Ca_v1.2 and Ca_v1.3 Are Differentially Coupled to Glucagon-Like Peptide-1 Potentiation of Glucose-Stimulated Insulin Secretion in the Pancreatic β -Cell Line INS-1

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ABSTRACT

The incretin peptides, glucose-dependent insulintropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), potentiate glucose-stimulated insulin secretion (GSIS) and β -cell proliferation and differentiation. Ca²⁺ influx via voltage-gated L-type Ca²⁺ channels is required for GLP-1 and GIP potentiation of GSIS. We investigated the role of the L-type Ca²⁺ channels Ca_v1.2 and Ca_v1.3 in mediating GLP-1- and GIP-stimulated events in INS-1 cells and INS-1 cell lines expressing dihydropyridine-insensitive (DHPi) mutants of either Ca_v1.2 or Ca_v1.3. Ca_v1.3/DHPi channels supported full potentiation of GSIS by GLP-1 (50 nM) compared with untransfected INS-1 cells. However, GLP-1-potentiated GSIS mediated by Ca_v1.2/DHPi channels was markedly reduced compared with untransfected INS-1 cells. In contrast, GIP (10 nM) potentiation of GSIS mediated by both Ca_v1.2/DHPi and Ca_v1.3/DHPi channels was similar to that observed in untransfected INS-1 cells. Disruption of intracellular Ca²⁺ release with thapsigargin, ryanodine, or

2-aminoethyldiphenylborate and inhibition of protein kinase A (PKA) or protein kinase C (PKC) significantly reduced GLP-1 potentiation of GSIS by Ca_v1.3/DHPi channels and by endogenous L-type channels in INS-1 cells, but not by Ca_v1.2/DHPi channels. Inhibition of glucose-stimulated phospholipase C activity with 1-(6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122) did not inhibit potentiation of GSIS by GLP-1 in INS-1 cells. In contrast, wortmannin, an inhibitor of phosphatidylinositol 3-kinase, and 2'-amino-3'-methoxyflavone (PD98059), an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase (ERK) kinase, both markedly inhibited GLP-1 potentiation of GSIS by endogenous channels in INS-1 cells and Ca_v1.3/DHPi channels, but not by Ca_v1.2/DHPi channels. Thus, Ca_v1.3 is preferentially coupled to GLP-1 potentiation of GSIS in INS-1 cells via a mechanism that requires intact intracellular Ca²⁺ stores, PKA and PKC activity, and activation of ERK1/2.

Upon ingestion of food, the incretin hormones glucagon-like peptide-1 (GLP-1) (Eissele et al., 1992) and gastric inhibitory peptide/glucose-dependent insulintropic polypeptide (GIP) (Meier et al., 2002) are secreted from intestinal L

and K cells into the bloodstream. These peptides bind to class B G-protein-coupled receptors (Thorens, 1992; Yamada et al., 1995) in multiple tissues and cell types (Usdin et al., 1993; Bullock et al., 1996), including the pancreatic β -cell, where they trigger multiple antidiabetogenic actions that range from the increase in glucose sensitivity and amplification of insulin secretion, to enhancement of insulin biosynthesis and enhancement of β -cell proliferation (MacDonald et al., 2002). GIP and GLP-1 potentiate insulin secretion in a glucose-dependent manner (Mojsov et al., 1987), a property that potentially reduces the risk of hyperglycemia, and has led to

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ABBREVIATIONS: GLP-1, glucagon-like peptide-1; GSIS, glucose-stimulated insulin secretion; DHPi, dihydropyridine-insensitive; ER, endoplasmic reticulum; L-VGCC, L-type voltage-gated Ca²⁺ channels; GIP, glucose-dependent gastrointestinal peptide; PKA, protein kinase A; PKC, protein kinase C; 2-APB, 2-aminoethyldiphenyl borate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PLC, phospholipase C; ERK, extracellular signal-regulated kinase; EPAC2, effector protein directly activated by cAMP 2; IBMX, isobutylmethylxanthine; IP₁, inositol-1-phosphate; IP₃, inositol-1,4,5-trisphosphate; PI-3K, phosphatidylinositol 3-kinase; PIP₂, phosphatidylinositol-4,5-bisphosphate; Bis, bisindolylmaleimide; RIM2, Rab3-interacting molecule 2; Rp-cAMPS, cyclic adenosine monophosphorothioate-Rp isomer; G418, (2*R*,3*S*,4*R*,5*R*,6*S*)-5-amino-6-[[1*R*,2*S*,3*S*,4*R*,6*S*]-4,6-diamino-3-[(2*R*,3*R*,4*R*,5*R*)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol; PD98059, 2'-amino-3'-methoxyflavone; U73122, 1-(6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione; U73343, 1-[6-(((17 β -3-methoxyestra-1,3,5[10]-trien-17-yl)amino)hexyl)-2,5-pyrrolidinedione.

great interest in these incretins as therapeutics for the treatment of type 2 diabetes. Glucose-stimulated insulin release from pancreatic β -cells (Devis et al., 1975), and its potentiation by both GIP and GLP-1 (Lu et al., 1993), require Ca^{2+} influx across the plasma membrane via L-type voltage-dependent Ca^{2+} channels.

Besides potentiation of GSIS, both GLP-1 and GIP modulate β -cell growth and differentiation. GLP-1 promotes differentiation from precursor cells into insulin-secreting β -cells, stimulates proliferation and increases mass of mature β -cells, and promotes β -cell survival (Buteau et al., 2003). In addition, both GIP (Böcker and Verspohl, 2001) and GLP-1 (Gomez et al., 2002; Arnette et al., 2003) stimulate activation of the MEK/ERK1/2 pathway, which is implicated in β -cell proliferation. Activation of the MEK/ERK1/2 pathway has also been shown to play a role in GSIS (Longuet et al., 2005). In glucose stimulation of MEK/ERK1/2 activation and its potentiation by GLP-1, it is clear that Ca^{2+} influx via L-type Ca^{2+} channels plays a crucial role (Gomez et al., 2002; Arnette et al., 2003).

