[Ca^{2+}]-reducing action of cAMP in rat pancreatic β-cells: involvement of thapsigargin-sensitive stores

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^{1}Department of Physiology and ^{2}First Department of Internal Medicine, Kagoshima University School of Medicine, 8–35–1 Sakuragaoka, Kagoshima 890; and ^{3}Laboratory of Intracellular Metabolism, National Institute for Physiological Sciences, Okazaki 444, Japan

Yaekura, Kazuo, and Toshihiko Yada. [Ca^{2+}]-reducing action of cAMP in rat pancreatic β-cells: involvement of thapsigargin-sensitive stores. Am. J. Physiol. 274 (Cell Physiol. 43): C513–C521, 1998.—In the present study, we examined the ability of adenosine 3',5'-cyclic monophosphate (cAMP) to reduce elevated levels of cytosolic Ca^{2+} concentration ([Ca^{2+}]_{i}) in pancreatic β-cells. [Ca^{2+}]_{i} and reduced pyridine nucleotide, NAD(P)H, were measured in rat single β-cells by fura 2 and autofluorescence microfluorometry. Sustained [Ca^{2+}]_{i} elevation, induced by high KCl (25 mM) at a basal glucose concentration (2.8 mM), was substantially reduced by cAMP-increasing agents, dibutyryl cAMP (DBcAMP, 5 mM), an adenylyl cyclase activator forskolin (10 μM), and an incretin glucagon-like peptide-1 (7–36) amide (10–30 μM), as well as by glucose (16.7 mM). The [Ca^{2+}]_{i}-reducing effects of cAMP were greater at elevated glucose (8.3–16.7 mM) than at basal glucose (2.8 mM). An inhibitor of protein kinase A (PKA), H-89, counteracted [Ca^{2+}]_{i} effects of cAMP but not those of glucose. Okadaic acid, a phosphatase inhibitor, at 10–100 nM also reduced sustained [Ca^{2+}]_{i} elevation in a concentration-dependent manner. Glucose, but not DBcAMP, increased NAD(P)H in β-cells. [Ca^{2+}]_{i}-reducing effects of cAMP were inhibited by 0.3 μM thapsigargin, an inhibitor of the endoplasmic reticulum (ER) Ca^{2+} pump. In contrast, [Ca^{2+}]_{i}-reducing effects of cAMP were not altered by ryanodine, an ER Ca^{2+}-release inhibitor, Na^+-free conditions, or diazoxide, an ATP-sensitive K^+ channel opener. In conclusion, the cAMP-PKA pathway reduces [Ca^{2+}]_{i} by sequestering Ca^{2+} in thapsigargin-sensitive stores. This process does not involve, but is potentiated by, activation of β-cell metabolism. Together with the known [Ca^{2+}]_{i}-increasing action of cAMP, our results reveal dual regulation of β-cell [Ca^{2+}]_{i} by the cAMP-signaling pathway and by a physiologic incretin.

cytosolic Ca^{2+} concentration; Ca^{2+} sequestration; endoplasmic reticulum; protein kinase A; glucagon-like peptide-1; glucose; adenosine 3',5'-cyclic monophosphate

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INSULIN RELEASE IN RESPONSE to glucose and other secretagogues is triggered by an increase in cytosolic Ca^{2+} concentration ([Ca^{2+}]_{i}) in pancreatic islet β-cells (25, 33). The [Ca^{2+}]_{i} signaling in response to glucose exhibits a dynamic change: an initial decrease, subsequent increase, and oscillation of [Ca^{2+}]_{i} (14). In the presence of stimulatory glucose, a rise in adenosine 3',5'-cyclic monophosphate (cAMP), an important regulator of islet β-cell functions (25, 33), also elicits [Ca^{2+}]_{i} oscillation (8, 12, 14). Simultaneous measurements of [Ca^{2+}]_{i} and insulin release in mouse islets revealed that [Ca^{2+}]_{i} oscillations are causally linked to oscillations of insulin release (3, 4, 10). We speculate that the time-coordinated [Ca^{2+}]_{i} signaling, including oscillations, is produced by an interplay between two mechanisms, one that increases and another that decreases [Ca^{2+}]_{i}. Although the former mechanism has been well elucidated in β-cells, the latter is yet poorly understood.

