Cyclic AMP potentiates glucose-stimulated insulin release by actions predominantly at a site, or sites, distal to the elevation of the cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]). Glucose also acts at a site, or sites, distal to the elevation of [Ca$^{2+}$], via the ATP-sensitive K$^{+}$ channel (K$^{+}_{ATP}$ channel)–independent signaling pathway. Accordingly, using rat pancreatic islets, we studied the location of the action of CAMP and its interaction with the glucose pathway. Forskolin, an activator of adenyl cyclase, raised intracellular CAMP levels and enhanced KCl-induced (Ca$^{2+}$-stimulated) insulin release in the presence, but not in the absence, of glucose. Thus, CAMP has no direct effect on Ca$^{2+}$-stimulated insulin release. The interaction between CAMP and glucose occurs at a step distal to the elevation of [Ca$^{2+}$], because forskolin enhancement of KCl-induced insulin release, in the presence of glucose, was demonstrated in the islets treated with diazoxide, a K$^{+}_{ATP}$ channel opener. The enhancement of insulin release was not associated with any increase in [Ca$^{2+}$]. Furthermore, the interaction between CAMP and glucose was unequivocally observed even under stringent Ca$^{2+}$-free conditions, indicating the Ca$^{2+}$-independent action of CAMP. This action of CAMP is physiologically relevant, because not only forskolin but also glucagon-like peptide 1, glucose-dependent insulinotropic polypeptide, and pituitary adenyl cyclase activating polypeptide exerted similar actions. In conclusion, the CAMP/protein kinase A pathway has no direct effect on Ca$^{2+}$-stimulated insulin exocytosis. Rather, it strongly potentiates insulin release by increasing the effectiveness of the K$^{+}_{ATP}$ channel–independent action of glucose.

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The pancreatic β-cell is regulated by several stimulatory, inhibitory, and modulatory signals that couple to insulin exocytosis (1,2). Among these, the CAMP/protein kinase A (PKA) system is an important enhancer of insulin secretion (3,4). Physiologically, the incretins enhance insulin secretion via this pathway, as is manifest when the pancreatic β-cell responds to the same degree of plasma glucose elevation with a greater amount of insulin output in the presence of incretins such as glucagon-like peptide (GLP)-1 and glucose-dependent insulinotropic polypeptide (GIP), peptides that are secreted mainly by the intestinal L cells after a meal (5–7). Administration of GLP-1 dramatically improves glycemic control in patients with type 2 diabetes, so the clinical importance of these peptides is well established (8–13). In addition, pituitary adenyl cyclase activating polypeptide (PACAP), a putative intrapancreatic enhancer of insulin release (14), exerts at least some of its effect via CAMP and PKA (15).

Despite the physiologic and pathophysiologic importance of the CAMP/PKA pathway in the β-cell, the underlying mechanism for CAMP enhancement of insulin secretion is not clearly understood. CAMP modulates the activity of the ATP-sensitive K$^{+}$ channels (K$^{+}_{ATP}$ channels) (16,17), L-type voltage-dependent Ca$^{2+}$ channels (18–20), and nonselective cation channels (18,21–23) on the β-cell plasma membrane, leading to a transient increase in glucose-stimulated Ca$^{2+}$ influx and elevation of the cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]). (16). More importantly, the CAMP/PKA pathway enhances insulin secretion at a distal site, beyond the elevation of [Ca$^{2+}$], in stimulus-secretion coupling. This enhancement has been demonstrated clearly by capacitance measurements in single mouse β-cells in which the insulin secretory response to a rise in [Ca$^{2+}$]$_{i}$ was strongly enhanced by cAMP (24–27). This distal site of CAMP action is thought to be responsible for most of the effect on secretion. These data and those of others (28–31) have led to the concept that CAMP sensitizes the exocytotic machinery to Ca$^{2+}$. Interestingly, most of the capacitance measurement experiments evaluating the effects of CAMP on the β-cell were performed in the presence of glucose at concentrations of 5–15 mmol/l (18,24–26), a concentration at which the K$^{+}_{ATP}$ channel–independent pathway of glucose signaling augments the rate of insulin secretion (32,33). Furthermore, both forskolin and GLP-1 failed to potentiate exocytosis on those occasions when the experiments were performed in the absence of glucose (17,34).
Thus cAMP could be acting on such glucose pathways to enhance insulin release. In view of this, we examined the effect of cAMP on Ca\(^{2+}\)-stimulated insulin secretion in the presence and absence of glucose. It was found that cAMP had no effect on Ca\(^{2+}\)-stimulated insulin release in the absence of glucose. We conclude that the effects of cAMP to enhance insulin release are mediated via an action on the K\(^{\text{ATP}}\) channel–independent, nonionic, glucose signaling pathway.

