Calbindin-D$_{28k}$ Controls [Ca$^{2+}$]$_i$ and Insulin Release

EVIDENCE OBTAINED FROM CALBINDIN-D$_{28k}$ KNOCKOUT MICE AND β CELL LINES

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The role of the calcium-binding protein, calbindin-D$_{28k}$, in potassium/depolarization-stimulated increases in the cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) and insulin release was investigated in pancreatic islets from calbindin-D$_{28k}$ nullmutant mice (knockouts; KO) or wild type mice and β cell lines stably transfected and overexpressing calbindin. Using single islets from KO mice and stimulation with 45 mM KCl, the peak of [Ca$^{2+}$]$_i$ was 3.5-fold greater in islets from KO mice compared with wild type islets (p < 0.01) and [Ca$^{2+}$]$_i$ remained higher during the plateau phase. In addition to the increase in [Ca$^{2+}$]$_i$, in response to KCl there was also a significant increase in insulin release in islets isolated from KO mice. Evidence for modulation by calbindin of [Ca$^{2+}$]$_i$ and insulin release was also noted using β cell lines. Rat calbindin was stably expressed in βTC-3 and βHC-13 cells. In response to depolarizing concentrations of K$^+$, insulin release was decreased by 45–47% in calbindin expressing βTC cells and was decreased by 70–80% in calbindin expressing βHC cells compared with insulin release from vector transfected βTC or βHC cells (p < 0.01). In addition, the K$^+$-stimulated intracellular calcium peak was markedly inhibited in calbindin expressing βHC cells compared with vector transfected βHC cells (225 nM versus 1,100 nM, respectively). Buffering of the depolarization-induced rise in [Ca$^{2+}$]$_i$ was also observed in calbindin expressing βTC cells. In summary, our findings, using both isolated islets from calbindin-D$_{28k}$ KO mice and β cell lines, establish a role for calbindin in the modulation of depolarization-stimulated insulin release and suggest that calbindin can control the rate of insulin release via regulation of [Ca$^{2+}$]$_i$.

Calbindin-D$_{28k}$ is a 28,000 $M_r$ calcium-binding protein initially identified in avian intestine and was the first known target of vitamin D action (1). Calbindin has since been reported in many other tissues including kidney, bone, and tissues that are not primary regulators of serum calcium such as brain and pancreas (2–4). This calcium-binding protein has been conserved during evolution and is regulated by a number of different hormones and factors (3, 4). Calbindin-D$_{28k}$, a predominantly cytosolic protein, is a member of a family of high affinity calcium-binding proteins that includes calmodulin, S100 protein, and parvalbumin (5). It has been suggested that the role of calbindin in kidney and intestine is to facilitate transcellular calcium diffusion (6, 7). In brain, calbindin is not vitamin D-dependent and its proposed function is to buffer calcium, resulting in protection against calcium-mediated neurotoxicity (8, 9).

In 1979 the discovery in the pancreas of a high affinity receptor for the hormonally active form of vitamin D, 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), was the first demonstration of a nonclassical target tissue to contain vitamin D receptors (10). Further autoradiographic and immunohistochemical analyses have shown that vitamin D receptors and calbindin-D$_{28k}$ are both localized in the β cell (11–13). Although these studies and others (14–17) established a link between the pancreatic β cell and the vitamin D endocrine system and although the importance of calcium in insulin secretion is well known, there is still little information available concerning the exact mechanism whereby vitamin D may affect β cell function. It has been suggested that the role of vitamin D in calcium metabolism of the β cell may involve a genomic effect of 1,25(OH)$_2$D$_3$, including the production of calbindin.

Although isolated islets and perfused pancreas from vitamin D-deficient animals have previously been used to study the effects of 1,25(OH)$_2$D$_3$ on β cell function (14–17), recently we reported that the β cell line RIN1046-38 contains both calbindin and receptors for 1,25(OH)$_2$D$_3$ and suggested that β cell lines may provide a useful in vitro system for studying the effects of the vitamin D endocrine system on β cell function (18, 19). Although interesting data have been generated in numerous studies using RIN cells, the RIN cell line may not be the best model because these cells have little or no response to glucose and the insulin content of these cells is only approximately 0.1% of the insulin content found in the normal β cell.

