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Differential Modulation of $Ca_v1.2$ and $Ca_v1.3$-Mediated Glucose-Stimulated Insulin Secretion by cAMP in INS-1 Cells: Distinct Roles for Exchange Protein Directly Activated by cAMP 2 (Epac2) and Protein Kinase A

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ABSTRACT

Using insulin-secreting cell line (INS)-1 cells stably expressing dihydropyridine-insensitive mutants of either $Ca_v1.2$ or $Ca_v1.3$, we previously demonstrated that $Ca_v1.3$ is preferentially coupled to insulin secretion and $[Ca^{2+}]_i$ oscillations stimulated by 11.2 mM glucose. Using the same system, we found that insulin secretion in 7.5 mM glucose plus 1 mM 8-bromo-cAMP (8-Br-cAMP) is mediated by both $Ca_v1.2$ and $Ca_v1.3$. Treatment of INS-1 cells or INS-1 cells stably expressing $Ca_v1.2$/dihydropyridine-insensitive (DHPi) channels in the presence of 10 μM nifedipine, with effector-specific cAMP analogs 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-cAMP [8-pCPT-2′-*O*-Me-cAMP; 100 μM; Exchange Protein directly Activated by cAMP 2 (Epac2)-selective] or N^6-benzoyl-cAMP [50 μM; Protein Kinase A (PKA)-selective] partially increased insulin secretion. Secretion stimulated by a combination of the two cAMP analogs was additive and comparable with that stimulated by 1 mM 8-Br-cAMP. In INS-1 cells stably expressing $Ca_v1.3$/DHPi in the presence of 10 μM nifedipine, N^6-benzoyl-cAMP, but not 8-pCPT-2′-*O*-Me-cAMP, significantly increased glucose-stimulated insulin secretion. However, the combination of N^6-benzoyl-cAMP and 8-pCPT-2′-*O*-Me-cAMP significantly increased glucose-stimulated secretion compared with N^6-benzoyl-cAMP alone. In INS-1 cells, 8-Br-cAMP potentiation of insulin secretion in 7.5 mM glucose is blocked by thapsigargin (1 μM) and ryanodine (0.5 μM). In contrast, ryanodine has no effect on insulin secretion or $[Ca^{2+}]_i$ oscillations stimulated by 11.2 mM glucose in INS-1 cells. Our data suggest that both $Ca_v1.2$ and $Ca_v1.3$ mediate insulin secretion stimulated by 7.5 mM glucose and cAMP via a mechanism that requires internal stores of Ca^{2+}. Furthermore, cAMP modulation of secretion mediated by $Ca_v1.2$ seems to involve both Epac2 and PKA independently. In contrast, cAMP modulation of $Ca_v1.3$-mediated secretion depends upon PKA activation, whereas the contribution of Epac2 is dependent upon PKA activation.

Metabolism of glucose to ATP in pancreatic β-cells results in the closure of K_{ATP} channels, which depolarizes the plasma membrane and activates voltage-dependent calcium channels (VDCCs) (Rajan et al., 1990). Calcium influx via L-type VDCCs causes an elevation of intracellular calcium concentration ($[Ca^{2+}]_i$) and triggers exocytosis of insulin-containing granules (Wollheim and Sharp, 1981). Glucagon-like peptide (GLP)-1 is an insulin secretagogue hormone secreted by intestinal L cells that can potentiate insulin secretion from β-cells in response to elevated blood glucose (Schmidt et al., 1985). The insulinotropic effect of GLP-1 has been widely recognized and intensively studied because of its potential therapeutic application in the treatment of noninsulin-dependent (type II) diabetes mellitus (Nauck et al., 1993a,b).

The glucose dependence of the insulinotropic action of GLP-1 implies that there is cross-talk between the L-type

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In memory of Claire E. Hockerman and Nathan G. Hockerman.

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ABBREVIATIONS: VDCC, voltage-dependent calcium channel; GLP, glucagon-like peptide; PKA, protein kinase A; GEF, guanine nucleotide exchange factor; INS-1, insulin-secreting cell line-1; Epac2, exchange protein directly activated by cAMP 2; CICR, Ca^{2+}-induced Ca^{2+} release; 8-Br-cAMP, 8-bromo-cAMP; H89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl; $Ca_v1.2$/DHPi, $Ca_v1.2$ channel insensitive to dihydropyridines; $Ca_v1.3$/DHPi, $Ca_v1.3$ channel insensitive to dihydropyridines; Rp-cAMPS, adenosine 3′,5′-cyclic monophosphorothioate (Rp-isomer); GFP, green fluorescent protein; DHPi, dihydropyridine-insensitive; KRBH, Krebs-Ringer-bicarbonate HEPES; ANOVA, analysis of variance; ER, endoplasmic reticulum; RyR, ryanodine receptor; IP_3R, inositol 1,4,5-triphosphate receptors.

VDCC-mediated secretion and the GLP-1 signaling pathways (Holz et al., 1992). After post-translational modification, the active form of GLP-1 [GLP-1 (7–36)] binds to the G-protein-coupled GLP-1 receptor in the pancreatic β-cell, which activates adenylate cyclase and results in elevated cAMP production (Holz et al., 1995). Although the prosecretory properties of GLP-1 in β-cells are believed to be mediated by PKA (Thorens and Waeber, 1993), recent studies have revealed alternative cAMP signal transduction pathways that seem to be PKA-independent. One such pathway involves a newly characterized family of cAMP-binding proteins designated as the cAMP-guanine nucleotide exchange factors (GEFs or Epac) (Kawasaki et al., 1998).

In β-cells, the increase in $[Ca^{2+}]_i$ in response to glucose and GLP-1 has recently been reported to be mediated by cAMP-GEFII (referred to here as Epac2). This PKA-independent action of GLP-1 is linked to the release of ryanodine-sensitive intracellular Ca^{2+} stores and is facilitated by the interaction of Epac2 with the monomeric G-protein Rab3 (Ozaki et al., 2000; Kashima et al., 2001). Glucose alone can induce an oscillatory or sustained $[Ca^{2+}]_i$ increase, and L-type calcium channels expressed in the plasma membrane of β-cells play an essential role in these $[Ca^{2+}]_i$ changes (Liu et al., 2004). GLP-1-induced cAMP elevation can further enhance $[Ca^{2+}]_i$ responses in β-cells compared with glucose alone. This alteration in $[Ca^{2+}]_i$ response is critical for the potentiation of insulin secretion induced by cAMP (Lu et al., 1993). Single-cell $[Ca^{2+}]_i$ imaging studies in rat and human pancreatic β-cells indicate that cAMP induces $[Ca^{2+}]_i$ oscillations via Ca^{2+}-induced Ca^{2+} release (CICR) from intracellular Ca^{2+} stores (Holz et al., 1999). Although the specific receptor(s) mediating intracellular Ca^{2+} release remains uncertain, Ca^{2+} influx through L-VDCC is required for the stimulatory action of both GLP-1 (Yada et al., 1993) and cAMP (Holz et al., 1999) on $[Ca^{2+}]_i$ in pancreatic β-cells.

Pancreatic β-cells of rats and humans express two distinct L-VDCC subtypes, $Ca_v1.2$ and $Ca_v1.3$ (Seino et al., 1992; Horvath et al., 1998). Both channel subtypes contain four highly homologous transmembrane domains, with a higher degree of divergence in the intracellular loops linking each domain. The gating properties of these two channels are slightly different, and they are both sensitive to L-VDCC blockers (Bell et al., 2001; Scholze et al., 2001) such as the dihydropyridine nifedipine and the benzothiazepine diltiazem (Hockerman et al., 1997). Because $Ca_v1.3$ is the dominant L-VDCC isoform expressed in human and rat pancreatic β-cells (Seino et al., 1992), it was proposed that $Ca_v1.2$ and $Ca_v1.3$ play different roles in insulin secretion. We previously reported that $Ca_v1.3$ is preferentially coupled to glucose-stimulated insulin secretion (Liu et al., 2003) and glucose-stimulated $[Ca^{2+}]_i$ oscillations (Liu et al., 2004). However, the specific role of these two L-VDCC subtypes in the insulinotropic effect of cAMP has not been investigated. Identifying Ca^{2+} influx channel(s) responsible for cAMP-potentiated insulin secretion would further our understanding of the molecular mechanisms involved in the action of cAMP-elevating agents and may provide new insights into the therapeutic usage of GLP-1 to treat type II diabetes.