**RESEARCH DESIGN AND METHODS**

**Isolation of pancreatic islets.** Pancreatic islets were isolated from male Wistar rats weighing 300–400 g. The rats were killed by CO\(_2\) asphyxiation. Immediately after death, the pancreases were surgically removed, and islets were isolated by collagenase digestion (35).

**Measurement of insulin release.** Insulin release was measured at 37°C in perfusion and static incubation experiments using Krebs-Ringer bicarbonate buffer (KRBB) containing 120 mM NaCl, 5 mM NaHCO\(_3\), 4.8 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 2.5 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 0.2% bovine serum albumin, and 10 mM HEPES, pH adjusted to 7.4. Various concentrations of glucose were used as indicated below.

In perfusion experiments, 50 size-matched islets from the single batch were placed in 10 columns, and all columns were perfused in parallel at a flow rate of 1 ml/min (36,37). After a perfusion period of 30 min, perfusate was collected at intervals of 1, 2, or 5 min. When the effects of forskolin or phorbol 12-myristate 13-acetate (PMA) were examined, forskolin and PMA were present from the beginning of the perfusion period until the end of the experiment. Perfusion samples were stored at −20°C until assayed for insulin.

In static incubations, batches of five size-matched islets were incubated in 1 ml KRBB with various concentrations of glucose for 30 or 60 min (preincubation). Then the incubation medium was removed by aspiration, and 1 ml fresh KRBB containing test substances was introduced. Incubation was then continued for the indicated periods (test incubation). When diazoxide, forskolin, GLP-1 (7-36) amide, GIP, or PACAP27 was present, it was included in both preincubation and test incubation periods. At the end of the test incubation, the medium was aspirated and kept at −20°C until assayed for insulin. In some experiments, KRBB devoid of Ca\(^{2+}\) and containing 1 mM EGTA was used throughout the experiments (washing before the preincubation and during the preincubation and test incubation).

Insulin was measured by radioimmunoassay using rat insulin as standard.

**Measurement of [Ca\(^{2+}\)]\(_i\), in pancreatic islets.** Freshly isolated islets were placed in RPMI culture medium containing 11.1 mM glucose for 60 min before loading with indo-1 AM. Islets were loaded with 3 µmol/l indo-1 AM in KRBB supplemented with 11.1 mM glucose and 250 mM sodium pyruvate for 60–75 min at 37°C. The islets were washed after loading and kept in KRBB with 2.8 mM glucose until used for experiments. Single islets were placed on a 35-mm glass coverslip, which formed the bottom of a 1-ml Teflon chamber. The chamber was placed in a Narishige microunincubation chamber mounted on the stage of a Nikon Diaphot 200 inverted epifluorescence microscope. Excitation at 360 nm was achieved using a xenon lamp, and emission was monitored at 405 and 485 nm using a photometer (Photon Technology International). The ratio of detected light (405/485 nm) was calculated and displayed using OSCAR software (Photon Technology International) and a Dell Optiplex 433/L computer.

Islets were maintained under static conditions at 37°C throughout these experiments. Individual islets were maintained at 37°C in KRBB containing 200 mM diazoxide and supplemented, depending on the experimental conditions, with glucose and forskolin for 10–15 min before the beginning of the experiment. Basal fluorescence was recorded for 3 min before the addition of 25 µl of 1 mg/ml solution, bringing the final concentration of potassium in the incubation chamber to 30 mM. The experiment was continued for an additional 15 min.

