-1]-dependent VAC,t at varying incubation periods (10 s to 4,000 min) were computed over the range 0.001 to 100 nM [GLP-1] (Fig. 4 D). With 6- and 10-s applications, VAC,t nearly overlap one another virtually without any sign of receptor desensitization. When the duration of GLP-1 application was prolonged, the desensitization gradually developed and a steady state was obtained at ~2,000 min application, which gave ~25% of the control VAC,t at the saturating [GLP-1]. The extent of desensitization was more pronounced when stimulated with higher [GLP-1].

Cross-talk between glucose and GLP-1 signal pathways in determining [cAMP]. Experimental studies in Min6 and INS-1 beta-cells demonstrated that [cAMP] increased in phase with a temporal increase in [Ca2+] in the presence of GLP-1, whereas

Table 2. Reduction of GLP-1-dependent [cAMP] accumulation induced by prestimulation of receptors for different time periods

<table>
<thead>
<tr>
<th>Preincubation Period With 100 nM GLP-1</th>
<th>0.5 h</th>
<th>1 h</th>
<th>2 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Reduction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Simulation results</td>
<td>16</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>Experimental results</td>
<td>21</td>
<td>35</td>
<td>50</td>
</tr>
</tbody>
</table>

The [cAMP] accumulation caused by IBMX alone (Fig. 4 C) was subtracted from the [cAMP] responses to the second application of agonist, and the differences were normalized to the control value. The simulation results are compared with the experimental data in INS-1 cells (3). See text for definitions of abbreviations.
the relation between [cAMP] and [Ca\(^{2+}\)] was out of phase without agonist (19, 23, 38). The simulation in Fig. 5 examines mechanisms of [Ca\(^{2+}\)]-dependent regulation of [cAMP] in the absence or presence of GLP-1. In the absence of GLP-1, the total cAMP production rates \(V_{AC,G} + V_{AC,PDE}\) matched the \(V_{PDE}\) at a resting [cAMP] of 1.6 \(\mu\)M (black curve in Fig. 5A) at 100 nM [Ca\(^{2+}\)]. When [Ca\(^{2+}\)] was increased to 500 nM (grey curve), [cAMP] slightly decreased to 1.4 \(\mu\)M due to the facilitation of PDE activity by [Ca\(^{2+}\)] \(V_{Cd,PDE}\) (grey curve in Fig. 5C). After 30 min simulation with 100 nM GLP-1, [cAMP] increased to 4.1 \(\mu\)M (Fig. 5B) due to enhanced \(V_{AC,G}\) at 100 nM [Ca\(^{2+}\)]. Increasing [Ca\(^{2+}\)] to 500 nM further elevated [cAMP] in a reversible manner up to 5.4 \(\mu\)M because the activation of AC\(_{G}\) by [Ca\(^{2+}\)] \(V_{Cd,AC}\) (black curve in Fig. 5D) was relatively larger than that of PDE \(V_{Cd,PDE}\). These simulation results revealed the mechanisms underlying the in-phase or out-of-phase patterns of [cAMP] fluctuations in response to the cyclic changes in [Ca\(^{2+}\)] (19, 23, 38). To get a deeper insight into the physiological significance of [Ca\(^{2+}\)] in determining [cAMP], high localized cAMP microdomain may possibly be present.
at submembrane space, since production of cAMP by ACs is limited at the surface membrane (29, 38), while PDE-mediated degradation occurs diffusely within the cytosol (4, 54). Indeed, cAMP microdomain has been observed beneath the surface membrane in other cell types, such as cardiac myocytes (70) and HEK cells (61).

To simulate the distribution of [cAMP], intracellular diffusion of cAMP was calculated (Fig. 7). For simplicity, one-dimensional diffusion was assumed over a distance of 4 μm (estimated from Ref. 48) from the surface membrane toward the nucleus as indicated in Fig. 7A. The diffusion path (x) was separated into 200 compartments, and [cAMP] in each compartment at time t [C(x,t)] was calculated using the following equation:

\[ \frac{\partial C(x,t)}{\partial t} = D \cdot \frac{\partial^2 C(x,t)}{\partial x^2} \]  

(14)