The present study explored the roles of the L-VDCC subtypes $Ca_v1.2$ and $Ca_v1.3$ in cAMP-potentiated insulin secretion at a submaximal glucose concentration in the rat pancreatic β-cell line INS-1. We have established a pharmacological knockdown system with stable INS-1 cell lines expressing either of two drug-insensitive mutant channels, $Ca_v1.2$/DHPi or $Ca_v1.3$/DHPi. We further probed this system using two cAMP analogs, 8-(4-chloro-phenylthio)-2′-*O*-methyladenosine-cAMP and N^6-benzoyl-cAMP to examine the contribution of Epac2 and PKA, respectively (Christensen et al., 2003). In the presence of 7.5 mM glucose, both $Ca_v1.2$ and $Ca_v1.3$ were able to mediate cAMP-potentiated insulin secretion in INS-1 cells. This seems to require intracellular Ca^{2+} stores and the combined action of both Epac2 and PKA.

Materials and Methods

Materials. 8-(4-Chloro-phenylthio)-2′-*O*-methyladenosine-cAMP and N^6-benzoyl-cAMP were from BIOLOG Life Science Institute (Bremen, Germany). 8-Br-cAMP and H89 were from Sigma (St. Louis, MO). Adenosine 3′,5′-cyclic monophosphorothioate and Rp-isomer (Rp-cAMPS) were from Calbiochem (La Jolla, CA).

Cell Culture. INS-1 cells were cultured in RPMI 1640 medium supplemented with 10 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol as previously reported (Asfari et al., 1992). Cultures were incubated at 37°C in a humidified 95% air/5% CO_2 atmosphere. Characterization of the stable INS-1 cell lines expressing either $Ca_v1.2$/DHPi or $Ca_v1.3$/DHPi channels fused to GFP is described elsewhere (Liu et al., 2003). In brief, the presence of the mutant channels in these stable cell lines was confirmed by reverse transcription-polymerase chain reaction with two primer sets and Western blotting with an anti-GFP antibody. Current displaying the characteristic pharmacology of the DHPi channels (i.e., insensitivity to nifedipine and sensitivity to diltiazem; Hockerman et al., 2000) was detected in each cell line using the whole-cell patch-clamp technique. The whole-cell Ba^{2+} current density was not significantly different in the DHPi cell lines and untransfected INS-1 cells.

Insulin Secretion and Cellular Insulin Content. INS-1 cells were cultured in 12-well dishes and allowed to grow until 80 to 90% confluent. Cells were rinsed twice with modified Krebs-Ringer-bicarbonate HEPES (KRBH) buffer (115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 25 mM HEPES, and 0.5% bovine serum albumin, pH 7.4), and preincubated in 1 ml of KRBH buffer for 30 min at 37°C, 5% CO_2. After aspiration of the buffer, the cells were stimulated in KRBH buffer supplemented with different stimuli (+ or − indicated drugs) for 30 min at 37°C. The buffer containing secreted insulin was collected and centrifuged at 700*g* for 3 min to remove any detached cells. The cellular insulin was extracted by incubation in 1 ml of acid-ethanol (ethanol/H_2O/HCl, 14:57:3) overnight at 4°C and centrifuged at 700*g* for 3 min. Supernatants were diluted 10-fold with glycine-NaOH buffer (0.2 M glycine and 0.5% bovine serum albumin, pH 8.8) before insulin secretion assay. Assays were performed using the High Range Rat Insulin ELISA kit (ALPCO, Windham, NH) according to the manufacturer's instructions.

Measurement of $[Ca^{2+}]_i$. INS-1 cells were split into four-well, glass-bottomed chambers (Nalge Nunc International, Naperville, IL) and cultured in complete medium for 48 h before experiments. Cells were washed with KRBH buffer and incubated with 5 μM of the calcium indicator indo-1 AM (Molecular Probes, Eugene, OR) in KRBH buffer for 30 min in the dark. After washing with KRBH buffer, cells were incubated for an additional 30 min in KRBH buffer. Ca^{2+} imaging experiments were carried out at the Purdue University Cytometry Laboratories. Indo-1 AM-loaded cells in glass-bottomed chambers were observed via confocal laser scanning microscopy with a Bio-Rad MRC 1024 (Bio-Rad, Hercules, CA) system based on an inverted Nikon Diaphot 300 microscope (Nikon, Tokyo, Japan). The stage was thermostatically controlled to maintain a temperature of 37°C at the bottom of the chamber. The confocal