The [Ca\(^{2+}\)]\(_i\), (in nanomoles per liter) was determined from ratio values using a calibration curve. The basal [Ca\(^{2+}\)]\(_i\) was determined for each islet by averaging the calcium concentration over the 3-min basal period; peak [Ca\(^{2+}\)]\(_i\) was determined from the highest absolute value of calcium after depolarization with potassium; plateau [Ca\(^{2+}\)]\(_i\) was determined by averaging the [Ca\(^{2+}\)]\(_i\) over the final 10 min of the stimulation period.

**Measurement of cAMP.** Cyclic AMP content of the islets was measured after extracting it at the end of the static incubation experiments. Briefly, 500 µl of 0.2 N HCl was added to the glass tube containing five islets/100 µl KRBB, immediately after collecting the incubation buffer for insulin radioimmunoassay. The tube was sonicated at −20°C in boiling water for 5 min with occasional vortexing for extraction of cAMP. Then the contents of the tube were evaporated using a SpeedVac System (Savant, Farmingdale, NY) and reconstituted with 50 µl distilled water. cAMP was determined by using commercially available radioimmunoassay kits (Yamasa, Chiba, Japan).

**Materials.** Forskolin, PMA, diazoxide, sulfinpyrazone, GLP-1 (7-36) amide, and GIP were purchased from Sigma Chemical (St. Louis, MO). Indo-1 AM and EGTA were obtained from Molecular Probes (Eugene, OR). PACAP27 (Human 1-27 amide) was obtained from the Peptide Institute (Osaka, Japan).

**Data analysis.** All data are shown as means ± SE. The differences between the two conditions in perfusion experiments were tested by the repeated measures analysis of variance (ANOVA) and Fisher tests. The differences between the two conditions in static incubation experiments were tested by the nonparametric Mann-Whitney tests. The differences among the four groups were tested by the one-way ANOVA and Kruskal-Wallis tests. The effects of GLP-1, GIP, and PACAP were tested by the one-way ANOVA and Bonferroni/Dunn tests. A P value < 0.05 was considered statistically significant.

**RESULTS**

Different effects of forskolin and PMA on the temporal profiles of Ca\(^{2+}\)-stimulated insulin release. In the pancreatic β-cell, application of a depolarizing concentration of KCl causes Ca\(^{2+}\)-influx, a rise in [Ca\(^{2+}\)]\(_i\), and Ca\(^{2+}\)-stimulated insulin release. Thus, in the presence of 5 mM glucose, 50 mM KCl produced a sharp increase in insulin secretion that peaked at 2 min and was followed by a gradual decline toward the baseline rate (Fig. 1A). Treatment of the islets with 1 µmol/l forskolin, an activator of adenyl cyclase, approximately doubled the initial peak of the Ca\(^{2+}\)-stimulated insulin release without affecting the insulin release during the later period. More precisely, enhancement of insulin release by forskolin occurred during the first 7 min of stimulation with 50 mM KCl. Basal insulin secretion in the presence of 5 mM glucose and a normal (nondepolarizing) concentr...
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The concentration of KCl (4.8 mmol/l) was unaffected by forskolin (Fig. 1A). In the absence of glucose, 50 mmol/l KCl caused a transient monophasic insulin response, as shown in Fig. 1B, less than that seen in the presence of glucose. Under this glucose-free condition, exposure of the islets to 1 µmol/l forskolin did not enhance the Ca²⁺-stimulated insulin release (Fig. 1B). Similarly, in the presence of 0.1 mmol/l glucose, even 6 µmol/l forskolin had no effect on a temporal profile of insulin release induced by 50 mmol/l KCl (data not shown).

Fig. 1C shows the effects of PMA, an activator of several protein kinase C (PKC) isoforms, on Ca²⁺-stimulated insulin release in the presence of 5 mmol/l glucose. Activation of PKCs by PMA clearly enhanced not only the initial peak of insulin release but also insulin release during the later period of the KCl response. PMA also increased the rate of basal insulin release. The effects of PMA on the basal release and the release during the later period were quite different from those of forskolin. Furthermore, as shown in Fig. 1D, PMA enhanced the release response to KCl even in the absence of glucose. Therefore, the enhancement of insulin release by PMA is not dependent on the presence of glucose. In this respect, the action of PMA is totally different from that of forskolin.