In this study, to understand the role of calbindin-D$_{28k}$ in the pancreatic β cells, calbindin was transfected and overexpressed in βHC and βTC cells, pancreatic β cells that secrete insulin in a regulated manner and at levels more comparable with those of normal β cells. Both cell lines are derived from transgenic mice that express the SV40 T-antigen in β cells under the
control of the insulin gene regulatory region (20–22). In addition, calbindin-D$_{28k}$ null mutant (or knockout) mice were also used because they provide a good model in which to examine the effect of complete ablation of calbindin in the pancreatic islet on insulin release. This study, which is the first to address the role of calbindin in the β cell using both islets and β cell lines, suggests that calbindin has an important role in controlling depolarization-induced increases in intracellular calcium and therefore insulin release from the pancreatic β cell.

**EXPERIMENTAL PROCEDURES**

**Materials**—1-Isobutyl-3-methylxanthine (IBMX), $^{125}$I-0-tetra-decanoylphorbol-13-acetate (TPA), glucose, bovine serum albumin, proteinase K, and acrylamide were obtained from Sigma. Kodak BioMAX MR film, $[^{32}P]$CTP (1 mCi/ml), and Renaissance chemiluminescence Western blot kits were purchased from NEN Life Science Products. Tissue culture media for cell culture, antibiotics, agarose, LipofectAMINE, Taq polymerase, and all restriction enzymes were obtained from Life Technologies, Inc. Sera for culture and G418 were purchased from Gemini (Calabasas, CA). Collagenase was from Roche Molecular Biochemicals. Bradford protein reagent and nitrocellulose membranes were purchased from Bio-Rad. Primers for PCR were obtained from Operon (Alameda, CA). The Gene Amp PCR core kit was from Perkin-Elmer (Foster City, CA). DNA purification kits were obtained from Qiagen (Chatsworth, CA). Antibody against rat calbindin-D$_{28k}$ was generated and characterized as described previously (23). All chemicals and reagents were used at the highest grade.

Calbindin-D$_{28k}$ Null Mutant (Knockout, KO) Mice—The calbindin-D$_{28k}$ KO mice were generated in the laboratory of Dr. Michael Meyer as described previously (24). For these studies colonies of calbindin-D$_{28k}$ null mutant and wild type mice (C57BL6) were maintained on Purina mouse chow and water ad libitum in the barrier room of the Research Animal Facility at New Jersey Medical School. Genotypes were determined by Southern blot analysis of PCR using genomic DNA obtained from the tails of mice at 3 weeks of age. For Southern analysis (25), DNA was digested with HindIII, and an external probe that recognizes a 7.8-kilobase wild type band and 5.5-kilobase mutant band was used (24). PCR was performed to determine the genotypes using the primers Cs3 (common primer; sequence 5'-GCAAGTAACTAATG-TGCAGCGGCTAGTTT-3') and KO mice (fed, 105.7 ± 0.5 mg/dl) versus 95.6 ± 0.5 mg/dl (p < 0.05). Additionally, there were no differences between WT and KO mice in the levels of serum insulin under these conditions. There were also no differences in blood pressure between WT and KO mice. The phenotype expressed in the calbindin KO mice is characteristic of the normocalcemic phenotype described previously (23).

**Insulin and Calcium Assays**—Insulin was determined by radioimmunoassay using rat insulin as a standard (rat Insulin$^{125}$I kit, Incstar, St. Louis, MO) or pBSR-CB28 (vector containing calbindin-D$_{28k}$ cDNA isolated by PCR from cDNA prepared from rat renal distal tubular mRNA (28) to create an expression plasmid designated pBSR-CB28), were mixed with 50 μl LipofectAMINE, added to cells in Opti- mem (Life Technologies, Inc.) and 2% fetal bovine serum and incubated for 20 h at 37 °C. Normal growth medium was added the next day. Three days after transfection, selection began with increasing amounts of G418 to a final concentration of 800 μg/ml. After 8 weeks of G418 selection, colonies were picked under sterile conditions and grown in 24-well plates.

**Western Blot Analysis and Calbindin-D$_{28k}$ Radioimmunoassay**—Cell extracts of various clones were prepared by short (<5 s) burst sonication in phosphate-buffered saline (0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4) and 5 mM phenylmethylsulfonyl fluoride. The supernatant generated after centrifugation at 14,000 g for 15 min at 4 °C was used for protein estimation with the Bradford method (29). 10 μg of protein was analyzed by Western blot analysis using a Chemiluminescence Western blotting kit (NEN Life Science Products). Calbindin protein levels were determined by radioimmunoassay using antisera against rat renal calbindin and purified rat renal calbindin-D$_{28k}$ as a standard as described previously (30).