With boundary condition of

\[ \frac{\partial C}{\partial x} \bigg|_{x=0.4} = 0 \]

where the diffusion coefficient (D) was 0.3 μm²/ms based on experimental measurements of 0.27 μm²/ms (6) and 0.33 μm²/ms (32). The cAMP production by AC stimulated by a [GLP-1] of 10 nM was assumed only in the first compartment (29, 38), whereas cAMP degradation by PDE was calculated in all compartments (4, 54). A quasi-steady-state concentration gradient of [cAMP] was established within 100 ms after the onset of AC activation, and the difference in [cAMP] over the diffusion path of 4 μm was only about 0.05 μM. Theoretically, but unrealistically, a much larger gradient (4 μM) was developed only when V_{PDE} was increased by 100 times. The distributions of [cAMP] relative to [cAMP] in the submembrane space simulated using the control and high V_{PDE} are shown in Fig. 7, B and C, respectively. This finding agrees with simulation results demonstrated by Oliveira et al. (47) (see Discussion for more detail). The flat distribution in Fig. 7B may justify the use of average [cAMP] in estimation of active fractions of cAMP effectors that are distributed throughout the cytosol of pancreatic β-cells.

With the use of the GLP-1 receptor signal transduction model, the dose-dependent activities of PKA at the end of 30 min application of various concentrations (0.001–100 nM) of GLP-1 under high [Ca^{2+}] condition were simulated (Fig. 6A). The [cAMP]-dependent PKA activation was calculated using a
$K_d$ and $nH$ (see Appendix I) obtained by Dao et al. (9). A significant fraction ($\sim 26\%$) of PKA was already active under the control condition of 1.4 $\mu$M [cAMP] at 500 nM [Ca$^{2+}$] without GLP-1 stimulation. As shown in Fig. 6A, the activity of the enzyme dose dependently increased, and the application of 100 nM [GLP-1] induced nearly the saturating activation of PKA ($\sim 70\%$) through an increase in [cAMP] to 5.4 $\mu$M. With the same experimental conditions as in Fig. 6A, the active fractions of Epac1 and 2, other cAMP effectors, were also simulated using the half-maximal values of cAMP for the activation of each enzyme (Appendix I). GLP-1 at 100 nM activates Epac1 from 4.4% of basal activity to 15.3%, whereas active Epac2 was about 6.4% at rest and was increased to $\sim 21.3\%$ (Fig. 6B).

**Systems analysis on the balance between the production and degradation of cAMP.** The level of [cAMP] is determined by the balance between AC and PDE activities (Eq. 8). Depression of $V_{PDE}$ below $V_{AC}$ will cause collapse of [cAMP] regulation, leading to a continuous accumulation of cAMP within cytosol. In both experiments and computer model simulations, quasi-steady-state [cAMP] levels were obtained even when the PDE activity was depressed by a saturating dose ($>200$ $\mu$M) of IBMX. This is because balancing $V_{AC}$; with $V_{PDE}$ was established with the IBMX-insensitive PDE fraction ($\sim 20\%$ total PDE activities) under this extreme condition. To examine the robustness of the [cAMP] homeostasis in our model, the $V_{AC}$; devoid of desensitization was compared with $V_{PDE}$, and the steady-state level of [cAMP] was defined by the intersection of $V_{AC}$; with the [cAMP]-$V_{PDE}$ curve in Fig. 8. When the $V_{AC}$; was maximized at 500 nM [Ca$^{2+}$] using the saturating concentration of 100 nM [GLP-1], the equilibrium [cAMP] was obtained at $\sim 115.7$ $\mu$M with 20% $V_{PDE}$ (grey sigmoidal curve) as indicated by the arrow a. This analysis determines that depression of $V_{PDE}$ below $\sim 14\%$ will cause collapse of the [cAMP] homeostasis when $V_{AC}$; was maximized. Under physiological conditions, the steady-state [cAMP] was maintained at 7.9 $\mu$M (arrow b) with intact $V_{PDE}$ (black sigmoidal curve) even when AC was fully activated. The lower limit of [cAMP] in the absence of GLP-1 stimulation, on the other hand, is determined by the activity of the G protein-insensitive component of AC, and the [cAMP] was balanced at 1.4 $\mu$M (arrow c). It should be noted that this range of [cAMP] change (1.4–7.9 $\mu$M) well fits the dynamic range of the PKA activation ($K_1/2 = \sim 3$ $\mu$M). This analysis revealed that the intact systems of PKA and PDE activities also elucidated the fractions of [Ca$^{2+}$]-sensitive components of these enzymes. Considering that [Ca$^{2+}$] is raised by [glucose] stimulation via the enhanced electrical activity of $\beta$-cells, the activation of GLP-1 signaling is a powerful amplifier for promoting the insulin release in the presence of stimulating concentrations of glucose.