It is known that cAMP increases [Ca^{2+}]_{i} in a glucose-dependent manner by enhancing Ca^{2+} influx through the L-type Ca^{2+} channel (8, 12, 16, 31, 35), in which activation of an Na^+-permeable channel has been suggested (16). In our previous study, which was designed to determine whether cAMP has a direct Ca^{2+} channel agonist action, we examined the effect of cAMP-increasing agents on sustained [Ca^{2+}]_{i} elevation induced by high KCl. cAMP not only failed to further increase [Ca^{2+}]_{i} (38), it rather, to our surprise, reduced the elevated [Ca^{2+}]_{i}. It was also previously reported that cAMP reduces the glucose-induced [Ca^{2+}]_{i} increase in suspension of islet cells (28). In the present study, we attempted to elucidate the underlying mechanism by which cAMP-increasing agents reduce [Ca^{2+}]_{i} elevation. We also examined the effect of glucagon-like peptide-1 (GLP-1), a physiological incretin that stimulates the cAMP-signaling pathway (16, 23, 32, 35). [Ca^{2+}]_{i} and reduced pyridine nucleotide, NAD(P)H, as an indicator of energy metabolism (9, 21, 24), were measured in rat pancreatic β-cells. We found that cAMP attenuates [Ca^{2+}]_{i} elevation by promoting Ca^{2+} sequestration in the endoplasmic reticulum (ER) in pancreatic β-cells and that GLP-1 mimics the cAMP effect.

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MATERIALS AND METHODS

Preparation of single islet cells and selection of β-cells. Islets of Langerhans were isolated, by collagenase digestion, from Wistar rats aged 8–12 wk. Animals were anesthetized by intraperitoneal injection of pentobarbital sodium at 80 mg/kg. The abdomen was opened, and collagenase (3 mg/ml) dissolved in 6 ml of 5 mM Ca^{2+}-containing Krebs-Ringer bicarbonate buffer (KRB) solution was injected into the common bile duct at the distal end after ligation of the duct proximal to the pancreas. The pancreas was dissected out and incubated at 37°C for 17 min. Islets were collected and immediately dispersed into single cells in Ca^{2+}-free KRB. The single cells were plated on coverslips and maintained in short-term culture for up to 3 days in Eagle’s minimum essential medium containing 5.6 mM glucose supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a 95% air-5% CO2 atmosphere. During the culture period, no appreciable change was observed in the frequency or the pattern of [Ca^{2+}]_{i} responses to glucose, tolbutamide, KCl, hormones, or cAMP-increasing agents.

β-Cells were selected among single islet cells according to the previously reported procedure (34, 35). Briefly, single islet cells that had a diameter of 12.5–17.5 μm on coverslips and responded to glucose (8.3 or 16.7 mM) and tolbutamide (300
mM) with increases in \([\text{Ca}^{2+}]\), were found to be immunocytochemically positive for insulin (37). Data were taken from the cells that fulfilled these morphological and physiological criteria for β-cells.

**Solutions and chemicals.** Measurements were carried out in KRB solution composed of (in mM) 129 NaCl, 5.0 NaHCO₃, 4.7 KCl, 1.2 KH₂PO₄, 1.0 CaCl₂, 1.2 MgSO₄, and 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid at pH 7.4 supplemented with 0.1% bovine serum albumin. Na⁺-free KRB was made by replacement of NaCl with equimolar N-methyl-D-glucamine (NMDG; Nacalai Tesque, Kyoto, Japan). Fura 2 and fura 2-acetoxymethyl ester (AM) were obtained from Dojin Chemical (Kumamoto, Japan), and dibutyryl cAMP (DBcAMP) was obtained from Boehringer Mannheim (Indianapolis, IN). H-89 \([\text{N-[2-[p-bromocinnamylamino]ethyl]-5-isouquinolinesulfonamide}]\) was provided by Dr. H. Hidaka and also obtained from Seikagaku Kogyo (Tokyo, Japan). All other chemicals were from Sigma (St. Louis, MO).