**Insulin Release: Perfusion and Static Incubation**—Pancreatic islets from 2–3-month-old mice (KO and WT) were isolated by collagenase dispersion (31) and handpicked under a stereomicroscope. Insulin release from islets was measured under perfusion conditions. The dynamics of insulin release from pancreatic islets were examined using a perfusion system as described previously (32) with minor modifications. Twenty size-matched islets were placed in each 0.7-ml perfusion chamber and perfused with glucose-free Kreb's-Ringer bicarbonate (KRB) buffer (129 mM NaCl, 5 mM NaHCO$_3$, 4.8 mM KCl, 1.2 mM KH$_2$PO$_4$, 2.0 mM CaCl$_2$, 1.2 mM MgSO$_4$, 10 mM HEPES, 0.1% bovine serum albumin, pH 7.4) at the rate 0.8 ml/min at 37 °C. Experiments were started after a 60-min perfusion equilibration period. Perifusate samples for insulin radioimmunoassay were collected every 1 or 2 min. Data were obtained from five chambers for each group of three independent experiments. Insulin release from βTC-3 or 8HT-13 cells was measured by static incubation as described previously (20, 33).

**Measurement of [Ca$^{2+}$]$_{i}$**—[Ca$^{2+}$]$_{i}$ was measured using isolated islets as described previously (34) with some modification. Islets were loaded with 5 μM indo-1 acetoxyethyl ester (Molecular Probes, Eugene, OR) in KRB buffer containing 250 μM sulfinpyrazone (to inhibit transport of fura-2 into the cell) and 20 mM glucose for 90–120 min. After loading twice in fresh KRB buffer, the islets were kept in buffer plus 20 μM glucose and 250 μM sulfinpyrazone at 37 °C until used in experiments. For measurement of [Ca$^{2+}$]$_{i}$, individual islets were placed on a glass coverslip in the Teflon chamber of a Narishige micrincubation system mounted on the stage of a Nikon Diaphot 200 inverted epiilluminescence microscope. The islets were maintained at 37 °C on the microscope stage. Each islet was incubated in glucose-free KRB buffer for at least 10 min prior to stimulation. KCl (45 mM) was added to the incubation chamber by pipette. The single islets were excited at 360 nm using a 75 watt xenon lamp and emission monitored at 405 and 485 nm using a photomultiplier (Photon Technology International). The ratio of detected light (405 nm/485 nm) was calculated and displayed using FELIX software (Photon Technology International) and a Dell Optiplex 430L computer. Changes in [Ca$^{2+}$], were represented by changes in the fluorescence ratio (405 nm/485 nm). All calcium tracings were obtained for single islets. All fluometry experiments were performed under static incubation.

[Ca$^{2+}$]$_{i}$, in βTC-3 and 8HT-3 cells were measured in cell suspensions as described previously (33). Cells dispersed by collagenase were cultured in a KRB buffer containing 1 μM fura-2 acetoxyethyl ester (Molecular Probes, Eugene, OR) and 250 μM sulfinpyrazone at a concentration of 2 × 10$^{-6}$ cells/ml and then loaded with fura2 by incubation at 37 °C for 30 min with continuous shaking. Sulfinpyrazone was added to diminish the transport of fura-2 out of the cells and thus increase the precision of the fluorescence measurements. After loading, the cells were washed and resuspended...
in KRB buffer containing 250 µM sulfipyrazone; 3 ml of the suspension was placed in quartz cuvettes with continuous stirring; and the temperature was maintained at 37 °C. Calcium was measured using a spectrofluorimeter (Perkin-Elmer LS-5) at an excitation wavelength of 340 nm and an emission wavelength of 510 nm. [Ca\(^{2+}\)] was calculated by the use of values of auto- and extracellular fura-2 emitted fluorescence as described previously (35).

**Statistical Analysis**—Results are presented as the means ± S.E. Data were analyzed with Student’s t test or one-way analysis of variance. The post-hoc analysis used with one-way analysis of variance was Tukey’s method.