**DISCUSSION.** In the present study, a minimal model of GLP-1 receptor signal transduction was developed mostly based on experimental data reported in $\beta$-cells or related cell lines. The model successfully reconstructed the experimental findings of dynamic changes in [cAMP] during agonist stimulation in the absence or presence of IBMX at two representative levels of [Ca$^{2+}$] (Fig. 3). The model predicted the activity of cAMP effectors PKA and Epac during GLP-1 stimulation (Fig. 6). The simulation also demonstrated that the GLP-1 receptor desensitization kinetics applied to the model successfully explained the fast and very slow inactivation steps, which have significant effects on the decay kinetics of [cAMP] during the continuous GLP-1 stimulation (Fig. 3) as well as reduced [cAMP] production after preconditioning of the receptor (Fig. 4 and Table 2). The cross talk between glucose- and GLP-1-dependent signal cascades in synergistic synthesis of cAMP was well reconstructed by incorporating the direct regulation of both AC and PDE by [Ca$^{2+}$] (see Table I). Models of the [Ca$^{2+}$]-dependent AC and PDE activities also elucidated the fractions of [Ca$^{2+}$]-sensitive components of these enzymes. Considering that [Ca$^{2+}$] is raised by [glucose] stimulation via the enhanced electrical activity of $\beta$-cells, the activation of GLP-1 signaling is a powerful amplifier for promoting the insulin release in the presence of stimulating concentrations of glucose.

The insulinotropic effect of the cAMP signal involves the activation of PKA and Epac, and subsequent modulation of ion channel functions (26, 31, 35, 41, 42, 57) and Ca$^{2+}$ release from ER (27, 36, 64), in addition to their direct effects on the exocytotic machinery. The effects on ion channels, for example, include the enhancement of Ca$^{2+}$ influx via L-type Ca$^{2+}$ currents (26, 57) and inhibition of K$^+$ currents (26, 31, 35, 41, 42), which may in turn promote the insulin release via an increase in the membrane excitability. These comprehensive mechanisms of the insulinotropic effect of GLP-1 will be analyzed by incorporating the GLP-1 receptor signaling cascade model into appropriate whole $\beta$-cell models in the future.

**Fig. 8. Systems analysis on the robustness of GLP-1 signaling system in [cAMP] regulation.** The black sigmoidal curve, the control $V_{PDE}$, plotted as a function of [cAMP] at 500 nM [Ca$^{2+}$] (see Eq. 13). Grey curve, 20% of $V_{PDE}$ under the presumptive presence of IBMX. Dotted curve, 14% $V_{PDE}$. The three horizontal lines from top to bottom indicate the sum of ($V_{max,AC} + V_{max,AC,o}$) and two levels of $V_{AC}$, at 100 nM and 0 [GLP-1], respectively. To evaluate maximum possible levels of [cAMP] increase, the desensitization of the receptor was removed in this calculation. The arrows a, b, and c indicate intersections of $V_{PDE}$ and $V_{AC}$ curves. Note that [cAMP] can vary between the minimum level given by the arrow c and the maximum level given by the arrow b under the physiological condition. With 20% $V_{PDE}$, [cAMP] could increase to an extreme level (arrow a). Inhibition of $V_{PDE}$ exceeding 14% would cause the "break-down" of the signaling system leading to a continuous [cAMP] accumulation, indicating that the intact PDE has an excessive capacity to balance against the production of cAMP by AC under physiological condition.

[Figure 8: Graph showing the systems analysis on the robustness of GLP-1 signaling system in [cAMP] regulation.]
limited at the submembrane space, while PDE-mediated degradation occurs diffusely within the cytosol. Our finding is, however, different from the localized cAMP micromanifold observed in other cell types [cardiac myocytes (70) and HEK293 cells (61)]. The theoretical study by Oliveira et al. (47) demonstrated a large [cAMP] gradient (~5 μM) in HEK293 cells upon stimulation with PGE1. They concluded that the PDE4D activity enhanced by PKA-mediated phosphorylation was necessary and sufficient for generating the cAMP micromanifold observed by Terrin et al. (61), and no physical barrier was required against the cAMP diffusion. If compared with our simulation, the cAMP diffusion constant used in their study was essentially the same magnitude as in our calculations. Interestingly, our diffusion model also generated a similar [cAMP] gradient of ~4 μM (Fig. 7C) when simulated using the \( V_{\text{PDE}} \) equivalent to that used in the Oliveira et al. (47). However, their \( V_{\text{PDE}} \) was much higher (~100 fold) than that determined by the model fitting to the published experimental data in the present study. If we adopt the higher PDE activity, the model failed to reconstruct experimental records of [cAMP] changes during GLP-1 stimulation. Our simulation results strongly suggest that the distribution of cAMP is homogeneous in pancreatic β-cells because of relatively low PDE activities. These results, however, do not necessarily exclude the possibility of a functional coupling among AC, PKA, and/or Epac and effector proteins by AKA near the membrane, which has been suggested in the heart and brain (12, 46). On the other hand, the rapid diffusion of [cAMP] could possibly be an essential factor in the GLP-1 receptor signaling for a proper modulation of insulin release, since PKA and Epac, the target proteins of cAMP, are widely distributed throughout the intracellular space (12, 40, 49). For activation of all these enzymes, the rapid access of cAMP might be critical to fulfill the basic needs of subsequent modulatory actions on ion channels on the surface and ER membrane as well as exocytotic machinery in concert within the entire intracellular space.