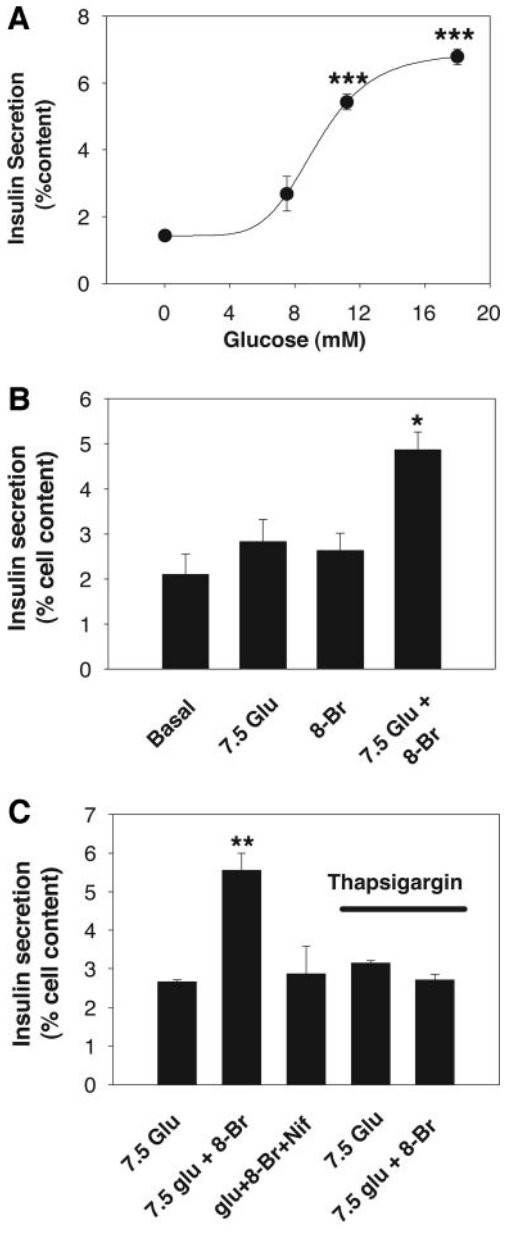

Fig. 1. L-type VDCC activity is required for 8-bromo-cAMP-potentiated glucose-stimulated insulin secretion. A, effect of different glucose concentrations on insulin secretion in INS-1 cells. Glucose (11.2 or 18 mM) stimulated significantly higher level of insulin secretion compared with 0 mM glucose (*, $p < 0.05$, one-way ANOVA). B, 8-Br-cAMP-potentiated insulin secretion in INS-1 cells. Either 7.5 mM glucose (Glu) or 1 mM 8-Br-cAMP (8-Br) did not significantly induce insulin secretion compared with basal level (2 mM glucose). However, in the presence of 7.5 mM glucose, 1 mM 8-Br-cAMP (Glu + 8-Br) significantly potentiated insulin secretion. Basal, 2.1 ± 0.45% ($n = 3$); Glu, 2.83 ± 0.48% ($n = 3$); 8-Br, 2.63 ± 0.68% ($n = 3$); and Glu + 8-Br, 4.87 ± 0.39% ($n = 3$) (*, $p < 0.05$ compared with basal level; one-way ANOVA with Tukey post hoc test). C, nifedipine, L-type VDCC blocker, or thapsigargin, ER Ca^{2+}-ATPase inhibitor, completely inhibited 8-Br-cAMP-potentiated insulin secretion. INS-1 cells were preincubated with KRBH buffer or 1 μM thapsigargin for 30 min. Cells were then incubated with KRBH buffer containing indicated reagents for another 30 min before insulin assay. 8-Br-cAMP (1 mM) together with 7.5 mM glucose (glu + 8-Br) significantly potentiated glucose-induced insulin secretion. Nifedipine (10 μM) completely inhibited this effect (glu + 8-Br + Nif). glu, 2.67 ± 0.05% ($n = 3$); glu + 8-Br, 5.56 ± 0.44% ($n = 3$); glu + 8-Br + Nif, 2.87 ± 0.71% ($n = 3$). After thapsigargin pretreatment, 1 mM 8-Br-cAMP can no longer potentiate insulin secretion in INS-1 cells (glu, 3.1 ± 0.02%, $n = 3$; glu + 8-Br, 2.71 ± 0.14%, $n = 3$). **, $p < 0.01$ compared with Glu, one-way ANOVA with Tukey post hoc test.

system was equipped with a 60× PlanApo 1.4 NA oil immersion objective lens and 100-mW argon ion water-cooled laser (Coherent Inc., Santa Clara, CA). Single cells or small clusters of cells, isolated optically by means of a diaphragm, were studied by indo-1 fluorescence. Indo 1 AM-loaded cells were excited at 363 nM, and the emissions at wavelengths of 405 nm (F_{405}) and 460 nm (F_{460}) were used to calculate the fluorescence ratio (F_{405}/F_{460}). Cells were excited at a frequency of 1 Hz, and the fluorescence images were collected simultaneously. $[Ca^{2+}]_i$ was calculated from F_{405}/F_{460} using a standard curve generated with a Ca^{2+} concentration buffer kit with Mg^{2+} (Molecular Probes).

Data Analysis. Insulin secretion data were analyzed using Sigma Plot 8.01. The time courses of the fluorescence values (F_{405} and F_{460}) from each cell were obtained using Lasersharp software (Bio-Rad). The ratios (F_{405}/F_{460}) were calculated, and final figures were presented using Sigma Plot 8.01. Results are shown as means ± S.E. for the number of observations as indicated. The statistical significance of differences among multiple experimental groups was determined using one-way ANOVA with Tukey or Dunnett's post hoc test, with $p < 0.05$ considered significant.

Results

Ca^{2+}-Induced ER Ca^{2+} Release Is Required for cAMP-Potentiated Insulin Secretion in Response to a Submaximal Glucose Concentration. Untransfected INS-1 cells secrete insulin in response to glucose in a dose-dependent fashion. The dose-response curve for glucose stimulation of insulin secretion from these cells is shown in Fig. 1A (EC_{50}, 9.4 mM). Note that although concentrations of 11.2 and 18 mM glucose clearly stimulate secretion, 7.5 mM glucose is not sufficient to significantly stimulate insulin secretion. Likewise, 1 mM 8-Br-cAMP alone also did not significantly increase insulin secretion above the basal level (Fig. 1B). In contrast, 7.5 mM glucose together with 1 mM 8-Br-cAMP significantly enhanced insulin secretion (Fig. 1B). Previously, single-cell $[Ca^{2+}]_i$ imaging studies suggested that the insulinotropic effect of cAMP is mediated by L-type Ca^{2+} channel-dependent CICR (Holz et al., 1999). Consistent with this result, pretreatment of cells with the ER Ca^{2+}-ATPase inhibitor thapsigargin (1 μM) abolished cAMP-potentiated insulin secretion (Fig. 1C), suggesting the involvement of ER Ca^{2+} release in this process. The 8-Br-cAMP-potentiated insulin secretion in response to 7.5 mM glucose in INS-1 cells was completely inhibited by the L-VDCC blocker nifedipine (Fig. 1C).

To address the possibility that Ca^{2+}-induced Ca^{2+} release is involved in the insulinotropic action of cAMP, we performed Ca^{2+} imaging experiments in INS-1 cells pretreated with ryanodine. Ryanodine receptors (RyRs) and inositol 1,4,5-triphosphate receptors (IP_3R) localized in the ER can mediate intracellular Ca^{2+} release and have both been identified in pancreatic β-cells (Blondel et al., 1993; Gamberucci et al., 1999). Because RyR is sensitive to elevated $[Ca^{2+}]_i$, it has been suggested that RyR may mediate cAMP-induced CICR in β-cells (Holz et al., 1999). In skeletal muscle, the Ca^{2+} permeability of RyR is modulated by ryanodine in a biphasic manner. Although low ryanodine concentrations (0.01–10 μM) potentiate RyR-mediated Ca^{2+} release, saturating ryanodine concentrations (10–300 μM) are inhibitory (Lattanzio et al., 1987).

We first tested whether ER Ca^{2+} release is required for high glucose-stimulated $[Ca^{2+}]_i$ oscillation in INS-1 cells. After pretreatment with either 0.5 or 20 μM ryanodine, the

glucose-stimulated $[Ca^{2+}]_i$ oscillations were still observed in untransfected INS-1 cells (Fig. 2A). We next examined the role of ER Ca^{2+} stores in insulin secretion in response to two different stimuli, high glucose alone (11.2 mM) or mildly elevated glucose (7.5 mM) together with 8-Br-cAMP. INS-1 cells maintained insulin secretion in response to high glucose after pretreatment with low (0.5 μM) or high (20 μM) ryanodine concentration (Fig. 2B). In the presence of mildly elevated glucose, 1 mM 8-Br-cAMP was still able to enhance insulin secretion in INS-1 cells pretreated with 20 μM ryanodine (Fig. 2C). However, this insulinotropic effect of cAMP was completely abolished in cells pretreated with 0.5 μM ryanodine (Fig. 2C), similar to the effect observed with thapsigargin pretreatment (Fig. 1C). Both the Ca^{2+} imaging and insulin secretion experiments indicate that, unlike the response to high glucose concentrations alone, ryanodine-sensitive intracellular Ca^{2+} stores seem to be required for the insulinotropic action of cAMP in the presence of mildly elevated glucose.