Pancreatic β -cells express two distinct L-type voltage-gated Ca^{2+} channels (L-VGCCs), $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ (Seino et al., 1992). Because these two channel subtypes share comparable sensitivity to small-molecule inhibitors of L-VGCC, their distinct roles in β -cell function has been difficult to delineate. A study with $\text{Ca}_v1.3^{-/-}$ mice revealed that silencing $\text{Ca}_v1.3$ resulted in hypoinsulinemia and glucose intolerance, as a consequence of reduced postnatal β -cell generation or proliferation. Glucose-stimulated insulin secretion from isolated $\text{Ca}_v1.3^{-/-}$ islets was maintained by a compensatory up-regulation of $\text{Ca}_v1.2$ expression (Namkung et al., 2001).

In the current study, we examined the roles of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ in mediating potentiation of GSIS by GLP-1 and GIP in the rat insulinoma cell line INS-1 (Asfari et al., 1992), by use of mutant $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels insensitive to the dihydropyridine class of L-VGCC inhibitors (DHPi), but normally sensitive to the benzothiazepine diltiazem (Hockerman et al., 2000). Thus, endogenous L-type channels can be blocked with a dihydropyridine drug such as nifedipine, and characteristics of the expressed mutant channels can be examined in isolation. Using this system, we previously reported that $\text{Ca}_v1.3$ is preferentially coupled to glucose-stimulated insulin secretion (Liu et al., 2003) and $[\text{Ca}^{2+}]_{\text{in}}$ oscillations (Liu et al., 2004) in INS-1 cells, and that $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ are differentially coupled to potentiation of GSIS by an effector protein activated by cyclic AMP 2 (EPAC2)-selective analog of cAMP (Liu et al., 2006). Here, we report that $\text{Ca}_v1.3$ is preferentially coupled to GLP-1 potentiation of GSIS, but that $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ are not different in their ability to mediate GIP potentiation of GSIS. Experiments with pharmacological agents suggest that coupling of $\text{Ca}_v1.3$ to GLP-1 potentiation of GSIS depends on Ca^{2+} release from internal stores, the activation of both protein kinase A and C, phosphatidylinositol 3-kinase activity, and activation of the MEK/ERK1/2 pathway.

Materials and Methods

Reagents. All reagents used in this study, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO). The protein kinase A (PKA) and pan-protein kinase C (PKC) inhibitors cyclic adenosine monophosphorothioate-Rp isomer (Rp-cAMPS) and bisindolylmaleimide (Bis), as well as the endoplasmic reticulum (ER)

Ca^{2+} efflux inhibitors ryanodine and 2-aminoethyl-diphenyl borate (2-APB) were obtained from Calbiochem (San Diego, CA). The truncated and amidated human peptide GLP-1^{7-36NH₂}, simply denoted as GLP-1, was used for all insulin secretion assays.

Plasmids and Stable Cell Line Construction. The two amino acid substitutions (Thr1039Tyr/Gln1043Met for $\text{Ca}_v1.2$ and Thr1029Tyr/Gln1033Met for $\text{Ca}_v1.3$) that render the $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ pore domains dihydropyridine-insensitive were introduced by site-directed mutagenesis with use of the Quikchange method (Stratagene, La Jolla, CA) as described previously (Hockerman et al., 2000). The pcDNA3 constructs for $\text{Ca}_v1.2/\text{DHPi}$ and $\text{Ca}_v1.3/\text{DHPi}$ were subcloned into the pEGFP-N1 vector (Clontech, Mountain View, CA) bearing the neomycin-resistance gene, and transfected into INS-1 Cells by use of Geneporter II (Gene Therapy Systems, San Diego, CA). Cells were transferred to selection medium containing 100 $\mu\text{g}/\text{ml}$ G418 three days after transfection. G418-resistant colonies were expanded and validated by Western blot, reverse transcription-polymerase chain reaction, and electrophysiology.

Cell Culture. INS-1 cells originally obtained from Dr. Ming Li (Tulane University) were maintained at 37°C and 5% CO_2 in RPMI 1640 medium (pH 7.35) that contained 11.2 mM glucose, 48 mM NaHCO_3 , 20 mM HEPES, and 0.0007% (v/v) β -mercaptoethanol as specified previously (Asfari et al., 1992). The culture medium was supplemented with 10% v/v fetal bovine serum (HyClone Laboratories, Logan, UT), and 1% v/v penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) (Invitrogen, Carlsbad, CA).

Insulin Secretion Assays. Three days before the treatment with stimuli, INS-1 cells (between P20 and P55) were seeded in 12-well plates at ~90% confluence. After 72 h, cells were washed twice with isotonic phosphate-buffered saline and preincubated with modified Krebs-Ringer buffer (KRBH, 115 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2.5 mM CaCl_2 , 24 mM NaHCO_3 , 25 mM HEPES, 0.05% bovine serum albumin, pH 7.3, 295 mOsm) for 30 min at 37°C, 5% CO_2 . The preincubation buffer was removed and replaced with KRBH buffer containing the stimuli. After stimulation of cells for 1 h at 37°C, 5% CO_2 , the conditioned buffer was removed and stored at -20°C for assay of secreted insulin content. Insulin content was assayed by the Rat Insulin High Range ELISA kit (Alpco Diagnostics, Inc., Salem, NH), according to the manufacturer's instructions. For the neomycin experiments and the phospholipase C (PLC) inhibitor study, the cells were preincubated and stimulated in the presence of KRBH supplemented with 1.5 mM neomycin (Fisher BioReagents, Fair Lawn, NJ) or with 10 μM U73122/U73343 (Sigma-Aldrich). Secreted insulin was measured and normalized to amount of protein per well determined by BCA Assay (Pierce Chemical, Rockford, IL).

IP₁ Assay. WT INS-1 cells were cultured as described previously for insulin secretion assays. Cells were preincubated for 1 h in 10 mM HEPES, 1 mM CaCl_2 , 0.5 mM MgCl_2 , 4.2 mM KCl, 146 mM NaCl (preincubation buffer) at 37°C, 5% CO_2 . The preincubation buffer was removed and the cells were stimulated for 1 h at 37°C, 5% CO_2 , by use of preincubation buffer supplemented with 50 mM LiCl (pH = 7.4) to allow accumulation of inositol-1-phosphate (IP_1) as well as the indicated stimuli. All reagents were obtained from Sigma-Aldrich. IP_1 concentrations were determined by use of the IP-One ELISA kit (Cisbio Biosays, Bedford, MA) according to the manufacturer's instructions.