**Sensitivity of the model to varying parameters.** Although [R] was estimated in a previous study (66), [G] has not yet been determined in pancreatic β-cells. In the present study, [G] was referred by Post et al. (50), who suggested the expression of G_{α} protein in large excess relative to β-adrenergic receptor in cardiac myocytes and hypothesized that this stoichiometry of [G] to [R] will be applicable to other G protein-coupled hormone receptor systems. We found that the EC_{50} of [GLP-1]-dependent [cAMP] accumulation is dependent on the agonist-induced [GLP-1]-complex [LRG] = [G]·[LR]/[K_{d}], and thereby the EC_{50} can be adjusted by modifying either [G] or [K_{d}] of [G]·[LR] binding. Since both parameters have not been investigated in pancreatic β-cells, [GLP-1]-dependent [cAMP] accumulation was reconstructed by determining an appropriate K_{d} in the present study under the assumption that [G] is expressed to a similar extent as in the cardiac tissue (50). The fraction of [R_{a}] is one of signaling factors that have the strong influence on AC activities and thus [cAMP]. Under physiological conditions, [R_{a}] will largely fluctuate because of extensive desensitization (75% at the maximal, see Fig. 4D), which is expected to proceed during several hours of the meal digestion.

The maximum AC activity (\( V_{\text{max,AC,G}} = V_{\text{max,AC}} + V_{\text{max,AC,G}} \)) is much larger than the \( V_{\text{max}} \) of PDE in our model. The lower AC activity under physiological conditions is mostly due to the low sensitivity of AC to G_{α}GTP in addition to the low [G_{α}GTP] production, even with a saturating [GLP-1] (see Eq. 11). Other modulatory factors of the AC activity, such as the dependencies on the substrate ATP and the Ca^{2+}-dependent inactivation, are nearly saturated with the physiological level of [ATP] and [Ca^{2+}]. Although Ca_{α}CaM-dependent activation of V_{AC,G} is pronounced with increasing [GLP-1], [cAMP] production becomes partially compensated by the parallel activation of PDE. More specifically, [cAMP] level balances at 7.61 μM under the stimulation with 10 nM GLP-1 at 500 nM [Ca^{2+}] without desensitization processes, whereas it increased to 8.61 μM when the component of Ca_{α}CaM-dependent activation of \( V_{\text{PDE}} \) was excluded from the model. The PDE component showing the high cAMP sensitivity (K_{pala}) also plays a subtle role in regulation [cAMP], whereas the low cAMP sensitivity (K_{mal}) component virtually fulfills the physiological role in cAMP hydrolysis in the present model.

**Limitations.** The kinetics of the simple sequential transition of desensitized receptors from R_{D1} to R_{D2} was modeled to describe the time course of the delayed recovery from slow inactivation (Fig. 4). Indeed, the reaction scheme for receptor desensitization (Fig. 1) well simulated both of the fast and very slow desensitization observed in experimental studies (see RESULTS). Up to date, it is clear that the phosphorylation of the GLP-1 receptor is the key desensitization step, whereas Widmann and colleagues (67) have shown that neither PKA nor PKC are involved in the process. The involvement of β-Arrestin2 and GRK5 was suggested by Jorgensen et al. (34), whereas it is still highly controversial since a different group demonstrated the desensitization was independent of β-Arrestin2 (59). The model scheme will need to be improved when the molecular mechanisms are established in future experimental studies. It may also be examined whether the activation of PDE through phosphorylation by PKA is responsible for a small fraction of the spontaneous decay of [cAMP], although the present study attributed the decay only to desensitization.

