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

Yukari Takeda,1 Akira Amano,2 Akinori Noma,2 Yasuhiro Nakamura,1 Shimpei Fujimoto,1 and Nobuya Inagaki1

1Department of Diabetes and Clinical Nutrition, Graduate School of Medicine, Kyoto University, Kyoto; and 2Faculty of Bioinformatics, Ritsumeikan University, Kusatsu City, Japan

Submitted 3 March 2011; accepted in final form 28 June 2011

Takeda Y, Amano A, Noma A, Nakamura Y, Fujimoto S, 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+]) 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 were clarified by calculating the direct regulation of [cAMP], magnitudes of AC and PDE activities were compared in the presence or absence of GLP-1 and/or the PDE inhibitor IBMX.3 Adenylyl 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+] (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 glucose-dependent insulinoimpeptptide (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 adenylyl cyclases (ACs) through binding to its G protein-coupled receptor and increases [cAMP], the key signal underlying the insulinoimpeptotropic 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+] (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 insulinoimpeptotropic 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).5 Adenylyl cyclase; glucagon-like peptide-1; model stimulation; phosphodiesterase

Glossary

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLP-1</td>
<td>GLP-1 (ligand)</td>
</tr>
<tr>
<td>[R]</td>
<td>free GLP-1 receptor</td>
</tr>
<tr>
<td>[Rt]</td>
<td>total GLP-1 receptor</td>
</tr>
<tr>
<td>[R+]</td>
<td>active GLP-1 receptor</td>
</tr>
<tr>
<td>[RD1]</td>
<td>desensitized GLP-1 receptor in state 1</td>
</tr>
<tr>
<td>[RD2]</td>
<td>desensitized GLP-1 receptor in state 2</td>
</tr>
<tr>
<td>[LR]</td>
<td>GLP-1 receptor bound with ligand</td>
</tr>
</tbody>
</table>

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

Address for reprint requests and other correspondence: N. Inagaki, 54 Shogoin, Kawahara-cho, Sakyo-ku, Kyoto-shi, Kyoto, Japan.
[LRG]  GLP-1 receptor bound with ligand and Gs
[G]  total Gs protein
[Gs]  Gs complex
[Gs\beta\gamma]  β and γ subunit of Gs
[GsGTP]  total GTP-bound α subunit of Gs
[GsGDP]  GDP-bound α subunit of Gs
V_{AC,1}  total adenylate cyclase activity
V_C  activity of adenylate cyclase with G protein unbound
V_{max,AC,1}  Maximum activity of V_{AC}
V_{AC,G}  activity of adenylate cyclase with GsGTP
V_{max,AC,G}  maximum activity of V_{AC,G}
\frac{f_{Ca,AC}}{K_{Ca,AC}}  fraction of Ca\textsubscript{a}CaM-dependent V_{AC,G}
\frac{V_{Ca,AC}}{[CaM]}  Ca\textsubscript{a}CaM-dependent component of V_{AC,G}
[CaM]  Calmodulin
[Ca\textsubscript{a}CaM]  Calmodulin bound with Ca\textsuperscript{2+} ions ([Ca\textsubscript{a}CaM] = [Ca\textsubscript{a}CaM\textsubscript{1}] + [Ca\textsubscript{a}CaM\textsubscript{2}])
[Ca\textsubscript{3}CaM]  Calmodulin bound with 3 Ca\textsuperscript{2+} ions
[Ca\textsubscript{4}CaM]  Calmodulin bound with 4 Ca\textsuperscript{2+} ions
V_{PDE}  activity of phosphodiesterase
V_{max,PDE}  maximum activity of V_{PDE}
K_{mL}  low K_m of PDE
K_{mH}  high K_m of PDE
f  fraction of PDE with K_{mL}
\frac{f_{Ca,PDE}}{K_{Ca,PDE}}  fraction of Ca\textsubscript{a}CaM-dependent V_{PDE}
\frac{V_{Ca,PDE}}{[CaPDE]}  Ca\textsubscript{a}CaM-dependent component of V_{PDE}