Effects of forskolin and PMA on the augmentation of Ca²⁺-stimulated insulin release. It is well established that glucose augments Ca²⁺-stimulated insulin release by actions that are independent of the K⁺ATP channel and changes in Ca²⁺ influx (32,33,37–40). Accordingly, we examined the possibility that the action of cAMP to enhance insulin secretion was via an effect on the K⁺ATP channel–independent pathway of glucose signaling. To dissect out this distal effect of glucose, the K⁺ATP channel activator diazoxide was used. In the presence of a maximally effective concentration of diazoxide, glucose can no longer close the K⁺ATP channels to depolarize the β-cell membrane and increase Ca²⁺ influx and [Ca²⁺]i (33). Nevertheless, as shown by the results in Fig. 2A, glucose augments insulin release induced by KCl in a concentration-dependent manner. The half-maximal concentration (EC₅₀) of this glucose action was ~10 mmol/l. Under these conditions, in the presence of diazoxide, forskolin enhanced glucose augmentation of insulin release; that is, cAMP enhances the K⁺ATP channel–independent action of glucose. Similarly, PMA enhanced glucose augmentation of Ca²⁺-stimulated insulin release in the presence of diazoxide. In the presence of diazoxide, as in the absence of it, PMA, but not forskolin, augmented KCl-triggered insulin release even in the absence of glucose (Fig. 2B).

Effects of KCl on [Ca²⁺]i in the presence of diazoxide. To verify an assumption that 30 mmol/l KCl causes similar [Ca²⁺]i responses in the presence of diazoxide irrespective of the presence of glucose and forskolin, we measured [Ca²⁺]i in single pancreatic islets. In Fig. 3 is shown a representative result for the changes in [Ca²⁺]i in response to 30 mmol/l KCl in the absence of glucose and forskolin. An immediate rise in [Ca²⁺]i,

FIG. 2. Effect of 5 µmol/l forskolin (A) or 100 nmol/l PMA (B) on glucose augmentation of insulin release induced by 30 mmol/l KCl in the presence of 200 µmol/l diazoxide. Insulin release was measured in static incubation experiments. Forskolin or PMA was present throughout the experiments. Values are means ± SE from 6 (A) and 10 (B) replicates. *P < 0.05, **P < 0.01 vs. the respective values without test substances. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739.

FIG. 3. Temporal profile of [Ca²⁺]i in response to 30 mmol/l KCl in the presence of 200 µmol/l diazoxide. This experiment was performed in the absence of glucose and forskolin. [Ca²⁺]i monitored and shown here is from a single rat pancreatic islet representative of many such experiments.
TABLE 1
Quantitative analysis of changes induced by 30 mmol/l KCl in [Ca\(^{2+}\)]\(_i\) in single pancreatic islets

<table>
<thead>
<tr>
<th>Experiments</th>
<th>(n)</th>
<th>Basal</th>
<th>Peak</th>
<th>Plateau</th>
<th>Peak – basal</th>
<th>Plateau – basal</th>
</tr>
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<tbody>
<tr>
<td>0 mmol/l glucose + 200 µmol/l diazoxide</td>
<td>6</td>
<td>200 ± 22</td>
<td>464 ± 92</td>
<td>286 ± 39</td>
<td>264 ± 73</td>
<td>86 ± 19</td>
</tr>
<tr>
<td>0 mmol/l glucose + 5 µmol/l forskolin</td>
<td>6</td>
<td>172 ± 10</td>
<td>706 ± 85*</td>
<td>269 ± 25</td>
<td>534 ± 84*</td>
<td>97 ± 23</td>
</tr>
<tr>
<td>11.1 mmol/l glucose</td>
<td>6</td>
<td>160 ± 14</td>
<td>288 ± 29</td>
<td>183 ± 15</td>
<td>128 ± 21</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>11.1 mmol/l glucose + 5 µmol/l forskolin</td>
<td>6</td>
<td>164 ± 13</td>
<td>563 ± 110</td>
<td>228 ± 27</td>
<td>400 ± 102</td>
<td>64 ± 16</td>
</tr>
</tbody>
</table>

Data are means ± SE. [Ca\(^{2+}\)]\(_i\) of single rat pancreatic islets were monitored as described in Methods. Basal, average for 3 min immediately before stimulation with 30 mmol/l KCl; peak, highest value in [Ca\(^{2+}\)]\(_i\) after the stimulation with 30 mmol/l KCl; plateau, average [Ca\(^{2+}\)]\(_i\) for the final 10 min of observations; peak – basal, difference between the peak value and the basal value; plateau – basal, difference between the plateau value and the basal value. *Significantly different from the corresponding values in experiment 3 \((P < 0.01)\).