**RESULTS**

**Calbindin-D\(_{28k}\) KO Mice**—To verify the nullmutation, tissues from WT and KO mice were analyzed by Western blotting and immunocytochemistry for the presence of calbindin-D\(_{28k}\) (Fig. 1). Western analysis of extracts of kidney, pancreas, and brain (cortex and cerebellum) demonstrated that the immunoreactivity detected in WT mice by calbindin-D\(_{28k}\) antiserum was absent in tissues from calbindin KO mice (Fig. 1, left panel). In addition, immunocytochemistry indicated the presence of calbindin in the distal tubule of the kidney (Fig. 1, middle panel A), in the pancreatic islet (Fig. 1, right panel A), and in some cells but not others throughout the brain (not shown) in WT mice and the complete absence of calbindin in the kidney (Fig. 1, middle panel B), pancreas (Fig. 1, right panel B), and brain (not shown) in KO mice. In the WT mouse pancreas, immunostaining was cytoplasmic and diffuse (Fig. 1, right panel A), consistent with results of calbindin immunostaining in human and rat pancreas (13), and was specific for the pancreatic islet. Acinar tissue did not show any immunostaining.

Because previous studies in calbindin-D\(_{28k}\) KO mice indicated that calbindin in the nervous system has a role in modulation of depolarization-induced calcium transients (24), in this study we focused on the role of calbindin in the β cell in membrane depolarization-stimulated insulin release. Studies using perfused isolated islets showed that when voltage-gated Ca\(^{2+}\) channels were opened by depolarizing the β cell membrane by treatment with KRB containing 45 mM KCl, islets from both WT and KO mice showed increased insulin release with a sharp initial phase that peaked by 3 min after stimulation (Fig. 2). From the peak level, insulin release decreased, despite the continuing presence of high K\(^{+}\), to reach a sustained phase after 10 min. Insulin release declined gradually over time. The peak insulin release was of similar magnitude for WT and KO islets. However a significant potentiation in the sustained phase of KCl-induced insulin release was observed in islets isolated from calbindin KO mice as evident from the time for insulin release to decline to 25% of the peak release (45 ± 6 min for KO islets versus 29 ± 3 min for WT islets, p < 0.05).

To determine whether the increase in potassium-stimulated insulin release in the islets isolated from KO mice may be due, at least in part, to an increase in [Ca\(^{2+}\)], because of lack of Ca\(^{2+}\) buffering by calbindin, indo-1 was used to measure K\(^{+}\)-induced rises in [Ca\(^{2+}\)] in single pancreatic islets. In islets from both wild type and KO mice 45 mM KCl caused an immediate, significant increase in [Ca\(^{2+}\)], which then decreased over several minutes to reach a plateau concentration that remained elevated over the basal [Ca\(^{2+}\)], for the remainder of the time (Fig. 3). The peak [Ca\(^{2+}\)] was markedly increased in islets from KO as compared with those from WT mice (3.5-fold greater; p < 0.01). The plateau [Ca\(^{2+}\)] was also significantly increased. These findings show that calbindin acts as a modulator of induced calcium transients in the β cell and that calbindin may have a role in modulating sustained insulin release via regulation of [Ca\(^{2+}\)].