**METHODS**

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 (V_{max}) and half-maximal effective concentration (K_{1/2}) of substances for activation of enzymes, rate constants, and various magnitude factors of kinetic equations are listed in Appendix I. The time-based 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 secons 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 different conformations: free receptor (R), ligand (L)-bound form, and the Gs-bound LR complex (LGR). 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 (K_d) for GLP-1 binding to the receptor has been determined by fitting the [GLP-1]-dependent [cAMP] accumulation by 10.220.33.1 on June 25, 2017 http://ajpcell.physiology.org/ Downloaded from
(\(V_{AC,G}\)), a basal component (\(V_{AC}\)) was assumed to maintain the resting [cAMP] in the absence of agonists. Thus the total activity of ACs (\(V_{AC,G}\)) is given by a sum of \(V_{AC}\) and \(V_{AC,G}\) (Eqs. 9–11).

\[
V_{AC} = V_{AC,G} = V_{max,AC} \cdot \frac{[ATP]}{0.0004 + G_{\alpha}GTP \cdot [ATP] + 1.03}
\]

\[
V_{AC,G} = V_{max,AC,G} \cdot \frac{G_{\alpha}GTP \cdot [ATP]}{0.0004 + G_{\alpha}GTP \cdot [ATP] + 0.315} \times \left(1 - f_{C_p,AC}\right) + f_{C_p,AC} \cdot \frac{[Ca_{\alpha}CaM] + [Ca_{\alpha}CaM]}{[Ca_{\alpha}CaM] + [Ca_{\alpha}CaM] + 0.000348}\n\]

The \(G_{\alpha}GTP\)-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 \(V_{AC}\) and \(V_{AC,G}\) were adopted from Dessauer et al. (13). The term for \(Ca^{2+}\)-dependent regulation of \(V_{AC,G}\) in Eq. 11 was originally developed in Aplysia neurons (69) and was modified to fit the ACVIII activity (21) in the \(\beta\)-cell model by Fridlyand et al. (23). Eq. 11 contains both \(Ca_{\alpha}CaM\) ([\(Ca_{\alpha}CaM\])\(+\)[\(Ca_{\alpha}CaM\)])-mediated activation and \(Ca^{2+}\)-dependent inhibition. We additionally introduced \(f_{C_p,AC}\), the fraction of \(Ca_{\alpha}CaM\)-dependent \(V_{AC,G}\). A [\(Ca^{2+}\)] of 500 nM was assumed under a high-glucose condition and a resting [\(Ca^{2+}\)] of 100 nM for a lower glucose concentration used in experiments (18, 33, 60). An instantaneous equilibrium was assumed for the binding of \(Ca^{2+}\) to \(CaM\) using the association and dissociation rate constants given by Yu and colleagues (69). The \(V_{AC,1}\) was determined at 1.8 \(\mu\)M/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 \(\mu\)M and [glucose] > 20 mM. Based on this estimation, \(V_{max}\) of AC activities (\(V_{max,AC}\) and \(V_{max,AC,G}\)) and fractions (\(f\)) of the \(Ca^{2+}\)-dependent component of \(V_{AC,G}\) were optimized (see APPENDIX I) by reconstructing experimental findings with the whole reaction scheme.

In \(\beta\)-cells, it has been suggested that several PDE isoforms (1C, 3B, 4B, and 10A) are involved in regulation of insulin secretion (15, 51). However, the fractional contribution of each isoform 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.

\[
V_{PDE} = V_{max, PDE} \cdot \left(\frac{f \cdot [cAMP]}{[cAMP] + K_{m, PDE}} + \frac{(1 - f) \cdot [cAMP]}{[cAMP] + K_{m, PDE}}\right)
\]

The fitting (gray curve in Fig. 2) determined the \(K_m\) values (\(K_{m, PDE}\) and \(K_{m, I}\)) and \(f\) of the two components. \(V_{PDE}\) thus reflects the sum of all PDE activities.

Since \(Ca_{\alpha}CaM\)-sensitive PDE1C plays a functional role in degradation of cAMP in the \(\beta\)-cell line \(\beta\)TC3 (28) and MIN6 (38), a \(Ca^{2+}\)-dependent component was added to the PDE model (Eq. 13).

\[
V_{PDE} = V_{max, PDE} \cdot \left(\frac{f \cdot [cAMP]}{[cAMP] + K_{m, PDE}} + \frac{(1 - f) \cdot [cAMP]}{[cAMP] + K_{m, PDE}}\right)
\]