**Fig. 1. Adenosine 3',5'-cyclic monophosphate (cAMP)-increasing agents reduce sustained elevation of cytosolic \([\text{Ca}^{2+}]\) concentration \([\text{Ca}^{2+}]\) induced by high K⁺ at basal glucose as well as increasing \([\text{Ca}^{2+}]\) at stimulatory glucose in single β-cells.** KCl (25 mM) induced a sustained elevation of \([\text{Ca}^{2+}]\) in single β-cells. Five millimolar dibutyryl cAMP (DBcAMP) (A), 10 µM forskolin (B), and 1 nM glucagon-like peptide-1 (GLP-1)-(7–36) amide (C) attenuated the sustained \([\text{Ca}^{2+}]\) elevation induced by high K⁺ at 2.8 mM glucose (G2.8). These agents also increased \([\text{Ca}^{2+}]\) in the presence of 8.3 mM glucose (G8.3) and at normal K⁺. \([\text{Ca}^{2+}]\) responses to G8.3 and 300 µM tolbutamide (Tolb) are also shown. Glucose concentration is indicated at top. Bars above tracing specify period of exposure to agents specified. Dotted lines indicate beginning of exposure to cAMP-increasing agents. Results are representative of 17 of 21 single β-cells examined in A, 20 of 28 cells in B, and 16 of 21 cells in C.

**Fig. 2. cAMP-increasing agents reduce sustained \([\text{Ca}^{2+}]\) elevation induced by high K⁺ at stimulatory glucose in single β-cells.** In the presence of a moderate elevation of \([\text{Ca}^{2+}]\), induced by 8.3 and 16.7 mM glucose, 25 mM KCl produced a further elevation of \([\text{Ca}^{2+}]\) in a sustained manner in single β-cells. Five millimolar DBcAMP (A) and 10 µM forskolin (B) attenuated the sustained \([\text{Ca}^{2+}]\) elevation induced by high K⁺. Magnitude of attenuation of \([\text{Ca}^{2+}]\) elevation was greater at stimulatory glucose than at basal glucose (see Fig. 1). Results are representative of 23 of 26 cells in A and 28 of 30 cells in B.
Measurements of [Ca$^{2+}$] were measured by dual-wavelength fura 2 microfluorometry combined with imaging, as previously reported (36, 37). Briefly, cells on coverslips were incubated with 1 µM fura 2-AM for 30 min at 37°C in KRB containing 2.8 mM glucose. Cells were then mounted in a chamber and superfused at a rate of 1 ml/min at 37°C in KRB. Cells were excited at 340 and 380 nm alternately every 2.5 s, emission signals at 510 nm (F340 and F380, respectively) were detected with an intensified charge-coupled device camera, and ratio (F340/F380) images were produced by an Argus-50 system (Hamamatsu Photonics, Hamamatsu, Japan). Ratio values were converted to [Ca$^{2+}$] according to calibration curves.

Measurements of NAD(P)H. Autofluorescence of NAD(P)H in two to eight β-cells in cluster was measured under superfusion conditions identical to those used for [Ca$^{2+}$] measurements. Cells were excited at 360 nm every 1 s, and emission signals through a 470 ± 20-nm band-pass filter were detected by a high-sensitivity photomultiplier using a P101 system (Nikon, Tokyo, Japan).

Criteria for [Ca$^{2+}$]-reducing response and determination of response amplitude. The amplitude of [Ca$^{2+}$]-reduction in response to cAMP-increasing agents was determined at the time point of maximal reduction of [Ca$^{2+}$]. When the KCl-induced [Ca$^{2+}$] elevation was not flat but mildly declining or increasing at a stable rate, an extrapolated curve was drawn based on the [Ca$^{2+}$] levels before addition and 5–10 min after washing out cAMP agents, and the maximal deviation from the extrapolated curve was taken as the amplitude of [Ca$^{2+}$]-reduction. Only the deviation that was >0.1 ratio (F340/F380) unit from the extrapolated curve and which took place in a clear shape within 5 min upon exposure to cAMP agents was considered as the response.

Statistical analyses. All data are presented as means ± SE (n = number of observations). The statistical analysis was carried out by Student’s t-test. Differences were considered statistically significant when P < 0.05.