Epac and PKA Are Both Required for the L-VDCC-Mediated Augmentation of Insulin Secretion in Response to Submaximal Glucose Concentration and cAMP. We hypothesized that the insulinotropic action of cAMP is mediated by the cAMP targets PKA or cAMP-GEFII (Epac2) or both. However, 8-Br-cAMP does not discriminate between these two cAMP effectors (Christensen et al., 2003). To probe the downstream cAMP effector pathways responsible for our observations, we used cAMP analogs that specifically target PKA or Epac2, N^6-benzoyl-cAMP (K_i for PKA RIβ, 0.7 μM) and 8′-pCPT-2′-*O*-Me-cAMP (K_i for Epac1, 2.2 μM). Both analogs have been shown to have higher affinity than cAMP for PKA or Epac2, respectively (Christensen et al., 2003). In untransfected INS-1 cells, treatment with either 100 μM 8′-pCPT-2′-*O*-Me-cAMP or 50 μM N^6-benzoyl-cAMP partially increased insulin secretion in response to mildly elevated glucose compared with basal (Fig. 3A). A combination of both Epac- and PKA-selective cAMP analogs elicited a maximal response in INS-1 cells that is additive and comparable with that produced by 1 mM 8-Br-cAMP. Insulin secretion in the presence of mildly elevated glucose and the cAMP analogs, alone or in combination, is sensitive to nifedipine. These data suggest that the L-VDCC-mediated combined action of the cAMP effectors Epac2 and PKA is sufficient to account for the cAMP potentiation of insulin secretion in response to submaximal glucose concentration. The additive response of INS-1 cells to either targeted cAMP analogs suggested that Epac2 can mediate insulin secretion independently of PKA, albeit to a partial extent. To examine this possibility, we pretreated INS-1 cells with the PKA antagonist H89 (10 μM) and stimulated the cells with 7.5 mM glucose plus a combination of the Epac2- and PKA-selective cAMP analogs (Fig. 3A). Inhibition of PKA with H89 reduced the stimulated secretion under these conditions so that it was not significantly different from 7.5 mM glucose alone, suggesting cross-talk between the Epac2 and PKA signaling pathways. We further confirmed the specificity of 100 μM 8′-pCPT-2′-*O*-Me-cAMP and 50 μM N^6-benzoyl-cAMP for activation of Epac2 and PKA, respectively, using the cyclic nucleotide analog PKA inhibitor Rp-cAMPS (Rothermel and Parker Botelho, 1988). As expected, 100 μM Rp-cAMPS inhibited stimulation of insulin secretion mediated by 50 μM N^6-benzoyl-cAMP but did not inhibit insulin secretion stimulated by 100 μM 8′-pCPT-2′-*O*-Me-cAMP in untransfected INS-1 cells (Fig. 3, B and C).

$Ca_v1.2$ and $Ca_v1.3$ Can Mediate cAMP-Potentiated Insulin Secretion at Submaximal Glucose Concentrations. We established INS-1 cell lines stably expressing either $Ca_v1.2$/DHPi or $Ca_v1.3$/DHPi fused to GFP in an effort to examine the distinct contributions of $Ca_v1.2$ and $Ca_v1.3$ to cAMP-potentiated glucose-stimulated insulin secretion (Liu et al., 2003, 2004). Because both of these L-VDCC subtypes present in β cells are sensitive to L-type channel blockers, the endogenous L-type channels in these cell lines can be silenced with nifedipine, and the independent contribution of each subtype can be assessed. Mutation of two amino acids in the IIIS5 region of $Ca_v1.2$ or $Ca_v1.3$ results in a channel that

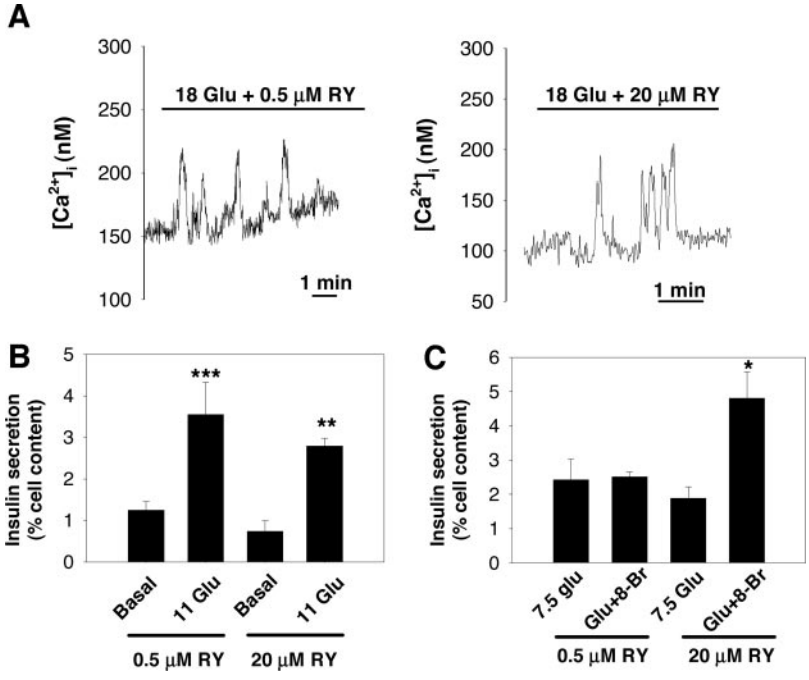

Fig. 2. Ca^{2+} release from internal stores is required for cAMP-potentiated insulin secretion but not high glucose-stimulated $[Ca^{2+}]_i$ oscillation and insulin secretion in INS-1 cells. A, $[Ca^{2+}]_i$ responses to glucose in untransfected INS-1 cells. Glucose (glu; 18 mM) triggered $[Ca^{2+}]_i$ oscillations, which are not inhibited by either 0.5 or 20 μM ryanodine. Traces shown are representative of 16 cells observed in each condition. B, insulin secretion stimulated by 11.2 mM glucose is not inhibited by either 0.5 or 20 μM ryanodine. INS-1 cells were preincubated with either 0.5 or 20 μM ryanodine in KRBH buffer for 30 min before stimulation with 11.2 mM glucose. Ryanodine (0.5 μM), basal, 1.25 ± 0.12% (n = 3); glu, 3.55 ± 0.44% (n = 3). Ryanodine (20 μM), basal, 0.74 ± 0.14% (n = 3); glu, 2.79 ± 0.11% (n = 3) (**, $p < 0.01$; ***, $p < 0.001$, compared with basal, one-way ANOVA with Tukey post hoc test). C, insulin secretion stimulated by 7.5 mM glucose plus 1 mM 8-Br-cAMP is inhibited by 0.5, but not 20, μM, ryanodine. Cells were preincubated with either 0.5 μM ryanodine (RY) or 20 μM ryanodine in KRBH buffer. Cells were then incubated with either 7.5 mM glucose (glu) or 7.5 mM glucose together with 1 mM 8-Br-cAMP (glu + 8-Br) for 30 min. Ryanodine (0.5 μM) preincubation inhibited the response to cAMP, whereas 20 μM ryanodine did not have any effect on cAMP-potentiated insulin secretion. RY (0.5 μM), glu, 2.42 ± 0.6% (n = 3); glu + 8-Br, 2.51 ± 0.15% (n = 3). RY (20 μM), glu, 1.88 ± 0.33% (n = 3); glu + 8-Br, 4.8 ± 0.78% (n = 3) (*, $p < 0.05$ compared with 7.5 glu in 20 μM ryanodine, one-way ANOVA with Tukey post hoc test).