The half-maximal value of \(Ca_{\alpha}CaM\) for the stimulation of PDE (68) was adopted from the PDE model developed for \(\beta\)-cells (23), and \(f_{C_p, DPE}\) reflects the fraction of the \(Ca_{\alpha}CaM\)-dependent component of the enzyme. Given the [cAMP] is determined under various experimental conditions (Table 1), \(V_{max, PDE}\) and \(f_{C_p, DPE}\) (APPENDIX I) were finely adjusted by reconstructing these experimental findings using 100 or 500 nM [\(Ca^{2+}\)] 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 \(\beta\)-cell line (BRIN-BD11 cells) was blocked by [IBMX] > 200 \(\mu\)M, and thus we assumed that the

The cAMP levels were indicated in units of fmol·10^3 cells^(-1). In the experimental work (11), and we converted these units to \(\mu\)M by assuming the cytoplasmic volume of a single \(\beta\)-cell [764 fl; (10)]. Administration of IBMX (250 \(\mu\)M) was simulated by decreasing PDE activity by 80%. See text for definitions of abbreviations.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Simulation Result</th>
<th>Simulation Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAMP levels, (\mu)M</td>
<td>1.4 mM Glucose</td>
<td>100 nM Ca^{2+}</td>
</tr>
<tr>
<td>Resting (control)</td>
<td>3.4</td>
<td>1.6</td>
</tr>
<tr>
<td>10 nM GLP-1</td>
<td></td>
<td>4.2</td>
</tr>
<tr>
<td>Resting w/ IBMX</td>
<td>10.5</td>
<td>12.0</td>
</tr>
<tr>
<td>10 nM GLP-1/w/IBMX</td>
<td>38.2</td>
<td>37.0</td>
</tr>
</tbody>
</table>

The \(\beta\)-cells and model simulations under control conditions and after 15 min stimulation with GLP-1 with or without IBMX.

Fig. 2. Determination of \(K_m\) values of phosphodiesterase (PDE) based on experimental Lineweaver-Burk plots. Filled circles and fitted (dotted and black) lines are reproduction of experimental data (54) determined in guinea pig islets. The properties of the PDE activity components were reevaluated by fitting the experimental results with Eq. 12 (solid gray line).

The fitting (gray curve in Fig. 2) determined the \(K_m\) values (\(K_{m, PDE}\) and \(K_{m, I}\)) and \(f\) of the two components. \(V_{PDE}\) thus reflects the sum of all PDE activities.

Table 1. Comparison of [cAMP] between experimental measurements in rat primary pancreatic \(\beta\)-cells and model simulations under control conditions and after 15 min stimulation with GLP-1 with or without IBMX.
IBMX-insensitive PDE8 may contribute 20% PDE activity in the presence of a high [IBMX].

**PKA and Epac activities.** Although pancreatic β-cells most likely express both PKA type I and II (2, 37, 39), the isomorph predominantly regulating the insulinotropic effect of GLP-1 has not been investigated. Since $K_d$ of PKA type I (2.9 μM (9)) and the half-maximal [cAMP] for the activation of type II [$K_{1/2} = 2–3$ μM (7)] were very similar, we included one hypothetical type of PKA in the present model, and the activity was calculated with a $K_d$ of 2.9 μM (Hill coefficient, $n_H = 1.4$) determined by Dao et al. (9). Distinct $K_{1/2}$ were reported for Epac1 and 2 (30 μM for Epac1 (20) and 20 μM for Epac2 (52, 63)), and thus the active fractions were separately determined.

**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. 4A). When the 2-h prestimulation protocol was applied (Fig. 4B), 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 GLP-1 decreases depending on the preincubation period. Figure 4C shows the [cAMP] response to the experimental protocol with varying pretreatment periods. It is evident, as predicted, that the longer the preincubation period, the more the [cAMP] response was reduced on 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.

Cross-talk between glucose and GLP-1 signal pathways in determining [cAMP]. Experimental studies in Min6 and INS-1 β-cells demonstrated that [cAMP] increased in phase with a temporal increase in [Ca²⁺] in the presence of GLP-1, whereas
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 $V_{AC_{i,j}}$ ($V_{AC} + V_{AC,G}$) matched the $V_{PDE}$ at a resting [cAMP] of 1.6 μM (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 μM 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 μM (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 μM 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], the changes in [cAMP] in response to increasing [Ca^{2+}] from 0.05 to 5.0 μM were simulated at varying [GLP-1]. The depression of [cAMP] response induced by increasing [Ca^{2+}] at a lower [GLP-1] was inverted at ~0.054 nM [GLP-1], and the enhancement of [cAMP] synthesis by [Ca^{2+}] was augmented further with increasing [GLP-1], saturating at ~2 μM [Ca^{2+}] (Fig. 5E). Note that with higher [Ca^{2+}] (>5 μM), cAMP response to GLP-1 starts to decrease, reflecting the [Ca^{2+}]-dependent inactivation (see Eq. 11).