RESULTS

KCl-induced sustained [Ca$^{2+}$] elevation and its attenuation by cAMP-increasing agents and GLP-1. To examine whether cAMP has an ability to reduce [Ca$^{2+}$] elevation, [Ca$^{2+}$] was damped at high levels and the effect of cAMP was examined. At a basal glucose concentration of 2.8 mM, KCl at a depolarizing concentration (25 mM) induced a sharp initial peak followed by a sustained elevation of [Ca$^{2+}$] in single β-cells (Fig. 1). The level of the sustained [Ca$^{2+}$] elevation either stayed constant or changed slowly and mildly at a stable rate. The KCl-induced sustained elevation of [Ca$^{2+}$] was reduced by membrane-permeable cAMP analogs 5 mM DBcAMP (Fig. 1A and Table 1) and 1 mM 8-bromo-cAMP (data not shown), by 10 µM forskolin (Fig. 1B), and by 1 nM GLP-1 (7–36) amide (GLP-1) (Fig. 1C). DBcAMP, forskolin, and GLP-1 evoked [Ca$^{2+}$]-reduction in ~80% of the single β-cells. When DBcAMP was combined with 0.3 mM 3-isobutyl-1-methylxanthine, the percentage of cells showing [Ca$^{2+}$]-reduction was further increased to ~90%. The reduction of [Ca$^{2+}$] started 0.5–1 min after administration of the cAMP-increasing agents, peaked at 2–5 min, and then gradu-

Table 1. [Ca$^{2+}$]-reducing action of cAMP in pancreatic β-cells, a phosphatase inhibitor, and effect of H-89, a PKA inhibitor

<table>
<thead>
<tr>
<th>Experiment</th>
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<td>11</td>
<td>84.6</td>
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<tr>
<td></td>
<td>DBcAMP (5 mM) + H-89 (40 µM)</td>
<td>13</td>
<td>11</td>
<td>84.6</td>
</tr>
<tr>
<td></td>
<td>DBcAMP (5 mM)</td>
<td>14</td>
<td>12</td>
<td>85.7</td>
</tr>
<tr>
<td></td>
<td>Okadaic acid (100 nM)</td>
<td>14</td>
<td>12</td>
<td>85.7</td>
</tr>
<tr>
<td>3</td>
<td>Okadaic acid (10 nM)</td>
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<td>7</td>
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<td></td>
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<td>85.7</td>
</tr>
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</table>

Values of response amplitude indicate mean amplitude of cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]) reduction averaged only for cells responding.