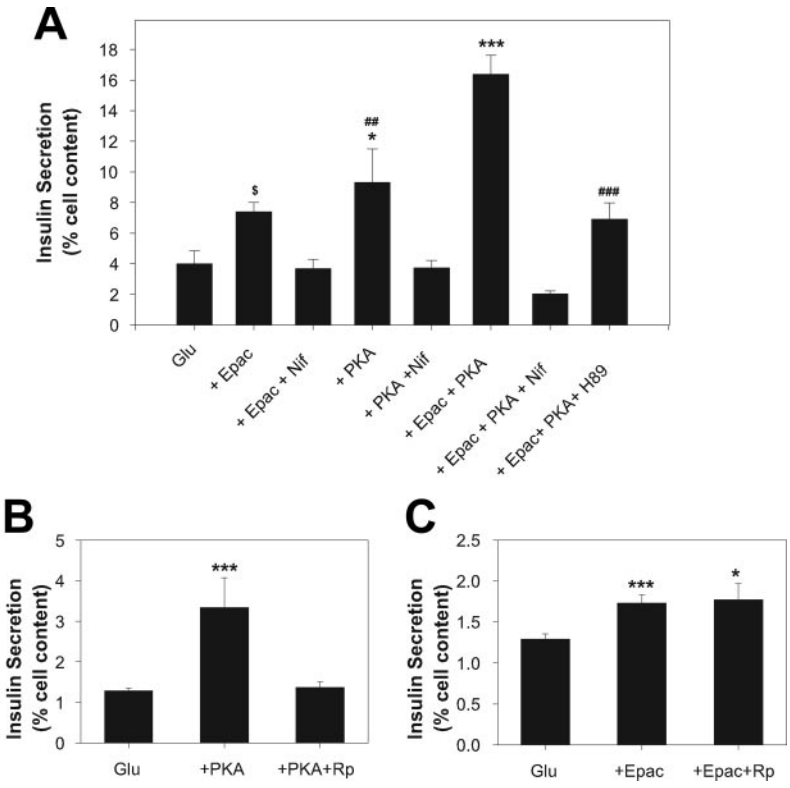

Fig. 3. cAMP-potentiated insulin secretion in response to 7.5 mM glucose involves both Epac2 and PKA. A, INS-1 cells were incubated with either 7.5 mM glucose (Glu) or 7.5 mM glucose together with 100 μM 8′-pCPT-2′-*O*-Me-cAMP (Glu + Epac) or 50 μM N^6-benzoyl-cAMP (Glu + PKA) or a combination of the three (Glu + Epac + PKA) in KRBH buffer for 30 min. Treatment with Epac-cAMP or PKA-cAMP alone resulted in a partial increase in insulin secretion relative to 7.5 mM glucose alone, but a combination of both analogs resulted in significantly greater response compared with either analog alone. In every case, stimulated secretion is completely blocked by 10 μM nifedipine (Nif). Inclusion of 10 μM of the PKA inhibitor H89 significantly reduced secretion stimulated by Glu + Epac + PKA. Glu, 4.00 ± 0.84 (n = 9); Glu + Epac, 7.40 ± 0.61% (n = 6); Glu + Epac + Nif, 3.69 ± 0.61% (n = 6); Glu + PKA, 9.31 ± 2.19% (n = 4); Glu + PKA + Nif, 3.73 ± 0.49% (n = 4); Glu + Epac + PKA, 16.4 ± 1.20% (n = 3); Glu + Epac + PKA + Nif, 2.04 ± 0.18 (n = 3); and Glu + Epac + PKA + H89, 6.9 ± 1.08% (n = 3). B, Rp-cAMPS (100 μM) inhibits the stimulation of insulin secretion by 50 μM N^6-benzoyl-cAMP in the presence of 7.5 mM glucose in INS-1 cells. Glu, 1.29 ± 0.06% (n = 21); Glu + PKA, 3.34 ± 0.73% (n = 6); and Glu + PKA + Rp-cAMPS, 1.37 ± 0.13% (n = 15). C, Rp-cAMPS (100 μM) does not inhibit the stimulation of insulin secretion by 100 μM 8′-pCPT-2′-*O*-Me-cAMP in the presence of 7.5 mM glucose in INS-1 cells. Glu, 1.29 ± 0.06% (n = 21); Glu + Epac, 1.73 ± 0.10% (n = 14); Glu + Epac + Rp-cAMPS, 1.77 ± 0.203% (n = 3). *, $p < 0.05$; ***, $p < 0.001$ compared with Glu, one-way ANOVA with Tukey post hoc test; $, $p < 0.05$ compared with Glu, one-way ANOVA with Dunnett's post hoc test; ##, $p < 0.01$, ###, $p < 0.001$ compared with Glu + Epac + PKA, one-way ANOVA with Tukey post hoc test.

is insensitive neutral DHPs such as isradipine and nifedipine but remains sensitive to the benzothiazepine diltiazem (Hockerman et al., 2000) (Fig. 4A). Using this system, we have shown previously that similar to untransfected INS-1 cells, insulin secretion in response to 11.2 mM glucose in the $Ca_v1.2$/DHPi cells was completely inhibited by nifedipine, whereas $Ca_v1.3$/DHPi cells demonstrated ~40% DHP-insensitive insulin secretion. These results indicated that $Ca_v1.3$, but not $Ca_v1.2$, is preferentially coupled to 11.2 mM glucose-stimulated insulin secretion (Liu et al., 2003).

To address the possibility that a particular L-VDCC subtype serves as the Ca^{2+} influx channel involved in glucose-stimulated Ca^{2+}-induced Ca^{2+} release in INS-1 cells, we pretreated $Ca_v1.2$/DHPi or $Ca_v1.3$/DHPi cells with an inhibitory concentration of ryanodine (0.5 μM) and examined $[Ca^{2+}]_i$ oscillations in response to high glucose. Similar patterns of glucose-stimulated $[Ca^{2+}]_i$ oscillations have been observed in untransfected INS-1 cells and $Ca_v1.2$/DHPi cells in our previous study (Liu et al., 2004). $Ca_v1.2$/DHPi cells responded to high glucose with nifedipine-sensitive $[Ca^{2+}]_i$ oscillations after 0.5 μM ryanodine pretreatment (Fig. 4B), consistent with the responses of these cells to L-VDCC antagonists without ryanodine pretreatment (Liu et al., 2004). However, in $Ca_v1.3$/DHPi cells, 11.2 mM glucose was still able to induce nifedipine-resistant $[Ca^{2+}]_i$ oscillations in the presence of ryanodine (Fig. 4C), indicating that $Ca_v1.3$ is preferentially coupled to glucose-stimulated $[Ca^{2+}]_i$ oscillation, as reported previously (Liu et al., 2004). These data are also consistent with our observation that ryanodine-sensitive intracellular Ca^{2+} stores are not involved in high glucose-stimulated $[Ca^{2+}]_i$ oscillations in untransfected INS-1 cells (Fig. 2A).

To further explore the specific roles of $Ca_v1.2$ and $Ca_v1.3$ in stimulus-secretion coupling, the cAMP-potentiated insulin secretion in response to mildly elevated glucose was measured in $Ca_v1.2$/DHPi and $Ca_v1.3$/DHPi cells (Fig. 5A). Treatment with 1 mM 8-Br-cAMP increased insulin secretion in response to 7.5 mM glucose in both stable cell lines. Moreover, both channels showed augmentation of insulin secretion that is insensitive to 10 μM nifedipine but blocked by 500 μM diltiazem. The detection of DHP-insensitive responses to 7.5 mM glucose plus 1 mM 8-Br-cAMP in these cell lines suggests that both $Ca_v1.2$ and $Ca_v1.3$ are able to mediate cAMP-potentiated insulin secretion under mildly elevated glucose conditions. It is interesting to note that unlike the submaximal (~40%) DHPi response of $Ca_v1.3$/DHPi to high glucose that we have previously reported (Liu et al., 2003), both $Ca_v1.2$/DHPi and $Ca_v1.3$/DHPi cells exhibited full responses to the synergistic effect of 7.5 mM glucose and 8-Br-cAMP in the presence of nifedipine.