Activation of PKA and Epac by GLP-1. Time courses of [cAMP] responses to GLP-1 were measured using a PKA-based biosensor in INS-1 cells (19). The fluorescent signals were detected using evanescent wave microscopy reflecting [cAMP] in the submembrane space. However, since the signals were not calibrated, extent of PKA activation by the GLP-1 stimulus was not obtained from the experimental results. We thus attempted to predict PKA activity using the GLP-1 receptor signal cascade model developed in the present study (Fig. 6). Toward this end, it was essential to examine the localization of cAMP under the surface membrane compared with the bulk [cAMP], since the PKA activities may vary within the cytosol due to an uneven distribution of cAMP. In pancreatic β-cells, highly localized cAMP microdomain may possibly be present.

Fig. 5. [cAMP] responses to changes in [Ca^{2+}]. A and B: [Ca^{2+}] (gray lines) was varied from 100 to 500 nM with a time constant of 30 s in the absence (A) or presence (B) of 100 nM GLP-1. [cAMP] (black lines) responses to the corresponding [Ca^{2+}]. C and D: [Ca^{2+}]-dependent AC_G ($V_{Cd_{AC}}$, black line) and PDE ($V_{Cd_{PDE}}$, grey line) activities under the conditions examined in A and B, respectively. E: [GLP-1]-[cAMP] response curves at varying [Ca^{2+}] (0.05, 0.1, 0.2, 0.5, 1.0, 2.0, and 5.0 μM). Dotted gray line indicates [cAMP] at [GLP-1] = 0.054 nM, where decreasing [cAMP] responses to increasing [Ca^{2+}] at a lower [GLP-1] is converted to increasing [cAMP].
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 \(\mu m\) (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:

\[
\frac{\partial C(x,t)}{\partial t} = D \cdot \frac{\partial^2 C(x,t)}{\partial x^2} \tag{14}
\]

With boundary condition of

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

where the diffusion coefficient \((D)\) was 0.3 \(\mu m^2/\text{ms}\) based on experimental measurements of 0.27 \(\mu m^2/\text{ms}\) (6) and 0.33 \(\mu m^2/\text{ms}\) (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 \(\mu m\) was only about 0.05 \(\mu M\). Theoretically, but unrealistically, a much larger gradient \((4 \mu M)\) was developed only when \(V_{\text{PDE}}\) was increased by 100 times. The distributions of [cAMP] relative to [cAMP] in the submembrane space simulated using the control and high \(V_{\text{PDE}}\) are shown in Fig. 7B 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 effecters that are distributed throughout the cytosol of pancreatic \(\beta\)-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 to 100 nM) of GLP-1 under high \([Ca^{2+}]\) condition were simulated (Fig. 6A). The [cAMP]-dependent PKA activation was calculated using a
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 µM) of IBMX. This is because balancing V_{AC,i} with V_PDE was established with the IBMX-insensitive PDE fraction (~20% total PDE activities) under this extreme condition. To examine the robustness of the [cAMP] homeostasis in our model, the V_{AC,i} devoid of desensitization was compared with V_PDE, and the steady-state level of [cAMP] was defined by the intersection of V_{AC,i} with the [cAMP]-V_PDE curve in Fig. 8. When the V_{AC,i} was maximized at 500 nM [Ca^{2+}] using the saturating concentration of 100 nM [GLP-1], the equilibrium [cAMP] was obtained at ~115.7 µM with 20% V_PDE (grey sigmoidal curve) as indicated by the arrow a. This analysis determines that depression of V_PDE below ~14% will cause collapse of the [cAMP] homeostasis when V_{AC,i} was maximized. Under physiological conditions, the steady-state [cAMP] was maintained at 7.9 µM (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 µM (arrow c). It should be noted that this range of [cAMP] change (1.4–7.9 µM) well fits the dynamic range of the PKA activation (K_{V/2} = ~3 µM). This analysis revealed that the intact PDE is highly capable of balancing V_{AC,i} and thus we conclude that the system of [cAMP] regulation is quite robust in pancreatic β-cells. The delayed desensitization of the GLP-1 receptor (Fig. 4D) may further strengthen the robustness of the system.