Data Analysis. Data were analyzed by use of SigmaPlot 11.0 for Windows (Systat Software Inc., Chicago, IL). Data are represented as mean values of at least three trials $\pm$ S.E.M. Statistical analyses of data sets were performed by use of one-way analysis of variance with the Student-Newman-Keuls post hoc comparison with SigmaPlot 11.0.

Results

Characterization of the $\text{Ca}_v1.2/\text{DHPi}$ and $\text{Ca}_v1.3/\text{DHPi}$ Cells. The mutation of a Thr to and a Gln in the transmembrane domain III S5 of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ (Fig. 1A)

Fig. 1. KCl-stimulated insulin secretion in INS-1, Ca_v1.2/DHPi, and Ca_v1.3/DHPi cells. **A**, diagram of the DHPi mutation. The native amino acids (T and Q) at the indicated positions in transmembrane domains IIIS5 were replaced with Y and M in both Ca_v1.2 and Ca_v1.3 to create the corresponding DHPi mutant channels. **B**, insulin secretion stimulated with 50 mM KCl in INS-1 cells. Secretion was completely blocked by 10 μ M nifedipine, a dihydropyridine L-type Ca²⁺ channel blocker. Insulin secretion stimulated with 50 mM KCl in Ca_v1.2/DHPi cells (**C**) and Ca_v1.3/DHPi cells (**D**). Note that the addition of 10 μ M nifedipine did not significantly inhibit secretion, but that the addition of 500 μ M diltiazem significantly inhibited secretion. Data shown are mean $\pm$ S.E. ($n = 9$ –27). ***, $P < 0.001$ compared with basal (KRBH); **, $P < 0.01$; \$\$, $P < 0.01$.

renders them insensitive to inhibition by the dihydropyridine calcium channel blocker nifedipine, but normally sensitive to the benzothiazepine calcium channel blocker diltiazem (Hockerman et al., 2000). We stably expressed these mutant channels termed Ca_v1.2/DHPi and Ca_v1.3/DHPi (dihydropyridine-insensitive) in INS-1 cells (Ca_v1.2/DHPi cells and Ca_v1.3/DHPi cells, respectively) and found that they were functionally expressed, and that Ca_v1.3/DHPi was preferentially coupled to GSIS (Liu et al., 2003). Whereas INS-1 cells secrete insulin in response to stimulation with 50 mM KCl in a manner that is blocked by 10 μ M nifedipine (Fig. 1B), both Ca_v1.2/DHPi cells and Ca_v1.3/DHPi cells secrete insulin in response to 50 mM KCl in a manner that is insensitive to 10 μ M nifedipine, but blocked by 500 μ M diltiazem (Fig. 1, C and D). Thus, the data in Fig. 1 demonstrate that the expected DHPi pharmacological profile is observed in L-type channel modulation of KCl-stimulated insulin secretion in Ca_v1.2/DHPi and Ca_v1.3/DHPi cells, and that both channels are capable of coupling Ca²⁺-influx to insulin secretion in response to stimulation with 50 mM KCl in INS-1 cells.

Characterization of GLP-1 and GIP Potentiation of GSIS in INS-1, Ca_v1.2/DHPi, and Ca_v1.3/DHPi Cells. We next examined the relative ability of GIP and GLP-1 to potentiate 7.5 mM GSIS in the rat insulinoma cell line INS-1. A fasting blood glucose concentration of 7.5 mM (135 mg/dl) is above the threshold for a diagnosis of diabetes (American Diabetes Association, 2009). Furthermore, this concentration is at the low end of the dose-response range for stimulation of insulin secretion in INS-1 cells (Merglen et al., 2004; Liu et al., 2006), but insulin secretion thus stimulated is strongly potentiated by cAMP analogs (Liu et al., 2006). In addition, 7.5 mM glucose is sufficient to trigger membrane potential depolarization in INS-1 cells (Merglen et al., 2004). GLP-1 (50 nM; $\sim 250 \times EC_{50}$; Moens et al., 1996) alone does not

stimulate insulin secretion, but markedly potentiates secretion in the presence of 7.5 mM glucose (Fig. 2A). In contrast, GIP (10 nM; $\sim 50 \times EC_{50}$; Moens et al., 1996) alone causes a slight but significant increase in insulin secretion (Fig. 2D). The combination of 7.5 mM glucose and 10 nM GIP potentiates insulin secretion ~ 3 -fold over 10 nM GIP alone. Moreover, in terms of nanograms of insulin secreted per well, 50 nM GLP-1 was ~ 2 times as efficacious in potentiating GSIS as 10 nM GIP. Potentiation of GSIS by both GLP-1 and GIP was blocked by the L-VGCC antagonist nifedipine (10 μ M), and the K_{ATP} channel opener diazoxide (200 μ M) (Fig. 2, A and D).

To determine the independent contribution of Ca_v1.2 and Ca_v1.3 to GLP-1-potentiated GSIS, we performed experiments with Ca_v1.2/DHPi and Ca_v1.3/DHPi expressing INS-1 cells. Figure 2B shows GLP-1 potentiation of GSIS in Ca_v1.2/DHPi cells. In the absence of nifedipine, 7.5 mM glucose + GLP-1 stimulate robust insulin secretion, to a magnitude that is similar to untransfected INS-1 cells. However, upon addition of 10 μ M nifedipine, insulin secretion in response to 7.5 mM glucose + 50 nM GLP-1 is significantly, but incompletely inhibited. This fraction of nifedipine-resistant secretion is attributable to the activity of the Ca_v1.2/DHPi channel as it is completely blocked by 500 μ M diltiazem, and amounts to $\sim 35\%$ of the secretion measured in the absence of nifedipine. The secretion mediated by the Ca_v1.2/DHPi channel is also inhibited by the K_{ATP} channel opener diazoxide (200 μ M), an agent that prevents membrane depolarizations required for L-VGCC activation. As shown in Fig. 2C, 50 nM GLP-1 robustly potentiated 7.5 mM GSIS in Ca_v1.3/DHPi cells, to an extent similar to untransfected INS-1 cells. In contrast to both untransfected INS-1 cells and Ca_v1.2/DHPi cells, 10 μ M nifedipine did not significantly block insulin secretion in response to 7.5 mM glucose + 50 nM GLP-1 in