In experiment 1, the effect of dibutyryl adenosine 3',5'-cyclic monophosphate (DBcAMP) on 25 mM KCl-induced [Ca$^{2+}$] elevation was examined. After the same cells had been treated for 20 min with H-89, the effect of DBcAMP was examined again. In experiment 2, the effects of DBcAMP and okadaic acid on 25 mM KCl-induced [Ca$^{2+}$] elevation were examined in the same cells. In experiment 3, the effects of increasing concentrations of okadaic acid on 25 mM KCl-induced [Ca$^{2+}$] elevation were examined in the same cells. PKA, protein kinase A. *P < 0.01 compared with 5 mM DBcAMP alone by paired t-test. No significant difference (P > 0.05) between 5 mM DBcAMP and 100 nM okadaic acid groups.
Fig. 4. Distinct properties between cAMP- vs. glucose-induced [Ca^{2+}]_i reduction. A: in presence of 25 mM KCl and sustained [Ca^{2+}]_i elevation, 5 mM DBcAMP and 16.7 mM glucose (G16.7) were administered in pulses in a single β-cell (left). The cell was then treated for 20 min with 40 µM H-89, a protein kinase A (PKA) inhibitor (arrow), followed by the recording shown on right. Note that [Ca^{2+}]_i-reducing effect of cAMP, but not that of glucose, was markedly suppressed by a PKA inhibitor. Treatment with H-89 had no effect on subsequent [Ca^{2+}]_i responses to stimulatory glucose (8.3 mM) and tolbutamide (300 µM). B: in presence of 25 mM KCl and sustained [Ca^{2+}]_i elevation, successive administration of 5 mM DBcAMP evoked [Ca^{2+}]_i-reducing responses two times sequentially without appreciable change in their amplitudes in a single β-cell. C: okadaic acid (OA), an inhibitor of phosphatase, at 10 and 30 nM attenuated the KCl-induced [Ca^{2+}]_i elevation in a concentration-dependent manner in a single β-cell. D: OA at 100 nM attenuated the 25 mM KCl-induced sustained [Ca^{2+}]_i elevation in a manner similar to 5 mM DBcAMP in a single β-cell. Treatment with OA had no effect on subsequent [Ca^{2+}]_i responses to stimulatory glucose and tolbutamide. E: effects of DBcAMP and glucose on NAD(P)H levels in β-cells. NAD(P)H autofluorescence at 470 nm in 3 β-cells in cluster is expressed as a percentage relative to the signal at the resting state with 2.8 mM glucose before KCl addition. Note that 16.7 mM glucose, but not 5 mM DBcAMP, increased fluorescence. Glucose concentration was 2.8 mM unless otherwise indicated in A–E. Results shown are representative of 13 of 17 cells in A, 8 of 12 cells in B, 25 of 28 cells in C, and 26 of 28 cells in D, whereas they represent 4 similar experiments in E.
Glucose dependence of $[Ca^{2+}]_{i}$-reducing effects of cAMP. At stimulatory glucose (8.3 and 16.7 mM) concentrations, DBCAMP and forskolin reduced KCl-induced $[Ca^{2+}]_{i}$ elevation to a greater extent than at 2.8 mM (Fig. 2, A and B). The amplitude of $[Ca^{2+}]_{i}$ reduction was significantly increased when glucose concentration was raised (Fig. 3).

$[Ca^{2+}]_{i}$-reducing effects of cAMP, glucose, and a phosphatase inhibitor, and selective inhibition of cAMP effects by a PKA inhibitor. The KCl-induced $[Ca^{2+}]_{i}$ elevation was attenuated both by DBCAMP and by high glucose (16.7 mM) in single $\beta$-cells (Fig. 4A). After the same cells had been treated for 20 min with 40 µM H-89, an inhibitor of protein kinase A (PKA), the amplitude of $[Ca^{2+}]_{i}$ reduction by DBCAMP was significantly suppressed, whereas that by glucose was unchanged (Fig. 4A and Table 1). H-89 at 10 µM also suppressed the effect of DBCAMP to reduce $[Ca^{2+}]_{i}$ to a lesser extent (data not shown). In control experiments, when DBCAMP was successively administered two times, the cells exhibited $[Ca^{2+}]_{i}$-reducing responses twice without appreciable change in their amplitudes (Fig. 4B). Okadaic acid, an inhibitor of phosphatase, at 10–100 nM also attenuated the KCl-induced $[Ca^{2+}]_{i}$ elevation in a concentration-dependent manner (Fig. 4, C and D, and Table 1). Okadaic acid at 100 nM, a maximal concentration, evoked $[Ca^{2+}]_{i}$ reduction in a similar pattern and with a similar amplitude to that induced by DBCAMP (Fig. 4D and Table 1).

Effects of cAMP and glucose on NAD(P)H levels in $\beta$-cells. Because the metabolic activity is known to control the level of $[Ca^{2+}]_{i}$ (7, 25), we next measured intracellular NAD(P)H as an indicator of the metabolic activity. The NAD(P)H autofluorescence from $\beta$-cells at basal glucose (2.8 mM) was not significantly altered by 25 mM KCl. In the presence of 25 mM KCl, DBCAMP produced no change, whereas 16.7 mM glucose rapidly and markedly increased fluorescence (Fig. 4E).