Epac2 Requires PKA Activation in the cAMP-Potentiated Insulin Secretion Mediated by $Ca_v1.3$ but Not $Ca_v1.2$. Our new finding that $Ca_v1.2$, as well as $Ca_v1.3$, can mediate insulin secretion in INS-1 cells in the presence of cAMP and 7.5 mM glucose led us to the hypothesis that this stimulus-secretion coupling via $Ca_v1.2$ is facilitated by the downstream cAMP effectors Epac2 and PKA, possibly in a VDCC subtype-selective manner. Therefore, we measured insulin secretion in response to 7.5 mM glucose and the Epac2- or PKA- selective cAMP analogs in both $Ca_v1.2$/DHPi and $Ca_v1.3$/DHPi cells in the presence of 10 μM nifedipine (Fig. 5B). In $Ca_v1.2$/DHPi cells treated with 7.5 mM glucose in combination with either 100 μM 8′-pCPT-2′-*O*-Me-cAMP or 50 μM N^6-benzoyl-cAMP, a significant augmentation of insulin secretion over basal levels was observed. Similar to untransfected INS-1 cells (Fig. 3), a combination of the Epac2- and PKA-selective cAMP analogs produced a significant increase in insulin secretion in response to 7.5 mM

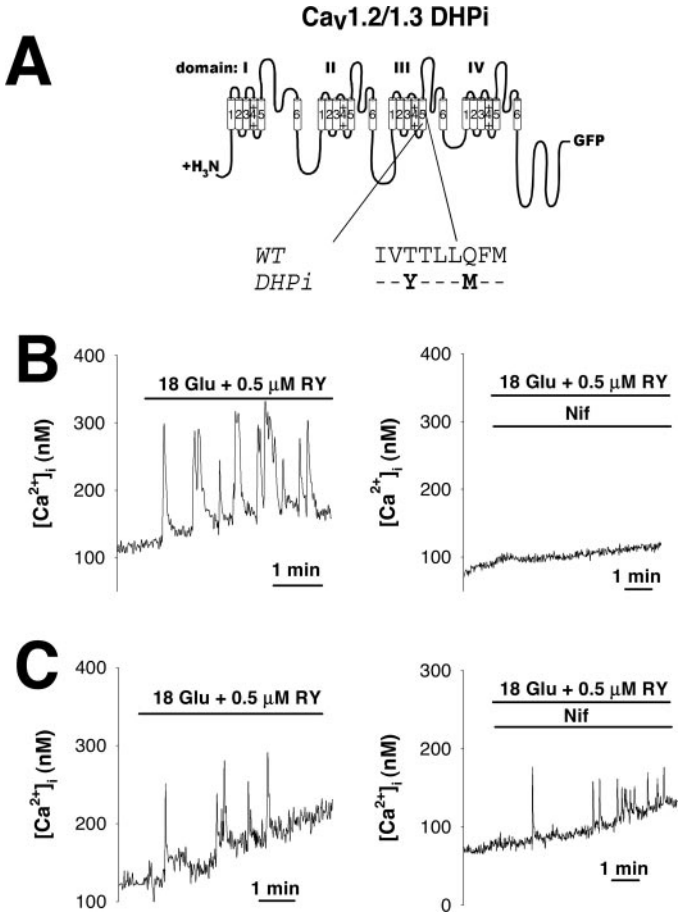

Fig. 4. $Ca_v1.3$-mediated $[Ca^{2+}]_i$ oscillations in INS-1 cells are not sensitive to ryanodine. A, $Ca_v1.2$/DHPi and $Ca_v1.3$/DHPi channels. Two amino acids in the transmembrane segment IIIS5 of $Ca_v1.2$ or $Ca_v1.3$ were mutated to generate channels insensitive to dihydropyridine block. The C termini of both mutant constructs were fused to GFP. B, effect of ryanodine pretreatment on glucose-stimulated $[Ca^{2+}]_i$ oscillation in $Ca_v1.2$/DHPi cells. Glucose (18 mM)-stimulated $[Ca^{2+}]_i$ oscillation was maintained after $Ca_v1.2$/DHPi cells were pretreated with 0.5 μM ryanodine. However, this $[Ca^{2+}]_i$ oscillation was completely inhibited by 10 μM nifedipine. C, effect of ryanodine pretreatment on glucose-stimulated $[Ca^{2+}]_i$ oscillation in $Ca_v1.3$/DHPi cells. After 0.5 μM ryanodine pretreatment, glucose-stimulated $[Ca^{2+}]_i$ oscillation maintained in $Ca_v1.3$/DHPi cells, and this oscillation is not sensitive to nifedipine. Traces shown in B and C are representative of 16 cells observed under each condition.

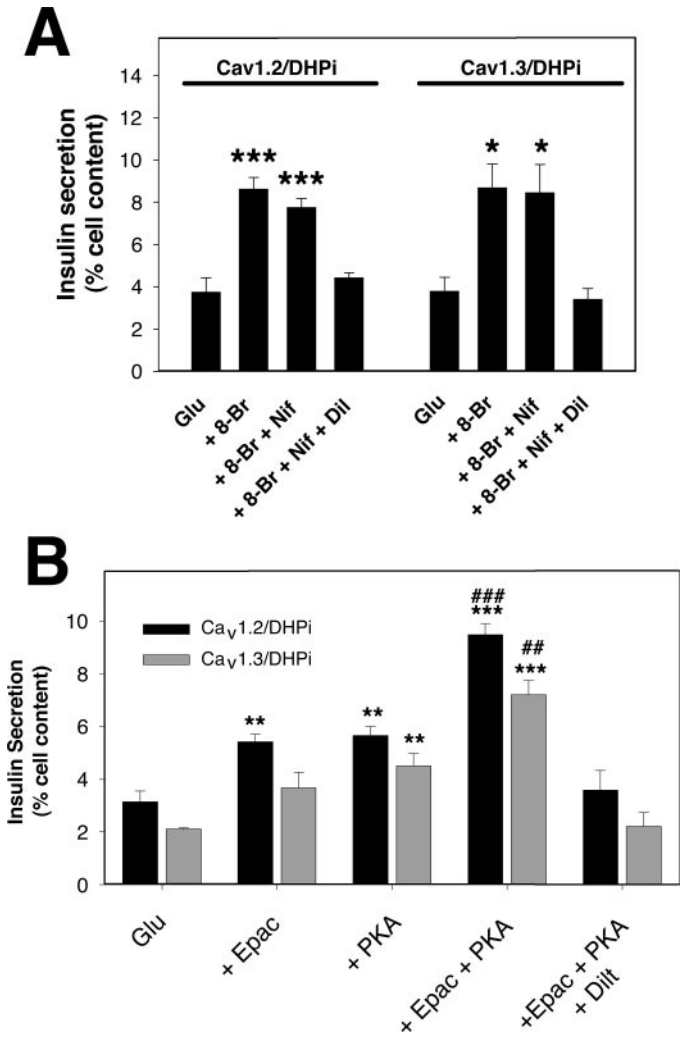

Fig. 5. Both $Ca_v1.2$ and $Ca_v1.3$ are able to mediate cAMP-potentiated insulin secretion in INS-1 cells. A, in the presence of 7.5 mM glucose (Glu), 1 mM 8-Br-cAMP-potentiated insulin secretion (+8-Br) in $Ca_v1.2$/DHPi cells or $Ca_v1.3$/DHPi cells was not inhibited by 10 μM nifedipine (+8-Br + Nif) but blocked by coapplication of 10 μM nifedipine + 500 μM diltiazem (+8-Br + Nif + Dil). In $Ca_v1.2$/DHPi cells, Glu, 3.75 ± 0.68%; +8-Br, 8.63 ± 0.55%; +8-Br + Nif, 7.76 ± 0.41%; +8- Br + Nif + Dil, 4.42 ± 0.24% (n = 4). In $Ca_v1.3$/DHPi cells, Glu, 3.79 ± 0.66%; +8-Br, 8.68 ± 1.14%; +8-Br + Nif, 8.46 ± 1.33%; +8- Br + Nif + Dil, 3.41 ± 0.52% (n = 6) (*, $p < 0.05$; ***, $p <$ 0.001 compared with Glu, one-way ANOVA with Tukey post hoc test). B, both Epac and PKA contribute to cAMP-potentiated coupling of $Ca_v1.2$ and $Ca_v1.3$ to insulin secretion. DHPi cells were incubated with either 7.5 mM glucose (Glu) or 7.5 mM glucose together with 100 μM 8′-pCPT-2′-*O*-Me-cAMP (+Epac) or 50 μM N^6-benzoyl-cAMP (+PKA) or a combination of the three (+Epac + PKA) in the presence of 10 μM nifedipine in KRBH buffer for 30 min. Diltiazem (500 μM) was added to inhibit the DHPi channels (+Epac + PKA + Dilt). In $Ca_v1.2$/DHPi cells, Glu, 3.15 ± 0.41%; +Epac, 5.40 ± 0.30%; +PKA, 5.66 ± 0.35%; +Epac + PKA, 9.49 ± 0.42%; +Epac + PKA + Dilt, 3.60 ± 0.74% (n = 5). In $Ca_v1.3$/DHPi cells, Glu, 2.10 ± 0.06%; +Epac, 3.67 ± 0.60%; +PKA, 4.51 ± 0.47%; +Epac + PKA, 7.21 ± 0.55%; and +Epac + PKA + Dilt, 2.22 ± 0.52 (n = 15). **, $p < 0.01$; ***, $p < 0.001$ compared with Glu; ###, $p < 0.001$ compared with Glu + PKA (one-way ANOVA with Tukey post hoc test).