Fig. 2. Potentiation of GSIS by GLP-1 and GIP in INS-1, $\text{Ca}_v1.2/\text{DHPi}$, and $\text{Ca}_v1.3/\text{DHPi}$ cells. **A**, GLP-1 (50 nM) robustly potentiates 7.5 mM GSIS in INS-1 cells. This potentiation is completely inhibited by either 10 μM nifedipine or 200 μM diazoxide. **B**, GLP-1 (50 nM) potentiation of 7.5 mM GSIS in $\text{Ca}_v1.2/\text{DHPi}$ cells. Potentiation is significantly, but incompletely, inhibited by 10 μM nifedipine. Addition of either 500 μM diltiazem or 200 μM diazoxide completely inhibits the nifedipine-insensitive portion of secretion. **C**, GLP-1 (50 nM) potentiation of 7.5 mM GSIS in $\text{Ca}_v1.3/\text{DHPi}$ cells. Addition of 10 μM nifedipine does not significantly inhibit potentiation of insulin secretion. However, addition of either 500 μM diltiazem or 200 μM diazoxide completely inhibits the nifedipine-insensitive secretion. **D**, GIP (10 nM) potentiation of 7.5 mM GSIS in INS-1 cells. GIP alone significantly increases insulin secretion, but also potentiates 7.5 mM GSIS. GIP potentiation of GSIS in INS-1 cells is completely inhibited by either 10 μM nifedipine or 200 μM diazoxide. **E**, GIP (10 nM) potentiation of 7.5 mM GSIS in $\text{Ca}_v1.2/\text{DHPi}$ cells. Addition of 10 μM nifedipine does not significantly reduce secretion stimulated by the combination of GIP and glucose. Addition of either 500 μM diltiazem or 200 μM diazoxide completely inhibits secretion. **F**, GIP (10 nM) potentiation of 7.5 mM GSIS in $\text{Ca}_v1.3/\text{DHPi}$ cells. Addition of 10 μM nifedipine does not significantly inhibit secretion stimulated by the combination of GIP and glucose. Addition of either 500 μM diltiazem or 200 μM diazoxide completely inhibits secretion. Data shown are mean $\pm$ S.E. ($n = 3-41$). ***, $P < 0.001$; **, $P < 0.01$ compared with 7.5 mM glucose alone; \$\$\$, $P < 0.001$; \$\$, $P < 0.01$ for the indicated comparison.

$\text{Ca}_v1.3/\text{DHPi}$ cells. However, both 500 μM diltiazem and 200 μM diazoxide completely blocked this nifedipine-resistant secretion. We also examined GIP potentiation of GSIS in $\text{Ca}_v1.2/\text{DHPi}$ and $\text{Ca}_v1.3/\text{DHPi}$ cells. In $\text{Ca}_v1.2/\text{DHPi}$ cells (Fig. 2E), 10 nM GIP potentiated GSIS to a similar extent as in untransfected INS-1 cells. However, in contrast to GLP-1, addition of 10 μM nifedipine did not significantly reduce GIP potentiation of GSIS in $\text{Ca}_v1.2/\text{DHPi}$ cells. Addition of either diltiazem or diazoxide blocked the nifedipine-resistant potentiation of GSIS in $\text{Ca}_v1.2/\text{DHPi}$ cells. In $\text{Ca}_v1.3/\text{DHPi}$ cells (Fig. 2F), GIP potentiated GSIS to a similar extent as in untransfected INS-1 cells, and addition of 10 μM nifedipine did not reduce this response. However, addition of diltiazem or diazoxide completely blocked the nifedipine-resistant potentiation of GSIS by GIP in $\text{Ca}_v1.3/\text{DHPi}$ cells. Thus, both $\text{Ca}_v1.2/\text{DHPi}$ and $\text{Ca}_v1.3/\text{DHPi}$ channels are capable of coupling to potentiation of GSIS by both GLP-1 and GIP. However, $\text{Ca}_v1.3/\text{DHPi}$ channels, in isolation, support GLP-1 potentiation of GSIS to a similar extent as untransfected INS-1 cells, whereas $\text{Ca}_v1.2/\text{DHPi}$ channels, in isolation, support only a small fraction of GLP-1-potentiated GSIS response observed in $\text{Ca}_v1.2/\text{DHPi}$ cells in the absence of nifedipine or in untransfected INS-1 cells.

Contribution of PKA and PKC Activity to GLP-1 Potentiation of GSIS mediated by $\text{Ca}_v1.2/\text{DHPi}$ and $\text{Ca}_v1.3/\text{DHPi}$. Because GIP potentiation of GSIS was not markedly different if it was mediated by $\text{Ca}_v1.2/\text{DHPi}$

or $\text{Ca}_v1.3/\text{DHPi}$, we investigated the mechanisms that underlay the preferential coupling of $\text{Ca}_v1.3/\text{DHPi}$ to GLP-1 potentiation of GSIS. GLP-1 stimulates cAMP in INS-1 cells in a glucose-independent manner (Jacobo et al., 2009), and cell-permeable analogs of cAMP can potentiate GSIS in INS-1 cells (Liu et al., 2006). Therefore, we examined the effect of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX; 100 μM) on 50 nM GLP-1 potentiation of 7.5 mM GSIS in INS-1 cells, and $\text{Ca}_v1.2/\text{DHPi}$, and $\text{Ca}_v1.3/\text{DHPi}$ cells in the presence of 10 μM nifedipine (Fig. 3A). In each case, IBMX significantly increased the potentiation of GSIS by GLP-1, suggesting that cAMP may play a role in GLP-1 potentiation of GSIS mediated by $\text{Ca}_v1.2/\text{DHPi}$ and $\text{Ca}_v1.3/\text{DHPi}$.