Effects of inhibitors of ER $Ca^{2+}$ pump and $Ca^{2+}$-release channel on $[Ca^{2+}]_{i}$-reducing action of cAMP. In the presence of 0.3 µM thapsigargin (TG), an inhibitor of the ER $Ca^{2+}$ pump (20, 30), KCl increased $[Ca^{2+}]_{i}$ to a level somewhat higher than that of control. CAMP-increasing agents failed to reduce the elevated levels of $[Ca^{2+}]_{i}$ in the majority of single $\beta$-cells (Fig. 5); thus the fraction of cells responding to CAMP-increasing agents with $[Ca^{2+}]_{i}$ reduction was dramatically reduced (for the response to DBCAMP, 21.7% with TG vs. 76.6% in control) (Table 2). The amplitude of the $[Ca^{2+}]_{i}$ reduction in the responding cells was also markedly attenuated [19.7 ± 2.5 nM (n = 5) with TG vs. 94.4 ± 4.1 nM (n = 23) in control, P < 0.0001] (Table 2). $[Ca^{2+}]_{i}$-reducing effects of glucose (8.3 and 16.7 mM) were also inhibited by TG, confirming previous reports (6, 13). In contrast, 20 µM ryanodine, an inhibitor of the ryanodine-sensitive $Ca^{2+}$-release channel in ER, affected neither the level of the KCl-induced $[Ca^{2+}]_{i}$ elevation nor the CAMP action to attenuate this level (Fig. 6A and Table 2).

Effects of inhibition or activation of plasma membraneNa$^{+}/Ca^{2+}$ exchanger and $K_{ATP}$ on $[Ca^{2+}]_{i}$-reducing
action of CAMP. It has been shown that the plasma membrane Na⁺/Ca²⁺ exchange serves as a Ca²⁺ extrusion mechanism in β-cells (15) and plays a greater role at elevated glucose than at basal glucose in the regulation of [Ca²⁺] (13, 38). Therefore, we assessed an involvement of Na⁺/Ca²⁺ exchange in the CAMP-induced [Ca²⁺] reduction at basal and elevated glucose concentrations by examining the effect of Na⁺-free conditions that inhibit Na⁺/Ca²⁺ exchange. Na⁺-free conditions were achieved by replacement of extracellular Na⁺ with NMDG. Under Na⁺-free conditions, high K⁺-induced sustained [Ca²⁺] elevation was attenuated by DBcAMP in a manner similar to control conditions, and this was observed at both basal and elevated glucose concentrations (Fig. 6, B and C, and Table 2).

It is also possible that CAMP reduces [Ca²⁺] by influencing the ATP-sensitive K⁺ channel (KₘATP), which is the major determinant of the β-cell resting membrane potential (2). This possibility was examined by testing the effect of CAMP under conditions in which KₘATP was fixed in either a maximally open or closed state. A sustained [Ca²⁺] elevation induced by KCl plus the KₘATP opener diazoxide at 0.4 mM, a concentration that fully opens this channel (2, 10), was attenuated by DBcAMP (Fig. 6D and Table 2) and by forskolin (data not shown). The KₘATP blocker tolbutamide at 1 mM, a supramaximal concentration for complete closure of this channel (2, 9), induced sustained elevation of [Ca²⁺], which was attenuated by DBcAMP (Fig. 6E). These results indicate that neither Na⁺/Ca²⁺ exchange nor KₘATP is involved in the [Ca²⁺]-reducing action of CAMP.