**DISCUSSION**

In the present study, a minimal model of GLP-1 receptor signal transduction was developed mostly based on experimental data reported in β-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 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+}] (Table 1). 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 β-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 β-cell models (24, 44) in the future.

In the present study, the distribution of [cAMP] within the cytosol of pancreatic β-cells was estimated (Fig. 7). It was suggested that the localization of cAMP beneath the surface membrane is insignificant even though production of cAMP is...
limited at the submembrane space, while PDE-mediated degradation occurs diffusely within the cytosol. Our finding is, however, different from the localized cAMP microdomain 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 microdomain 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 $V_{PDE}$ equivalent to that used in the Oliveira et al. (47). However, their $V_{PDE}$ 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 AKAP 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 exocytic 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 Gl 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 [LRG] 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 maximum, see Fig. 4D), which is expected to proceed during several hours of the meal digestion.

The maximum AC activity ($V_{max,AC}$ = $V_{max,AC} + V_{max,AC,G}$) is much larger than the $V_{max}$ of PDE in our model. The lower AC activity under physiological conditions is mostly due to the low sensitivity of AC to G$_{o}$GTP in addition to the low [G$_{o}$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$_{a}$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$_{a}$CaM-dependent activation of $V_{PDE}$ was excluded from the model. The PDE component showing the high cAMP sensitivity ($K_{cat}$) also plays a subtle role in regulation [cAMP], whereas the low cAMP sensitivity ($K_{cat}$) 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 ([cAMP]$_{total}$ = [cAMP] + [cAMP-PKA] + [cAMP-Epac]). Similarly, the mass conservation should also be consid-
Parameters Determined in the Present Study by Fitting the Experimental Records or Measurements in References

B) Parameters Determined in the Present Study by Fitting Specific Equations from the present model.

Parameters Determined in Published Experimental Studies

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

   Total amount of receptor, \([R]\) = 0.00434 μM (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 Gs, \([G]\) = 2.83 μM (Ref. 50)

   \(G_{\text{GTP}}\text{-dependent AC}_G\) activation \(K_{1/2} = 0.4 \mu M\) (Ref. 58)

   ATP-dependent AC activity \(K_m = 1.03 \text{mM}\) (Ref. 13)

   ATP-dependent AC activity \(K_m = 0.315 \text{mM}\) (Ref. 13)

   Ca,CaM-dependent AC,\(_G\) activity \(K_{1/2} = 0.348 \mu M\) (Ref. 21)

   \(Ca^{2+}\text{-dependent AC}\) 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 \(K_d = 2.9 \mu M_{M_1}\) = 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 Gs protein

   \(k_5 = 16 \text{s}^{-1}, k_6 = 1 \text{s}^{-1}, k_7 = 1, 200, 000 \text{mM} / \text{s}\) (Refs. 5, 56)

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

Desensitization rate constants

\(k_1 = 0.0025 \text{s}^{-1}, k_2 = 0.005833 \text{s}^{-1}\) (Fig. 3)

\(k_3 = 0.0002833 \text{s}^{-1}, k_4 = 0.000005 \text{s}^{-1}\) (Fig. 4 and Table 3)

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

\(V_{AC,V_{\text{max},AC}} = 0.0006173 \text{mM/s}\)

\(V_{AC,GV_{\text{max},AC,G}} = 0.01738 \text{mM/s}\)

\(f_{\text{Ca,AC}} = 0.6\)

\(V_{\text{PDE}} = 0.015 \text{mM/s}\)

\(f_{\text{Ca,PDE}} = 0.2\)

\(K_{\text{ml}} = 0.4148 \mu M, K_{\text{mlh}} = 53.98 \mu M\)

\(f = 0.012\)

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)

ACKNOWLEDGMENTS

The authors acknowledge insightful discussions with Dr. T. Powell and valuable suggestions made by Dr. G. G. Holz. The authors also thank Drs. T. Shimayoshi, C. Cha, Y. Himeno, and other members in the Biosimulation Project for helpful discussion as well as technical supports.

REFERENCES


57. Tsalkova T, Blumenthal DK, Mei FC, White MA, Cheng X. Mechanism of Epac activation: structural and functional analyses of Epac2 hinge