on addition of KCl peaked within 30 s. The peak of [Ca\(^{2+}\)]\(_i\) was followed by an abrupt decrease and then a sustained elevation. This pattern of [Ca\(^{2+}\)]\(_i\) change was seen in all conditions tested. A quantitative analysis of [Ca\(^{2+}\)]\(_i\) responses under four conditions (with or without glucose and with or without forskolin) is shown in Table 1. The presence of glucose slightly lowered the peak and the average values of the plateau level, although the differences were not significant (experiment 1 vs. 3 in Table 1). This difference reaches statistical significance after subtracting the basal [Ca\(^{2+}\)]\(_i\) levels from peak levels [Ca\(^{2+}\)]\(_i\), levels. It should be noted that insulin release in the two conditions was greatly different, the one with glucose being 13 times greater (see Fig. 2A). Presence of forskolin gave rise to higher peak [Ca\(^{2+}\)]\(_i\) on KCl depolarization, although the differences were not statistically significant. Thus, the proximal effects of cAMP cannot be completely ruled out.

**Comparison of the effects of forskolin on insulin release and cAMP content.** In Fig. 4A is shown a comparison of the effects of different concentrations of forskolin on islet cAMP content in the absence and presence of glucose. As expected, forskolin increased cAMP content in a concentration-dependent manner under both conditions. With 0, 1, and 3 µmol/l forskolin, cAMP content was slightly greater in the presence of 11 mmol/l glucose than in its absence. At higher concentrations, forskolin produced similar increases in islet cAMP in the presence and absence of glucose.

The relationship between islet cAMP content and insulin release was next compared by plotting the individual data obtained in the experiments shown in Fig. 4A. In the presence of 11 mmol/l glucose, cAMP content correlated well with the amount of released insulin when the islet cAMP content was between 0 and 100 fmol/islet \((r = 0.416, P < 0.0001)\) (Fig. 4B). Higher concentrations of forskolin produced larger increases in cAMP but did not further enhance insulin release (Fig. 4B), indicating that the maximally effective concentration of cAMP is approximately 100 fmol/islet. In contrast, there was no correlation between cAMP content and insulin release in the absence of glucose. These data confirm that cAMP does not directly augment Ca\(^{2+}\)-stimulated insulin release. Instead, cAMP manifests its insulinotropic effect by increasing the effectiveness of the K\(^{\text{ATP}}\) channel–independent pathway of glucose signaling.

**Effects of forskolin on glucose augmentation of PMA-triggered insulin release.** PMA stimulates insulin release in the presence and absence of extracellular Ca\(^{2+}\), and both

FIG. 4. A: Effect of forskolin on cAMP content in the presence or absence of 11.1 mmol/l glucose. The experiments were performed in the presence of 200 µmol/l diazoxide. cAMP content was measured after 30-min stimulation with 30 mmol/l KCl in the presence of different concentrations of forskolin. Values are the means ± SE from 6 to 14 replicates. Differences of cAMP contents between the absence and presence of glucose reach statistical significance at forskolin concentrations of 0, 1, and 3 µmol/l \((P < 0.005)\). B: Comparison of the islet cAMP content and amount of KCl-induced insulin release in the presence or absence of 11.1 mmol/l glucose. Individual data obtained in the experiments shown in A were plotted in B. Conversion factor of picograms per islet per minute to nanomoles per islet per minute is 0.1739.
these stimulations can be augmented by glucose in a K$_{ATP}$ channel–independent manner (39,41). Accordingly, we looked to see if cAMP enhanced the glucose augmentation of PMA-stimulated insulin release in the presence of diazoxide. As shown in Fig. 5A, inclusion of glucose slightly augmented the PMA-stimulated release, and forskolin significantly enhanced it only when glucose was present; that is, cAMP is not directly enhancing the PMA-stimulated release, rather, it is acting on the augmentation effect of glucose. The cAMP content was not affected by glucose. In the absence of glucose, cAMP content was 15 ± 1 fmol/islet without forskolin and 81 ± 35 fmol/islet with 5 µmol/l forskolin. In the presence of 11 mmol/l glucose, it was 17 ± 1 fmol/islet without forskolin and 79 ± 15 fmol/islet with 5 µmol/l forskolin.