We next examined the role of cAMP-dependent PKA and PKC in GLP-1 potentiation of GSIS mediated by $\text{Ca}_v1.2/\text{DHPi}$ and $\text{Ca}_v1.3/\text{DHPi}$. Both PKA (Gromada et al., 1998), a major effector of cAMP signaling, and PKC (Suzuki et al., 2006) are implicated in GLP-1 potentiation of GSIS. In INS-1 cells pretreated with the nucleotide PKA inhibitor Rp-cAMPS (100 μM), insulin secretion was significantly blocked by ~60% (Fig. 3B). Treatment of INS-1 cells with the pan-PKC inhibitor Bis (2.4 μM) similarly resulted in ~60% inhibition of insulin secretion in response to 7.5 mM glucose + GLP-1 (Fig. 3B). The combination of Rp-cAMPS and Bis did not further inhibit GLP-1 potentiation of 7.5 mM GSIS beyond the extent observed with either compound alone. In

Fig. 3. Inhibitors of PKA and PKC reduce GLP-1 potentiation of GSIS in INS-1 cells and Ca_v1.3/DHPi cells. **A**, inhibition of phosphodiesterase activity with 100 μ M IBMX significantly enhanced 50 nM GLP-1 potentiation of 7.5 mM GSIS in INS-1 cells, and nifedipine (10 μ M)-resistant GLP-1 potentiation of GSIS in Ca_v1.2/DHPi and Ca_v1.3/DHPi cells. ***, $P < 0.001$ compared with KRBH; \$\$\$, $P < 0.001$; \$\$\$, $P < 0.01$ for the indicated comparison. **B**, the nucleotide PKA inhibitor Rp-cAMPS (100 μ M), or the pan-PKC inhibitor Bis (2.4 μ M) both significantly inhibited GLP-1 potentiation of GSIS in INS-1 cells. The combination of Rp-cAMPS and Bis did not further inhibit secretion compared with either compound alone. Comparable results for insulin secretion assays were obtained with the non-nucleotide PKA inhibitor H-89 (10 μ M). **C**, nifedipine (10 μ M)-resistant 50 nM GLP-1 potentiation of 7.5 mM GSIS in Ca_v1.2/DHPi cells is insensitive to inhibition of both PKA and PKC. **D**, nifedipine (10 μ M)-resistant 50 nM GLP-1 potentiation of 7.5 mM GSIS in Ca_v1.3/DHPi is significantly inhibited by both 100 μ M Rp-cAMPS and 2.4 μ M Bis. The combination of Rp-cAMPS and Bis did not significantly decrease GLP-1 potentiation of GSIS beyond that observed with either compound alone. Data shown are mean $\pm$ S.E. ($n = 6-15$). ***, $P < 0.001$ compared with GLP-1 + glucose (INS-1 cells) or GLP-1 + glucose + nifedipine (Ca_v1.2/DHPi and Ca_v1.3/DHPi cells).

Ca_v1.2/DHPi cells, neither 100 μ M Rp-cAMPS, 2.4 μ M Bis, nor Rp-cAMPS + Bis significantly inhibited nifedipine-resistant insulin secretion stimulated by 50 nM GLP-1 and 7.5 mM glucose (Fig. 3C). In contrast, the nifedipine-resistant potentiation of 7.5 mM GSIS by 50 nM GLP-1 was significantly inhibited by both 100 μ M Rp-cAMPS and 2.4 μ M Bis, whereas the combination of Rp-cAMPS + Bis did not further inhibit secretion beyond the extent observed with either compound alone. Thus, although inhibition of cAMP hydrolysis by IBMX enhances potentiation of GSIS by GLP-1 mediated by either Ca_v1.2/DHPi or Ca_v1.3/DHPi channels, only secretion mediated by Ca_v1.3/DHPi channels is sensitive to both PKA and PKC inhibition in the presence of 10 μ M nifedipine.

Contribution of Internal Ca²⁺ Stores to GLP-1 Potentiation of GSIS Mediated by Ca_v1.2/DHPi and Ca_v1.3/DHPi. Ca²⁺-induced ER Ca²⁺ release is proposed to play a role in the potentiation of GSIS by GLP-1 (Kang and Holz, 2003). Therefore, we examined the contribution of ER Ca²⁺ stores to potentiation of GSIS by GLP-1 by use of pharmacological agents that disrupt release of Ca²⁺ from the ER. In INS-1 cells, depletion of ER Ca²⁺ stores with ryanodine (0.5 μ M) or the Ca²⁺-ATPase inhibitor thapsigargin (1 μ M), or antagonism of the inositol-1,4,5-trisphosphate (IP₃)-R with 2-APB (50 μ M) all significantly inhibited GLP-1 potentiation of GSIS (Fig. 4A). In contrast, nifedipine-resistant potentiation of GSIS GLP-1 in Ca_v1.2/DHPi cells was not significantly affected by 1 μ M thapsigargin, or 50 μ M 2-APB; however, 0.5 μ M ryanodine partially, but significantly, reduced nifedipine-resistant GLP-1 potentiation of GSIS in Ca_v1.2/DHPi cells (Fig. 4B). Finally, nifedipine-resistant GLP-1 potentiation of GSIS in Ca_v1.3/DHPi cells was significantly inhibited by 0.5 μ M ryanodine, 1 μ M thapsigargin, and 50 μ M 2-APB (Fig. 4C). Thus, as observed with

inhibitors of PKA and PKC, Ca_v1.3/DHPi channels mediate GLP-1 potentiation of GSIS with a pharmacological profile that closely mirrors that of untransfected INS-1 cells. In contrast, GLP-1 potentiation of GSIS by Ca_v1.2/DHPi seems to require neither activation of PKA or PKC nor release of Ca²⁺ from internal stores.