**β Cell Lines**—In addition to the studies with isolated islets from knockout and wild type mice, the role of calbindin in insulin secretion and modulation of [Ca\(^{2+}\)], transients was also examined using transfected β cell lines overexpressing calbindin. βTC cells were transfected with the pBSR\(_{α}\) vector or pBSR\(_{α}\)-CB28 and selected with G418. After transfection, hundreds of G418-resistant colonies were initially selected. Pooled colonies were screened by Western analysis (Fig. 4) and radiomunooassay. The clones that expressed the highest levels of calbindin were designated βTC-CaBP47 (expressing 515 ± 37 ng/mg protein) and βTC-CaBP54 (expressing 300 ± 17 ng/mg protein). Another G418-resistant βTC-3 clone designated βTC-CaBP14 (not shown), a low calbindin expressing clone (barely detectable levels of calbindin using Western blot analysis and levels below the limits of detection using the calbindin radiomunooassay (30), similar to vector transfected cells), was used as a control in addition to the vector transfected clone vector 8. The responses of βTC-3 cells to a variety of secretory stimuli have previously been characterized and shown to resemble the effects of those secretagogues in normal islets (20). Using the same treatment protocols previously reported for stimulation of insulin release from βTC-3 cells (20), we examined the effect of calbindin overexpression on insulin release stimulated by depolarizing concentrations of potassium as well as by TPA (a tumor promoting phorbol ester and activator of protein kinase C whose actions on the β cell have been linked with membrane depolarization, 35). In response to 45 mM potassium plus 0.5 mM IBMX insulin release was stimulated 26-fold over basal levels in the vector 8 transfected clones (Fig. 5), similar to the results observed previously using nontrans-
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Fig. 3. Intracellular calcium levels in islets isolated from WT and calbindin-D<sub>28k</sub> KO mice after depolarization with K<sup>+</sup>. A, representative tracing of KCl-induced changes in [Ca<sup>2+</sup>]<sub>i</sub>, levels in single islets loaded with indo-1. After a preincubation in 4.8 mM KCl (basal conditions) the KCl was increased to 45 mM (indicated by arrows). Basal [Ca<sup>2+</sup>]<sub>i</sub>, levels in islets from KO and WT mice were not significantly different (KO: 111 ± 17; WT: 125 ± 19 nM, n = 10; p > 0.5). B, graphical representation of the changes in [Ca<sup>2+</sup>]<sub>i</sub>, shown in A, from basal to peak and during the plateau phase after treatment with 45 mM KCl. There was a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> (basal to peak and in the plateau phase) in response to 45 mM KCl in islets isolated from calbindin-D<sub>28k</sub> KO mice compared with those from WT mice (p < 0.01).

Fig. 4. Western blot analysis of the expression of calbindin-D<sub>28k</sub> in βTC-3 cells. Proteins in 50 μg of cytoplasmic extract from βTC-3 cells transfected with pBSRr-CR28 (βTC-CaBP47 and βTC-CaBP54) and βTC-3 cells transfected with vector alone (βTC-vector 8) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. Cytoplasmic extract from rat kidney was used as a positive control. Calbindin-D<sub>28k</sub> protein was visualized by a chemiluminescent peroxidase method after incubation with a polyclonal rat calbindin-D<sub>28k</sub> antibody (30).

Fig. 5. Insulin release in response to secretagogues in βTC-3 cells overexpressing calbindin-D<sub>28k</sub>. Insulin release was measured from vector-transfected (V8) and calbindin-transfected (CaBP14, CaBP47, and CaBP54) βTC-3 cells. Cells were plated at a density of 4 × 10<sup>5</sup> cells/well in a 24-well dish, and insulin release was measured after a 1-h preincubation in control media and a 2-h incubation in the specific treatment media. KCl, 45 mM KCl + 0.5 mM IBMX; TPA, 1 μM TPA. Insulin release is reported as fold increase over basal. Under basal conditions insulin release (as percentages of insulin content) ranged from 2.5–2.7% among the different clones. Data represent the means ± S.E. for n = 3–4 experiments. In each experiment for each clone and specific treatment four replicates were analyzed at two different dilutions (** p < 0.01 compared with vector transfected).

Fig. 6. Increases in intracellular calcium levels in βTC-3 cells in response to depolarization with K<sup>+</sup> are attenuated by calbindin-D<sub>28k</sub>. Clones vector 8, CaBP14, and CaBP54 were treated with 25 mM KCl, and the change in [Ca<sup>2+</sup>]<sub>i</sub> from time 0 to time 0.2 min (basal to peak) is represented graphically as the average change in [Ca<sup>2+</sup>]<sub>i</sub>, in nM. Data are shown as the means ± S.E. (n = 3; p < 0.01 for CaBP54 versus vector 8). No significant change was found in the plateau phase (not shown).

In response to glucose (16.7 mM) preliminary results indicate that significant changes in insulin secretion in the calbindin overexpressing clones were not observed (data not shown). In addition, Northern analysis indicated that there was no change in insulin mRNA levels in calbindin-overexpressing cells as compared with vector-transfected cells in response to treatment with glucose (not shown). Under basal conditions changes in insulin mRNA were also not observed between vector-transfected and calbindin-transfected cells (not shown). This differs from calbindin overexpressing high passage RIN 1046-38 cells, which showed a marked increase in insulin mRNA under basal condition when compared with vector transfected cells (19). High passage RIN cells differ from βTC cells because they are not glucose-responsive, and the insulin content of RIN cells (0.1% of normal islets) is much lower than the insulin content of βTC cells (20–30% of that of normal islets; 20). Thus it is possible that calbindin may affect calcium-mediated regulation of components of the insulin transcription complex in the low insulin synthesizing RIN cells but not in βTC cells.