The active fractions of PKA as well as Epac1 and 2 were calculated by a use of biochemically determined K_{d} or K_{1/2}, half-maximal [cAMP] for the activation of these enzymes. However, especially for PKA activation, there has been obvious disagreement in published values of K_{1/2}, which vary over a nanomolar to micromolar range. It seems that the experimental K_{1/2} is highly related to the concentration of enzyme used in biochemical investigations (7). K_{1/2} was ~3 μM for more physiological concentration of the PKA isozyme II (400 nM), whereas it was significantly reduced to ~50 nM when tested on 1 nM holoenzyme. Indeed, other investigators (14) used low holoenzyme concentrations of 20~30 nM and obtained a K_{1/2} of 98 nM and 540 nM for PKAI and PKAII, respectively. In the present study, PKA activities were calculated with K_{1/2} of ~3 μM, yet, it may need to be reevaluated when more accurate experimental measurements of K_{1/2} values as well as concentrations of PKA isozyme become available.

cAMP is distributed within a β-cell in forms of free cAMP and PKA or Epac-bound form (cAMP-PKA and cAMP-Epac). In the present study, however, [cAMP] was calculated neglecting [cAMP-PKA] and [cAMP-Epac]. If the total amount of cAMP is comparable to PKA and/or Epac, it will be necessary to consider [PKA] and/or [Epac] in calculating the concentration of unbound cAMP (\( [\text{cAMP}]_{\text{total}} = [\text{cAMP}] + [\text{cAMP-PKA}] + [\text{cAMP-Epac}] \)). Similarly, the mass conservation should also be consid-
ferred for \([G_\alpha GTP]\) \(([G_\alpha GTP]_\text{total} = [G_\alpha GTP] + [G_\alpha GTP-AC])\) when \([AC]\) is available. Since the amounts of PKA, Epac, and AC are not determined in \(\beta\)-cells, we excluded these conservation equations from the present model.

APPENDIX I

A) Parameters Determined in Published Experimental Studies

1) Parameters determined based on GLP-1 signaling system in pancreatic \(\beta\)-cell and a \(\beta\)-cell line

   Total amount of receptor, \([R]\)_d = 0.00434 \(\mu\)M (Ref. 66)
   Binding between \([L]\) and \([R]\): \(K_d = 0.004 \, \mu\)M (Refs. 43, 65)

2) Parameters determined by biochemical investigations

   Total amount of \(G_s\), \([G_s]\) = 2.83 \(\mu\)M (Ref. 50)
   \(G_s\)-GTP\(_\text{off}\)-dependent AC\(_G\) activation \(K_{1/2} = 0.4 \, \mu\)M (Ref. 58)
   ATP-dependent AC activity \(K_m = 1.03 \, mM\) (Ref. 13)
   ATP-dependent AC\(_G\) activity \(K_m = 0.315 \, mM\) (Ref. 13)
   Ca\(_{CaM}\)-dependent AC\(_G\) activation \(K_{1/2} = 0.348 \, \mu\)M (Ref. 21)
   Ca\(_{CaM}\)-dependent AC\(_G\) inhibition \(K_{1/2} = 75 \, \mu\)M (Ref. 21)
   Ca\(_{CaM}\)-dependent PDE activation \(K_{1/2} = 0.348 \, \mu\)M (Ref. 68)
   cAMP-dependent PKA activation \(K_d = 2.9 \, \mu\)M, \(K_m = 1.4 \) (Ref. 9)
   cAMP-dependent Epac1 activation \(K_{1/2} = 30 \, \mu\)M (Ref. 20)
   cAMP-dependent Epac2 activation \(K_{1/2} = 20 \, \mu\)M (Refs. 52, 63)

   The kinetics for the activation and deactivation of \(G\) protein
   \(k_5 = 16 \, s^{-1}\), \(k_6 = 1 \, s^{-1}\), \(k_7 = 1, 200, 000 \, mM/s\) (Refs. 5, 56)

B) Parameters Determined in the Present Study by Fitting Specific Experimental Records or Measurements in References

1) Desensitization rate constants

   \(k_1 = 0.0025 \, s^{-1}\), \(k_2 = 0.005833 \, s^{-1}\) (Fig. 3)
   \(k_3 = 0.000283 \, s^{-1}\), \(k_4 = 0.00005 \, s^{-1}\) (Fig. 4 and Table 3)

2) AC and PDE activities (Figs. 3 and 6 and Table 2)

   \(V_{AC>V_{\text{max}}} = 0.0006173 \, mM/s\)
   \(V_{AC>G_{\text{max}}} = 0.01738 \, mM/s\)
   \(f_{\text{CAMP}} = 0.6\)
   \(V_{\text{PDE}} = 0.015 \, mM/s\)
   \(f_{\text{CAMP}} = 0.2\)
   \(K_{m} = 0.4148 \, \mu\)M, \(K_{m} = 53.98 \, \mu\)M
   \(f = 0.012\)

C) Parameters Determined in the Present Study by Fitting the Overall Model Scheme to Experimental Records or Measurements In References

   Binding between \([G]\) and \([LR]\): \(K_d = 0.372 \, \mu\)M (Ref. 66)

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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