Contribution of PIP₂ to GLP-1 Potentiation of GSIS in INS-1 Cells. Given our results that suggest a role for IP₃ receptor-mediated release of Ca²⁺ from internal stores in coupling of Ca_v1.3/DHPi channels to GLP-1 potentiation of GSIS, we examined whether glucose or GLP-1 can stimulate PLC activation and hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) to produce IP₃. As a proxy for IP₃, we measured the accumulation of IP₁, a metabolite of IP₃ that is stable in the presence of Li⁺. Stimulation of INS-1 cells with 500 μ M carbachol, an agonist at muscarinic acetylcholine receptors, in the absence of glucose, resulted in a modest, but significant increase in IP₁ that was completely blocked by 100 μ M atropine. The PLC inhibitor U73122 (10 μ M) not only blocked carbachol-stimulated IP₁ accumulation, but reduced IP₁ accumulation to a level significantly below basal, suggesting that PLC is chronically active in INS-1 cells in the absence of glucose (Fig. 5A). When INS-1 cells were stimulated with 7.5 mM glucose alone, IP₁ formation was stimulated significantly above basal levels, and this stimulation was completely blocked by the addition of 10 μ M nifedipine. As observed during inhibition of carbachol-stimulated IP₁ accumulation, glucose-stimulated IP₁ accumulation was inhibited by U73122 to a level below basal. In addition, 50 nM GLP-1 alone did not significantly affect IP₁ levels in INS-1 cells in the absence of glucose (Fig. 5A). Furthermore, 50 nM GLP-1 did not potentiate IP₁ accumulation stimulated by glucose (data not shown).

Fig. 4. Intact stores of intracellular Ca^{2+} are required for GLP-1-potentiation of GSIS in INS-1 cells and $\text{Ca}_v1.3/\text{DHPi}$ cells. **A**, 50 nM GLP-1 potentiation of 7.5 mM GSIS in INS-1 cells was significantly attenuated by 1 μM thapsigargin (Tg), 0.5 μM ryanodine (Ry), and 50 μM 2-APB. **B**, 10 μM nifedipine-resistant GLP-1 potentiation of GSIS is not inhibited by 1 μM Tg or 50 μM 2-APB, but is slightly inhibited by 0.5 μM Ry. **C**, 10 μM nifedipine-resistant GLP-1 potentiation of GSIS in $\text{Ca}_v1.3/\text{DHPi}$ is significantly inhibited by 0.5 μM Ry, 1 μM Tg, and 50 μM 2-APB. Data shown are mean $\pm$ S.E. ($n = 5-41$). ***, $P < 0.001$; **, $P < 0.01$ compared with control.

Because we found that 7.5 mM glucose significantly stimulates IP_1 accumulation in a nifedipine-sensitive manner, we examined whether inhibition of PLC with U73122 could inhibit GLP-1 potentiation of GSIS. Figure 5B shows that neither U73122 nor the inactive analog U73343 inhibited potentiation of 7.5 mM GSIS by 50 nM GLP-1. Finally, we examined the role of PIP_2 metabolism in GLP-1 potentiation of GSIS in INS-1 cells by sequestering PIP_2 with 1.5 mM neomycin (Fujiwara et al., 2005). Although pretreatment with neomycin did not significantly affect 7.5 mM GSIS, it significantly inhibited potentiation of 7.5 mM GSIS by 50 nM GLP-1 (Fig. 5C). Thus, glucose-stimulated IP_3 formation seems not to be required for GLP-1 potentiation of GSIS, but PIP_2 may be playing another role.

One possible link between PIP_2 and GLP-1 potentiation of insulin secretion might be the activation of phosphatidylinositol 3-kinase (PI-3K). PI-3K is implicated in modulation of GSIS (Li et al., 2006). Therefore, we examined the possibility that the preferential coupling of $\text{Ca}_v1.3/\text{DHPi}$ to GLP-1 potentiation of GSIS might involve PI-3K activity. In INS-1

cells, 1 μM wortmannin, an inhibitor of PI-3K, significantly, but incompletely, inhibited GLP-1 potentiated GSIS (Fig. 6A). ERK1/2 is a potential downstream effector of PI-3K, and inhibition of ERK1/2 activity is also reported to decrease GSIS in MIN6 cells and rat islets (Longuet et al., 2005). Therefore, we measured GLP-1 potentiation of GSIS in INS-1, $\text{Ca}_v1.2/\text{DHPi}$, and $\text{Ca}_v1.3/\text{DHPi}$ cells in the presence of the MEK inhibitor PD98059. 10 μM PD98059 completely

Fig. 5. PIP_2 , but not phospholipase C, is involved in GLP-1 potentiation of GSIS in INS-1 cells. **A**, carbachol (500 μM) and glucose (7.5 mM) both stimulate the accumulation of IP_1 in INS-1 cells. The stimulation of IP_1 accumulation by carbachol is blocked by either 100 μM atropine or 10 μM U73122. The stimulation of IP_1 accumulation by glucose was blocked by either 10 μM nifedipine or 10 μM U73122. GLP-1 (50 nM) alone did not stimulate IP_1 accumulation in INS-1 cells. **B**, inhibition of phospholipase C does not significantly reduce GLP-1 potentiation of GSIS in INS-1 cells. Neither 10 μM U73122 nor 10 μM U73343 (the inactive analog of U73122) affected GSIS or its potentiation by 50 nM GLP-1. **C**, preincubation of INS-1 cells with 1.5 mM neomycin markedly inhibits 50 nM GLP-1 potentiation of 7.5 mM GSIS. Data shown are mean $\pm$ S.E. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$ compared with basal; \$\$\$, $P < 0.001$; \$\$, $P < 0.01$ for the indicated comparison.