To determine whether cAMP enhances the glucose augmentation of PKC-triggered release in a Ca$^{2+}$-independent manner, similar experiments were performed under stringent Ca$^{2+}$-deprived conditions (Fig. 5B). First, the islets were incubated in Ca$^{2+}$-free buffer containing 1 mmol/l EGTA for 60 min at 37°C. Insulin secretion was then measured from the islets under static incubation conditions in fresh Ca$^{2+}$-free/EGTA buffer. Under these conditions, glucose did not increase [Ca$^{2+}$], (39), but the hexose augmented PMA-stimulated insulin secretion. Forskolin had no effect on PMA-stimulated insulin release in the absence of glucose but increased it markedly in the presence of glucose. These results imply that cAMP potentiates PKC-stimulated insulin release via its action on the K$_{ATP}$ channel–independent, Ca$^{2+}$-independent pathway of glucose signaling (40).

**Effects of GLP-1, GIP, and PACAP on glucose augmentation of insulin release.** To examine the physiologic relevance of the synergism between cAMP and the K$_{ATP}$ channel–independent action of glucose, we examined the effects of physiologic peptides such as GLP-1, GIP, and PACAP. All experiments were performed in the presence of diazoxide. In the absence of glucose, none of the peptides augmented KCl-stimulated release (data not shown), whereas in the presence of 11 mmol/l glucose, GLP-1 augmented KCl-stimulated release in a concentration-dependent manner from 3 to 100 nmol/l (Fig. 6A). GLP-1 at a concentration of 100 nmol/l induced a fourfold increase in insulin release. In Fig. 6B are shown the effects of GLP-1, GIP, and PACAP in the presence...
of 8.3 mmol/l glucose. GLP-1, GIP, and PACAP, each at 10 nmol/l, doubled the rate of KCl-stimulated insulin release. Therefore, the effects of cAMP on the K<sub>ATP</sub> channel–independent glucose signaling pathway observed in experiments with forskolin are also demonstrated when physiologic stimulators of adenylyl cyclase such as GLP-1, GIP, and PACAP are used.

**DISCUSSION**

Glucose acts at several steps to increase the rate of insulin exocytosis. One is on the K<sub>ATP</sub> channel, which closes on glucose stimulation. This closure leads to membrane depolarization, opening of voltage-dependent Ca<sup>2+</sup> channels, influx of Ca<sup>2+</sup>, and eventually elevation of [Ca<sup>2+</sup>]<sub>i</sub>, which triggers insulin release. In addition, glucose potently enhances Ca<sup>2+</sup>-stimulated insulin release even if the K<sub>ATP</sub> channel is kept open by diazoxide, this enhancement being now recognized as the K<sub>ATP</sub> channel–independent action of glucose (32,33,37,38). In the present study, we demonstrated that an activation of the cAMP/PKA pathway augments Ca<sup>2+</sup>-stimulated insulin release only in the presence of glucose, as was most clearly depicted in Fig. 4B. Namely, a positive correlation between cAMP accumulation and insulin release existed when the islet ß-cells were stimulated with a combination of high K<sup>+</sup>, forskolin, and glucose but not with the former two alone. Because such synergism between cAMP and glucose on Ca<sup>2+</sup>-stimulated insulin release was resistant to diazoxide, it is the K<sub>ATP</sub> channel–independent glucose action that is enhanced by cAMP signaling. That the lack of glucose per se was not preventing enhancement of insulin release—by, say, an effect to block phosphorylation—was demonstrated by the fact that PMA, which acts via phosphorylation of key signaling proteins, did enhance insulin secretion under glucose-free conditions. This enhancement of release similarly rules out any serious inhibitory effects on the exocytotic events. PMA might augment K<sub>Cl</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation leading to enhancement of insulin exocytosis. We further demonstrated that not only forskolin, a pharmacologic agent activating adenylyl cyclase, but also physiologic peptides including GLP-1, GIP, and PACAP augment such glucose action. These peptides are known to activate ß-cell adenylyl cyclase through the receptor-coupled heterotrimeric GTP-binding proteins (7,15,26).