**DISCUSSION**

The present study has shown that membrane-permeable CAMP analogs, forskolin and GLP-1, agents that increase CAMP, all reduce sustained [Ca²⁺] elevation induced by high K⁺ and tolbutamide, thereby revealing the [Ca²⁺]-reducing action of CAMP in pancreatic β-cells. The [Ca²⁺] reduction by CAMP was abolished or markedly inhibited by TG at the concentration that specifically inhibits glucose-induced [Ca²⁺] decrease, a process mediated by Ca²⁺ sequestration in ER (13). It is well established that TG inhibits the ER and sarcoplasmic reticulum (SR) Ca²⁺-ATPase (Ca²⁺-pumping ATPase) activity without influencing the plasma membrane Ca²⁺-ATPase and Na⁺-K⁺-ATPase in a variety of preparations (20, 30). In contrast, [Ca²⁺]-reducing effects of CAMP were observed similarly in the absence and presence of ryanodine. Therefore, it is unlikely that CAMP attenuates [Ca²⁺] elevation by inhibiting the ryanodine-sensitive Ca²⁺ release from ER. The [Ca²⁺] reduction could, alternatively, be due to alteration of ion transport systems in the β-cell plasma membrane. Na⁺/Ca²⁺ exchange functions as a Ca²⁺ extrusion mechanism in β-cells (15, 38). However, [Ca²⁺]-reducing effects of CAMP were unaltered under Na⁺-free conditions, irrespective of whether the glucose concentration was basal or stimulatory. Therefore, the CAMP-induced [Ca²⁺] reduction is not accounted for by stimulation of Na⁺/Ca²⁺ exchange at the plasma membrane. Another possibility is that CAMP reduces [Ca²⁺] via influencing KₘATP, the channel that mainly determines the resting membrane potential of β-cells. In the present study, however, in the presence of the KₘATP opener diazoxide at 0.4 mM, a supramaximal concentration that fully opens this channel (2), the KCl-induced [Ca²⁺] elevation was also reduced by CAMP. Likewise, the KₘATP blocker tolbutamide at 1 mM, a concentration that fixes this channel at the completely closed state (13), induced sustained [Ca²⁺] elevation, and it was also attenuated by CAMP. Thus KₘATP appears not to be involved in [Ca²⁺]-reducing effects of CAMP. Taken together, it is concluded that [Ca²⁺]-reducing effects of CAMP are due primarily to stimulation of Ca²⁺ sequestration in the TG-sensitive ER in β-cells.

Glucose also stimulates Ca²⁺ sequestration in the TG-sensitive ER in β-cells, accounting for the glucose-induced initial decrease in [Ca²⁺] (6, 11, 13, 26, 27, 36). It is likely that the glucose-induced [Ca²⁺] reduction is due to activation of β-cell metabolism for the following reasons. Glucose is metabolized by β-cells, resulting in production of ATP from ADP (2); the ATP/ADP ratio is a regulatory factor of the Ca²⁺-pumping ATPase; and the steady-state [Ca²⁺] varies inversely with the ATP/ADP ratio in the permeabilized insulinoma cells (7). It is

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**Table 2. Percentage of cells responding to CAMP with [Ca²⁺]-reducing action and mean amplitude of [Ca²⁺] reduction in single β-cells under conditions that alter endoplasmic reticulum and plasma membrane Ca²⁺ transport**

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<tr>
<th>Glucose, mM</th>
<th>Condition</th>
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<th>Examined</th>
<th>Responding</th>
<th>Frequency, %</th>
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<td>−87.5 ± 4.8</td>
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<tr>
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<td>23</td>
<td>5</td>
<td>21.7</td>
<td>−19.7 ± 2.5*</td>
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<td>86.9 ± 9.8</td>
<td>73.9</td>
<td>−169.8 ± 10.3</td>
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</table>

Values of response amplitude indicate the mean amplitude of [Ca²⁺], reduction averaged only for the cells responding. *P < 0.0001 compared with 5 mM DBcAMP alone by paired t-test. No significant difference (P > 0.05) between ryanodine, Na⁺ free, diazoxide, and control groups at 2.8 mM glucose and between Na⁺ free and control groups at 16.7 mM glucose.
possible that activation of β-cell metabolism is also involved in the cAMP-induced [Ca\(^{2+}\)] reduction. In the present study, however, the NAD(P)H level in β-cells, an indicator of metabolism, was not significantly altered by cAMP, whereas it was markedly increased by high glucose, as previously reported (9, 24). In contrast, the [Ca\(^{2+}\)]-reducing effect of cAMP, but not that of high glucose, was inhibited by pretreatment with the PKA inhibitor H-89. Furthermore, okadaic acid, an inhibitor of phosphatase, mimicked cAMP-increasing agents to reduce the KCl-induced [Ca\(^{2+}\)] elevation. These findings indicate that cAMP and glucose reduce [Ca\(^{2+}\)] elevation by distinct mechanisms and that PKA-mediated phosphorylation may be involved in the [Ca\(^{2+}\)]-reducing action of cAMP.