Fig. 6. Inhibition of GLP-1 potentiation of GSIS by MEK and PI-3K. A, in INS-1 cells, both 10 μ M PD98059 (PD; MEK inhibitor) and 1 μ M wortmannin (WORT; PI-3K inhibitor) significantly inhibit potentiation of 7.5 mM GSIS by 50 nM GLP-1. B, in Ca_v1.2/DHPi cells in the presence of 10 μ M nifedipine (Nif), neither PD nor WORT significantly affects GLP-1 potentiation of GSIS. C, in Ca_v1.3/DHPi cells in the presence of 10 mM nifedipine both PD and WORT significantly inhibit GLP-1 potentiation of GSIS. Data are shown as mean $\pm$ S.E. ($n = 3$). ***, $P < 0.001$; **, $P < 0.01$ compared with KRBH; \$\$\$, $P < 0.001$ for the indicated comparison.

inhibited GLP-1-potentiated GSIS in INS-1 cells (Fig. 6A). In contrast, neither 1 μ M wortmannin nor 10 μ M PD98059 inhibited nifedipine-resistant GLP-1 potentiation of GSIS in Ca_v1.2/DHPi cells (Fig. 6B). However, in Ca_v1.3/DHPi cells, both 1 μ M wortmannin and 10 μ M PD98059 significantly, but incompletely, inhibited nifedipine-resistant GLP-1 potentiation of GSIS (Fig. 6C). Thus, potentiation of GSIS by GLP-1 mediated by Ca_v1.2/DHPi channels is not sensitive to inhibition by wortmannin or PD98059, but potentiation of GSIS by GLP-1 mediated by Ca_v1.3/DHPi was inhibited by both compounds, as it was in untransfected INS-1 cells.

Discussion

Ca_v1.3/DHPi Is Preferentially Coupled to GLP-1 Potentiation of GSIS in INS-1 Cells. GLP-1 robustly potentiates glucose-stimulated insulin secretion in the rat insulinoma cell line INS-1, in a manner that requires Ca²⁺ influx via L-VGCC. Because INS-1 cells express two distinct L-VGCC subtypes, Ca_v1.2 and Ca_v1.3, we sought to elucidate the role of Ca_v1.2 and Ca_v1.3 in mediating GLP-1-potentiated GSIS in these cells by use of dihydropyridine-resistant mutants of these two channels. We have previously used this approach to demonstrate a preferential role for Ca_v1.3 in mediating GSIS (Liu et al., 2003) and glucose-stimulated [Ca²⁺]_i oscillations (Liu et al., 2004). In the present study, we find that both Ca_v1.2 and Ca_v1.3 can couple to GLP-1 potentiation of GSIS, but to different degrees. This conclusion is based on the observation that insulin secretion in response to GLP-1 and glucose is not different in the presence or absence of nifedipine in Ca_v1.3/DHPi cells, but is significantly inhibited by nifedipine in Ca_v1.2/DHPi cells. In contrast, potentiation of GSIS by GIP was completely resistant to nifedipine in both Ca_v1.2/DHPi cells and Ca_v1.3/DHPi cells, suggesting that either Ca_v1.2 or Ca_v1.3 in isolation is capable of fully mediating the potentiation of GSIS by GIP.

Differential Intracellular Signaling Pathways Contribute to Coupling of Ca_v1.2 and Ca_v1.3 to GLP-1 Potentiation of GSIS. Activation of the GLP-1 receptor initiates multiple signaling pathways including cAMP, PKC, and the release of Ca²⁺ from internal stores (MacDonald et al., 2002). We therefore examined the possibility that differential coupling of Ca_v1.2 and Ca_v1.3 to GLP-1 potentiation of GSIS might reflect the sum of distinct sets of intracellular

signaling pathways modulating either channel activity or events downstream of Ca²⁺ influx. The inhibition of GLP-1 potentiation of GSIS by Rp-cAMP in INS-1 cells and Ca_v1.3/DHPi cells in the presence of nifedipine suggest that PKA plays a prominent role in secretion stimulated by Ca²⁺ influx via Ca_v1.3. In addition, the inhibition of GLP-1 potentiation of GSIS by Bis in INS-1 cells and Ca_v1.3/DHPi cells in the presence of nifedipine suggests that PKC also plays a role in this process when Ca²⁺ influx occurs via Ca_v1.3. Because the effects of both PKA and PKC inhibitors were not additive, it seems that both PKA and PKC are required for, but not independently sufficient to mediate, GLP-1 potentiation of GSIS in either INS-1 cells or Ca_v1.3/DHPi cells in the presence of nifedipine.

In contrast to INS-1 cells and Ca_v1.3/DHPi cells, potentiation of GSIS in Ca_v1.2/DHPi cells in the presence of nifedipine is not significantly inhibited by either Rp-cAMPs or Bis, or the combination of the two. GLP-1 potentiation of GSIS mediated by Ca_v1.2 seems to involve cAMP, because IBMX, a broadly specific inhibitor of phosphodiesterases, strongly enhances it. It is likely that this cAMP-dependent, but PKA-independent effect of GLP-1 is mediated by EPAC2. This conclusion is consistent with our previous finding that 8-Br-cAMP potentiates GSIS mediated by either Ca_v1.2 or Ca_v1.3 channels, but that the EPAC2-specific cAMP analog 8-pCPT-2'-O-Me-cAMP independently potentiates GSIS mediated by Ca_v1.2 but not Ca_v1.3 (Liu et al., 2006). Biochemical evidence also supports the specific relationship between EPAC2 and Ca_v1.2. The intracellular loop between homologous domains II and III of Ca_v1.2, but not Ca_v1.3, binds to the scaffolding proteins Rab3-interacting molecule 2 (RIM2) and Piccolo in vitro (Shibasaki et al., 2004), and immunoprecipitates full-length RIM2 from INS-1 cells (Jacobo et al., 2009). RIM2 and Piccolo are both involved in EPAC2 mediated, cAMP-dependent potentiation of GSIS (Fujimoto et al., 2002). Experiments to test the role of EPAC2 in Ca_v1.2-mediated GLP-1 potentiation of GSIS will require manipulation of EPAC2 expression, because no specific inhibitors of EPAC2 activity are currently available.