glucose over that observed with either cAMP analog alone, which is blocked by diltiazem. In contrast, in $Ca_v1.3$/DHPi cells, only N^6-benzoyl-cAMP, but not 8′-pCPT-2′-*O*-Me-cAMP, elicited a significant increase in insulin secretion in response to 7.5 mM glucose above basal levels. Interestingly, a combination of these cAMP analogs elicited a synergistic response that was significantly greater than either 7.5 mM glucose alone or 7.5 mM glucose plus N^6-benzoyl-cAMP. These observations suggest that at mildly elevated glucose concentrations, Epac2 can directly augment insulin secretion mediated by $Ca_v1.2$. However, Epac2 seems to be unable to mediate cAMP-dependent stimulus-secretion coupling via $Ca_v1.3$ independently of PKA activation. In both DHPi cell lines, the maximal response elicited by mildly elevated glucose in combination with both of the selective cAMP analogs is also comparable with the DHP-insensitive response produced by 8-Br-cAMP (Fig. 5A). Thus, although PKA stimulation can directly potentiate secretion mediated by either $Ca_v1.2$ or $Ca_v1.3$, it seems that PKA-independent potentiation of secretion by Epac2 is restricted to $Ca_v1.2$-mediated secretion.

Discussion

Ca^{2+} Influx via Either $Ca_v1.2$ or $Ca_v1.3$ Can Mediate Insulin Secretion in Response to 7.5 mM Glucose and cAMP. Previously, we found that the VDCC $Ca_v1.3$, but not

$Ca_v1.2$, is preferentially coupled to insulin secretion (Liu et al., 2003) and $[Ca^{2+}]_i$ oscillations (Liu et al., 2004) in response to high glucose concentrations in INS-1 cells. Here, we investigated the role of L-VDCCs in stimulus-secretion coupling in INS-1 cells in response to mildly elevated glucose concentrations in combination with cAMP. In contrast to our studies using high glucose concentrations alone, we found that both $Ca_v1.2$ and $Ca_v1.3$ are able to mediate insulin secretion in response to 7.5 mM glucose and cAMP. What can account for this difference in channel selectivity in the face of these two distinct stimuli? Our data in Figs. 2 and 4 (and our previous studies) suggest that the Ca^{2+} influx across the plasma membrane mediated by $Ca_v1.3$ drives both insulin secretion and $[Ca^{2+}]_i$ oscillations in response to 11.2 mM glucose in INS-1 cells because neither are inhibited by unloading of intracellular Ca^{2+} stores by thapsigargin or 0.5 μM ryanodine. In contrast, cAMP potentiation of mildly elevated glucose-stimulated insulin secretion seems to involve release of Ca^{2+} from internal stores because both thapsigargin and 0.5 μM ryanodine, which empties ER Ca^{2+} stores via activation of RyR, inhibit secretion under these conditions. Furthermore, the release of Ca^{2+} from internal stores can be triggered by Ca^{2+} influx across the plasma membrane via either $Ca_v1.2$ or $Ca_v1.3$. The DHP-insensitive insulin secretion observed in $Ca_v1.3$/DHPi cells in response to 11.2 mM glucose was ~40% of that observed in the absence of nifedipine (Liu et al., 2003). In contrast, both $Ca_v1.2$/DHPi and $Ca_v1.3$/DHPi cells exhibit insulin secretion in response to 7.5 mM glucose and 1 mM 8-Br-cAMP in the presence of nifedipine that is not significantly different from that observed in the absence of nifedipine (Fig. 5A). This observation further supports the notion that, in the presence of mildly elevated glucose and high cAMP concentrations, Ca^{2+} influx via $Ca_v1.2$ or $Ca_v1.3$ serves as a trigger for Ca^{2+} release from internal stores and does not directly drive insulin secretion.

Previous studies have also implicated CICR in cAMP-potentiated-insulin secretion. Measurements of $[Ca^{2+}]_i$ changes in response to endogenous cAMP elevation in β-cells is reported to require CICR (Holz et al., 1999; Kang et al., 2001). The intracellular Ca^{2+} release channel responsible for cAMP-induced CICR has not been clearly identified. However, some studies have indicated the involvement of RyR in this event (Holz et al., 1999). RyR2 is expressed in INS-1 cells (Gamberucci et al., 1999) and is able to mediate Ca^{2+} release from ER (Gamberucci et al., 1999; Bruton et al., 2003). Similar to thapsigargin, we found that 0.5 μM ryanodine pretreatment emptied ER Ca^{2+} stores and prevented the cAMP-dependent CICR and, therefore, abrogated insulin secretion in response to mildly elevated glucose (Fig. 2C). However, 20 μM ryanodine, which decreases the Ca^{2+} permeability of RyR (Lattanzio et al., 1987), did not influence the action of 8-Br-cAMP (Fig. 2C). Our results suggest that the pool of Ca^{2+} required for insulin secretion stimulated by 7.5 mM glucose and cAMP is sensitive to ryanodine but is not released via RYR2 in INS-1 cells. In neurons, it is reported that ER acts as a single functional Ca^{2+} store shared by both RyR and IP_3R (Solovyova and Verkhratsky, 2003). Recently, the IP_3 receptor was implicated in CICR in mouse β-cells (Berggren et al., 2004); thus, it is an attractive candidate as the mediator of CICR in INS-1 cells.

cAMP Potentiation of 7.5 mM Glucose-Stimulated Insulin Secretion Involves the Combined Action of Epac2 and PKA. Epac2 has been shown previously to modulate the cAMP-dependent insulin secretion (Kashima et al., 2001) and CICR (Kang et al., 2003) in β-cells independently of PKA. In our present study, we found that Epac2 triggered cAMP-dependent insulin secretion in response to 7.5 mM glucose independently of PKA (Figs. 3 and 5B). Although this potentiation is significantly different from basal, the maximal response under these conditions is seen only when PKA is also activated. The observation that, in untransfected INS-1 cells, inhibition of PKA with H89 reduces secretion stimulated by 7.5 mM glucose plus both selective cAMP analogs to a level that is not significantly different from that of 7.5 mM glucose alone (Fig. 3A) may reflect the ratio of L-VDCC subtypes present in INS-1 cells. $Ca_v1.3$ is prevalent in this cell line, whereas $Ca_v1.2$ is present in a much smaller amount (Horvath et al., 1998). Thus, the small population of $Ca_v1.2$ channels present in untransfected INS-1 cells may mediate a small amount of Epac2-dependent, PKA-independent secretion that becomes statistically insignificant in the presence of H89.