An essential control for these studies was the measurement of [Ca<sup>2+</sup>]<sub>i</sub>, because of the possibility that the enhanced responses to cAMP seen in the presence of glucose were due to an increase in the stimulatory [Ca<sup>2+</sup>]<sub>i</sub> signal. Thus [Ca<sup>2+</sup>]<sub>i</sub> was measured in the islets. The experiments were carried out under the same conditions as those in the insulin release experiments. Namely, islets were stimulated with a depolarizing concentration of K<sup>+</sup> in the presence of diazoxide, and the islet responses to forskolin were determined in the presence and absence of glucose. Basal and stimulated [Ca<sup>2+</sup>]<sub>i</sub> levels were not significantly different in the presence or absence of glucose. Forskolin did not significantly enhance the K<sup>+</sup> depolarization–induced elevation in [Ca<sup>2+</sup>]<sub>i</sub>, irrespective of the presence or absence of glucose. Thus, the glucose-dependent augmentation of Ca<sup>2+</sup>-stimulated insulin release by cAMP occurs with little, if any, additional elevation of [Ca<sup>2+</sup>]<sub>i</sub>. In fact, if anything, [Ca<sup>2+</sup>]<sub>i</sub> was slightly lower in the presence of glucose. Moreover, glucose-dependent augmentation of insulin release by cAMP was clearly demonstrated under stringent Ca<sup>2+</sup>-free conditions. Namely, PMA-stimulated insulin release in Ca<sup>2+</sup>-free, EGTA-containing buffer was strongly enhanced by forskolin only in the presence of glucose. We have previously shown that [Ca<sup>2+</sup>]<sub>i</sub> is not raised by these stimuli under this condition (39). The mechanism by which PMA stimulates insulin release under Ca<sup>2+</sup>-free conditions is unknown. Activation of PKC, unlike that of PKA, may directly trigger fusion of the secretory granules and the plasma membrane even in the absence of Ca<sup>2+</sup>.

Our data do not directly deny the fact that cAMP has a proximal effect to augment the nutrient-induced rise in [Ca<sup>2+</sup>]<sub>i</sub>. In fact, KCl-induced [Ca<sup>2+</sup>]<sub>i</sub> elevation tends to be greater in the presence of forskolin. However, our data strongly suggest that most of the enhancement of nutrient-stimulated insulin release by cAMP is through its distal effect on the K<sub>ATP</sub> channel–independent augmentation pathway. The importance of distal effects of cAMP has been proposed based on the fact that activation of PKA enhances Ca<sup>2+</sup>-stimulated increases in membrane capacitance, an indicator of insulin exocytosis (18,24–27). Accordingly, it was considered that the activation of PKA directly sensitizes the Ca<sup>2+</sup>-responsive exocytotic process. However, all of those experiments were performed in the presence of 5 mmol/l glucose, a concentration at which the K<sub>ATP</sub> channel–independent pathway is activated (32,33,39). Based on our findings, it seems likely that they were actually seeing the action of cAMP/PKA exerted via the K<sub>ATP</sub> channel–independent glucose signaling pathway on exocytosis.

Augmentation of insulin release by cAMP signaling is an extremely important mechanism for glucose homeostasis in vivo. Mice with targeted disruption of the GLP-1 receptor exhibit glucose intolerance and decreased insulin secretion in response to oral or intraperitoneal glucose (42). This reaction confirms the tonic regulation of ß-cell function via cAMP and an incretin such as GLP-1. Clinical observations also support this. Chronic subcutaneous injection or even an overnight intravenous infusion of GLP-1 dramatically improves ß-cell glucose sensitivity in patients with type 2 diabetes irrespective of the route of glucose administration (9–13). Thus, the classic incretin concept needs to be reconsidered, because GLP-1 is not simply a postmeal ß-cell potentiator, but is a tonic regulator of ß-cell exocytosis.

In summary, we have discovered the pathway on which cAMP acts to enhance insulin secretion. cAMP acts on the K<sub>ATP</sub> channel–independent pathway of glucose signaling to potently enhance Ca<sup>2+</sup>-stimulated insulin release.

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