Inhibition of PKC and disruption of release of Ca²⁺ from internal stores also differentially affected GLP-1 potentiation of GSIS mediated by Ca_v1.2 or Ca_v1.3. PKC inhibition and unloading of ER Ca²⁺ stores both markedly inhibited Ca_v1.3

coupling to GLP-1 potentiation of GSIS. Apparently, release of Ca^{2+} from the ER via IP_3R contributes to this process, as 2-APB, an IP_3R antagonist, also markedly inhibits GLP-1 potentiation of GSIS mediated by $\text{Ca}_v1.3/\text{DHPi}$ channels. In contrast, among the three pharmacological inhibitors of ER Ca^{2+} release examined, only ryanodine significantly inhibited GLP-1 potentiation of GSIS mediated by $\text{Ca}_v1.2/\text{DHPi}$ channels, albeit to a lesser degree than in $\text{Ca}_v1.3/\text{DHPi}$ cells. Thus, release of internal stores of Ca^{2+} does not seem to play a major role in $\text{Ca}_v1.2/\text{DHPi}$ -mediated GLP-1 potentiation of GSIS. This result is in contrast to a previous report that EPAC2 mediates release of Ca^{2+} from internal stores (Kang et al., 2005). However, EPAC2 is also reported to augment GSIS by increasing the insulin granule density near the plasma membrane via a Rap1-dependent mechanism (Shibasaki et al., 2007). Thus, our finding that disruption of intracellular Ca^{2+} stores does not inhibit GLP-1 potentiation of GSIS by GLP-1 mediated by $\text{Ca}_v1.2/\text{DHPi}$ is not inconsistent with a role for EPAC2 in this process.

What might account for these differences in $\text{Ca}_v1.2/\text{DHPi}$ - and $\text{Ca}_v1.3/\text{DHPi}$ -mediated GLP-1 potentiation of GSIS? Glucose-induced Ca^{2+} influx across the plasma membrane in β -cells activates PLC and PIP_2 hydrolysis, which is coupled to activation of PKC (Suzuki et al., 2006). We examined the possibility that potentiation of GSIS mediated by $\text{Ca}_v1.3$ preferentially involves PIP_2 -dependent pathways. However, we found that, whereas 7.5 mM glucose stimulated IP_1 accumulation in a nifedipine-sensitive manner, addition of GLP-1 did not further increase IP_1 accumulation, nor did inhibition of PLC with U73122 significantly inhibit either GSIS or potentiation of GSIS by GLP-1. Nonetheless, sequestration of PIP_2 with 1.5 mM neomycin inhibited potentiation of GSIS by GLP-1 in INS-1 cells, suggesting a role for PIP_2 . Mounting evidence suggests that PI-3K plays a key role in GSIS and the actions of GLP-1. GSIS is markedly impaired in PI-3K γ knockout mice (Li et al., 2006). Exendin 4, an agonist at the GLP-1 receptor, inhibits voltage-dependent K^+ currents in a cAMP-, PKC-, and PI-3K-dependent manner in rat β -cells (MacDonald et al., 2003). Indeed, wortmannin, an inhibitor of PI-3K, markedly inhibited GLP-1 potentiation of GSIS in INS-1 cells and nifedipine-resistant GLP-1 potentiation of GSIS in $\text{Ca}_v1.3/\text{DHPi}$ cells but not $\text{Ca}_v1.2/\text{DHPi}$ cells. This suggests that activation of PI-3K can modulate $\text{Ca}_v1.3$ activity, or a process that requires influx via $\text{Ca}_v1.3$ channels, preferentially over $\text{Ca}_v1.2$ channel activity or $\text{Ca}_v1.2$ -dependent processes in pancreatic β -cells.

Our results suggest that the preferential coupling of $\text{Ca}_v1.3$ to GLP-1 potentiation of GSIS may involve the activation of the MEK/ERK1/2 pathway. Activation of the proliferative MEK/ERK1/2 pathway by GLP-1 and glucose in cultured pancreatic β -cell lines requires activation of PI-3K and PLC (Buteau et al., 2003), intact stores of intracellular Ca^{2+} (Arnette et al., 2003), and Ca^{2+} influx via L-type Ca^{2+} channels (Gomez et al., 2002). Our results are consistent with a report that GSIS in MIN6 cells is markedly inhibited by both PD98059 and siRNA-mediated knockdown of ERK1 and ERK2 (Longuet et al., 2005). Furthermore, Longuet et al. found that ERK stimulation of insulin secretion is mediated by a mechanism that involves phosphorylation of synapsin I. Synapsin I is also phosphorylated by p21-activated kinases in a CDC42-dependent manner in response to bradykinin receptor stimulation (Sakurada et al., 2002). CDC42 is a

small Rho family GTPase that is required for second-phase GSIS (Wang et al., 2007). The GTPase activity of CDC42 is enhanced by CdGAP, and the activity of CdGAP is decreased upon phosphorylation by ERK1/2 (Tcherkezian et al., 2005). Thus, ERK1/2 may modulate insulin secretion via phosphorylation of synapsin I or by regulation of CDC42 activity. Because activation of synapsin I is associated with vesicle movement from the reserve pool to the readily releasable pool (Fdez and Hilfiker, 2006), and CDC42 is associated with the second phase of insulin secretion (Wang et al., 2007), our results led us to speculate that, in INS-1 cells in the presence of GLP-1, $\text{Ca}_v1.3$ channels may facilitate the later, sustained phase of GSIS via activation of the MEK/ERK1/2 pathway. On the other hand, GLP-1 potentiation of GSIS mediated by $\text{Ca}_v1.2$ channels does not require activation of the MEK/ERK1/2 pathway. Together with our previous studies showing that $\text{Ca}_v1.2$ does not mediate GSIS (Liu et al., 2003) but can mediate cAMP potentiation of GSIS (Liu et al., 2006) in INS-1 cells, this observation suggests that perhaps $\text{Ca}_v1.2$ may be recruited to augment the early phase of secretion by GLP-1 receptor activation.

In conclusion, we have shown that both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ couple to GLP-1 potentiation of GSIS in INS-1 cells, but via signaling pathways. GLP-1 potentiation of GSIS mediated by $\text{Ca}_v1.3$ depends on intact internal stores of Ca^{2+} , PKC, PKA, and PI-3K activity, and the activation of the MEK/ERK1/2 pathway. In contrast, none of these signaling pathways seems to be required for $\text{Ca}_v1.2$ -mediated GLP-1 potentiation of GSIS. It will be of interest to determine whether $\text{Ca}_v1.3$ does, in fact, preferentially mediate the activation of the MEK/ERK1/2 pathway by glucose and GLP-1 in β -cells.

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