Although the [Ca\(^{2+}\)]-reducing effect of cAMP does not involve activation of β-cell metabolism, it was substantially enhanced as the glucose concentration was in-
increased. Thus interactive effects of cAMP and glucose were demonstrated. This interaction may be accounted for by the following hypothetical mechanism. The cAMP-dependent phosphorylation pathway stimulates Ca\(^{2+}\) sequestration in ER by upregulating the active form of Ca\(^{2+}\)-ATPase (Ca\(^{2+}\) pump); because Ca\(^{2+}\)-ATPase is an ATP-dependent enzyme, higher ATP levels at higher glucose concentrations make more Ca\(^{2+}\)-ATPase in the active form, thereby allowing a larger potentiation of the Ca\(^{2+}\)-ATPase activity by cAMP. However, precise mechanisms for the interaction between cAMP and glucose in reducing [Ca\(^{2+}\)]\(i\) are yet to be elucidated. Because the [Ca\(^{2+}\)]\(i\)-reducing action of cAMP is blocked by an ER Ca\(^{2+}\) pump inhibitor and a PKA inhibitor and mimicked by a phosphatase inhibitor, a possible involvement of PKA-mediated phosphorylation in the TG-sensitive Ca\(^{2+}\) sequestration in ER is suggested. It has been shown that glucagon stimulates Ca\(^{2+}\) uptake in ER of liver cells (I, S), cAMP mimics this effect (I, 29), and Ca\(^{2+}\)-pumping activity is enhanced by PKA (22). In cardiac muscles, phospholamban, a membrane-associated protein in SR, is a substrate of PKA, and the ratio of phosphorylated to unphosphorylated phospholamban correlates with the Ca\(^{2+}\) transport enzyme activity and the rate of Ca\(^{2+}\) uptake in SR (17, 18, 22). However, phospholamban or related proteins have not yet been demonstrated in pancreas. The mechanisms that link PKA to the ER Ca\(^{2+}\) sequestration in pancreatic \(\beta\)-cells remain to be investigated.

It should be noted that administration of cAMP-increasing agents induced large transients of [Ca\(^{2+}\)]\(i\) in 6.6% of \(\beta\)-cells (data not shown). These [Ca\(^{2+}\)]\(i\)-transients took place usually before, and occasionally during, the [Ca\(^{2+}\)]\(i\) reduction. The duration of a transient was 30–120 s. A previous study (19) reported that cAMP induces the pronounced transients of [Ca\(^{2+}\)]\(i\) in glucose- or KCl-stimulated \(\beta\)-cells and suggested a mechanism by which cAMP sensitizes the inositol 1,4,5-trisphosphate (IP\(_3\)) receptor to stimulate mobilization of intracellular Ca\(^{2+}\). Therefore, our [Ca\(^{2+}\)]\(i\)-transients could reflect the IP\(_3\)-mediated Ca\(^{2+}\) mobilization. However, the time resolution of our [Ca\(^{2+}\)]\(i\) measurements may not be fast enough to detect short transients evoked by IP\(_3\). It should be emphasized that [Ca\(^{2+}\)]\(i\)-reducing effects of cAMP were observed irrespective of whether the [Ca\(^{2+}\)]\(i\)-transients occurred. A link between the cAMP-induced [Ca\(^{2+}\)]\(i\) reduction and [Ca\(^{2+}\)]\(i\)-transients remains unknown.

The [Ca\(^{2+}\)]\(i\)-reducing action of cAMP may play a role in optimizing the amplitude of [Ca\(^{2+}\)]\(i\) signals, thereby finely controlling Ca\(^{2+}\)-dependent functions in \(\beta\)-cells. It is also suggested that the [Ca\(^{2+}\)]\(i\)-reducing action of cAMP takes part in buffering an excessive rise in [Ca\(^{2+}\)]\(i\), a putative cytotoxic signal which leads to \(\beta\)-cell dysfunction and death. On the other hand, it is well established that cAMP increases [Ca\(^{2+}\)]\(i\) in a glucose-dependent manner in rat \(\beta\)-cells. Thus the present study reveals a dual action of cAMP on [Ca\(^{2+}\)]\(i\). The dual function of the cAMP-signaling pathway may play a role in producing frequency-coded [Ca\(^{2+}\)]\(i\) signals, such as oscillations of [Ca\(^{2+}\)]\(i\), in pancreatic \(\beta\)-cells.

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