Epac2 and PKA Differentially Modulate $Ca_v1.2$- and $Ca_v1.3$-Coupled Insulin Secretion in Response to 7.5 mM Glucose and cAMP. Because we found that both $Ca_v1.2$ and $Ca_v1.3$ are able to mediate insulin secretion in response to 7.5 mM glucose and cAMP (Fig. 5A), we examined the contribution of Epac2 and PKA in the potentiation of secretion in both of the DHPi cell lines. We reasoned that, although both $Ca_v1.2$ and $Ca_v1.3$ seem to mediate CICR in the presence of elevated cAMP concentrations, they could use distinct cAMP effector pathways. We found that, similar to untransfected INS-1 cells, $Ca_v1.2$-coupled insulin secretion is partially enhanced by Epac2 and PKA independently of each other (Fig. 5B). However, $Ca_v1.3$-coupled secretion seems not to be enhanced by Epac2 in the absence of PKA activation (Fig. 5B). In all cases examined, the combined action of Epac2 and PKA seems sufficient to account for the insulinotropic action of 8-Br-cAMP (Fig. 5B). It is interesting that Epac2 exerts PKA-independent activity only in $Ca_v1.2$/DHPi cells because it has been previously reported that RIM 2, a downstream target of Epac2 (Ozaki et al., 2000), binds to the intracellular II-III loop of $Ca_v1.2$, but not $Ca_v1.3$ (Shibasaki et al., 2004). Phosphorylation of $Ca_v1.3$ may be necessary for Epac2 to modulate secretion mediated by that channel. Indeed, inspection of the II-III loop region of $Ca_v1.3$ reveals two putative PKA phosphorylation sites that are not present in $Ca_v1.2$. On the other hand, Epac2 modulation of $Ca_v1.3$-mediated secretion is consistent with the presence of a putative PKA phosphorylation site on Epac2 (Kawasaki et al., 1998). Several studies have suggested possible Epac2- or PKA-mediated regulatory mechanisms at the level of channel gating and protein-protein interactions. Previous studies have suggested that in human embryonic kidney 293 cells, the phosphorylation of $Ca_v1.2$ by PKA (Gromada et al., 1998) causes a leftward shift in the $V_{1/2}$ of activation, requiring a smaller depolarization to initiate channel gating. Likewise, HIT-T15 insulinoma cells transfected with the $G\alpha_s$-coupled GLP-1 receptor demonstrate an increase in the VDCC currents, presumably due to a leftward shift in the $V_{1/2}$ of activation (MacDonald et al., 2002). A rightward shift in the voltage dependence of steady-state inactivation VDCC in mouse pancreatic β-cells is observed upon endogenous cAMP elevation by treatment with GLP-1 (Britsch et al., 1995). In addition to modulation of L-type channel activity, PKA acti-

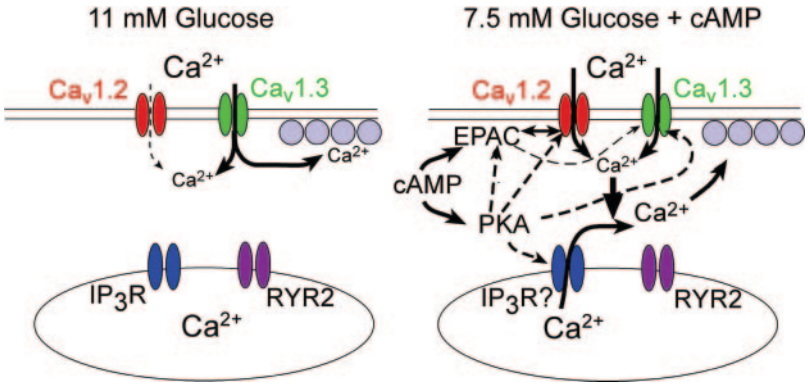

Fig. 6. Proposed models for the involvement of $Ca_v1.2$ and $Ca_v1.3$ in insulin secretion in response to either 11.2 mM glucose alone or 7.5 mM glucose together with cAMP. Left, $Ca_v1.3$ is preferentially coupled to glucose-stimulated insulin secretion in INS-1 cells. Upon 11.2 mM glucose stimulation, Ca^{2+} influx through $Ca_v1.2$ and $Ca_v1.3$ form a local high $[Ca^{2+}]_i$ domain beneath each channel. $Ca_v1.3$ is preferentially coupled to the insulin-containing granules, probably via $Ca_v1.3$ II-III loop, which may position insulin-containing granules within the $Ca_v1.3$-mediated high $[Ca^{2+}]_i$ domain and induce insulin secretion. Alternatively, the lower activation voltage of $Ca_v1.3$ versus $Ca_v1.2$ may result in preferential activation of $Ca_v1.3$ by glucose. Under these conditions, Ca^{2+} release from internal stores is not involved. Right, both $Ca_v1.2$ and $Ca_v1.3$ are able to mediate cAMP-potentiated insulin secretion. In the presence of cAMP, 7.5 mM glucose-induced membrane depolarization activates Ca^{2+} entry via $Ca_v1.2$ and $Ca_v1.3$. The Ca^{2+} influx through both channels is able to trigger Ca^{2+}-induced Ca^{2+} release from internal stores. This release of Ca^{2+} is not mediated by RYR, but the required pool of Ca^{2+} is sensitive to both thapsigargin and 0.5 μM ryanodine. The IP_3R, or some other ryanodine-insensitive mechanism, probably mediates the Ca^{2+} release. cAMP-induced PKA activity is required for this action and is proposed to affect the Ca^{2+} sensitivity of the Ca^{2+} release channels on the ER membrane and/or the Ca^{2+} influx channels ($Ca_v1.2$ and/or $Ca_v1.3$) on the plasma membrane. Epac2 or PKA activation can independently potentiate $Ca_v1.2$-mediated insulin secretion. PKA activation can independently potentiate $Ca_v1.3$-mediated insulin secretion, whereas PKA activity is required for Epac2 potentiation of $Ca_v1.3$-mediated insulin secretion.

vation has been shown recently to amplify CICR from internal stores via IP_3 receptors in rat pancreatic β cells (Dyachok and Gylfe, 2004). Finally, cAMP also plays a role in replenishing ER stores of Ca^{2+}, by stimulating the thapsigargin-sensitive reuptake of Ca^{2+}, via a PKA-dependent mechanism in rat pancreatic β-cells (Yaekura and Yada, 1998). Thus, priming of CICR and ER Ca^{2+} refilling, in contrast to increasing Ca^{2+} influx via L-type channels, are potential mechanisms to explain why insulin secretion in response to mildly elevated glucose concentrations plus cAMP involves CICR, but secretion in response to elevated glucose concentrations alone does not.

A model for the roles of $Ca_v1.2$ and $Ca_v1.3$ in glucose-stimulated or cAMP-potentiated insulin secretion based upon our data in INS-1 cells and the literature is proposed in Fig. 6. $Ca_v1.3$ is preferentially coupled to 11.2 mM glucose-stimulated insulin secretion, whereas both $Ca_v1.2$ and $Ca_v1.3$ are able to mediate cAMP-potentiated insulin secretion in the presence of 7.5 mM glucose. This insulinotropic action of cAMP that is synergistic with submaximal glucose concentrations is facilitated by the downstream effectors Epac2 and PKA and is dependent on CICR. Although insulin secretion mediated by $Ca_v1.2$ can be stimulated by Epac2 independently of PKA, secretion mediated by $Ca_v1.3$ is stimulated by Epac2 only in concert with PKA activation. Although the INS-1 cell line has proven to be an excellent system for the study of insulin secretion mechanisms, it is a transformed cell line; thus, our model must be tested in primary β-cells. A previous study using a $Ca_v1.3$ knockout mouse showed normal insulin secretion in pancreatic β-cells (Schulla et al., 2003), but the roles of $Ca_v1.2$ and $Ca_v1.3$ in insulin secretion have not been investigated in rat and human pancreatic β-cells. It will be interesting to determine the molecular basis for the distinct roles of $Ca_v1.2$ and 1.3 in mediating insulin secretion and the downstream targets of both Epac2 and PKA that mediate the potentiation of insulin secretion.

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