Because a significant inhibitory effect of calbindin on insulin secretion was observed in response to K<sup>+</sup>-induced depolarization, similar to the studies in isolated islets, we asked whether the effect of calbindin on potassium-stimulated insulin release may similarly be related to regulation of [Ca<sup>2+</sup>], by calbindin. βTC-3 cell clones (vector 8, βTC-CaBP14, and βTC-CaBP54) were treated with a depolarizing concentration of 25 mM KCl (this concentration was found to be optimal for βTC-3 cells to minimize variation in measurements of [Ca<sup>2+</sup>]). Note that the peak change in [Ca<sup>2+</sup>] in response to KCl was significantly lower in the calbindin-expressing clone βTC-CaBP54 compared with vector transfected cells (vector 8; p < 0.01; see Fig. 6). Although the change in [Ca<sup>2+</sup>] in response to KCl was lower in clone βTC-CaBP14 compared with the vector transfected clone, the difference was not significant (p > 0.5). These findings...
clone, designated bindin in islets. Although both the peak and plateau \([\text{Ca}^{2+}]\) in calbindin-overexpressing isolated insulin release (Fig. 8). Calcium rises were also buffered method after incubation with a polyclonal rat calbindin-D 28k antibody to an increase in \([\text{Ca}^{2+}]\). This is compared with a rise to 225 nM for calbindin transfected expressing calbindin (Fig. 7). As shown in Fig. 8 potassium-stimulated insulin release from both vector transfected and \(\beta\)-HC-13 cells was found to markedly impair \(K^+\)-stimulated insulin release (Fig. 8). Calcium rises were also buffered in calbindin-overexpressing \(\beta\)-HC-13 cells in response to \(K^+\) stimulation (Fig. 9). Vector transfected cells (vector 1) showed a rapid increase in \([\text{Ca}^{2+}]_i\) levels immediately after treatment with KCl, from a resting level of 200 nM to a level of 1100 nM. This is compared with a rise to 225 nM for calbindin transfected cells (\(\beta\)-HC-CaBP40) from a resting level of 200 nM. Basal \([\text{Ca}^{2+}]_i\), levels in \(\beta\)-HC cells transfected with vector or overexpressing calbindin (\(\beta\)-HC-CaBP40) were not significantly different. These results agree with the results obtained from isolated islets and \(\beta\)-TC-3 cells and suggest that calbindin has a role in modulating depolarization-induced calcium transients and therefore insulin release from the pancreatic \(\beta\) cell.

In addition to \(\beta\)-TC-3 cells, \(\beta\)-HC-13 cells (22) were also transfected with the pBSRα vector or pBSRα-CB28 and selected. For studies using the \(\beta\)-HC-13 cells the highest calbindin expressing clone, designated \(\beta\)-HC-CaBP40 (expressing 583 ± 90 ng calbindin-D 28k/mg protein) and vector-transfected clone vector 1 were used for all subsequent experiments (Fig. 7). As shown in Fig. 8 potassium-stimulated insulin release from both vector transfected and \(\beta\)-HC-13 cells overexpressing calbindin (\(\beta\)-HC-CaBP40) in a concentration-dependent manner, with half-maximal values between 30 and 40 mM KC for both cell types. Similar to the finding in \(\beta\)-TC-3 cells, overexpression of calbindin in \(\beta\)-HC-13 cells was found to markedly impair \(K^+\)-stimulated insulin release (Fig. 8). Calcium rises were also buffered in calbindin-overexpressing \(\beta\)-HC-13 cells in response to \(K^+\) stimulation (Fig. 9). Vector transfected cells (vector 1) showed a rapid increase in \([\text{Ca}^{2+}]_i\), levels immediately after treatment with KCl, from a resting level of 200 nM to a level of 1100 nM. This is compared with a rise to 225 nM for calbindin transfected cells (\(\beta\)-HC-CaBP40) from a resting level of 200 nM. Basal \([\text{Ca}^{2+}]_i\), levels in \(\beta\)-HC cells transfected with vector or overexpressing calbindin (\(\beta\)-HC-CaBP40) were not significantly different. These results agree with the results obtained from isolated islets and \(\beta\)-TC-3 cells and suggest that calbindin has a role in modulating depolarization-induced calcium transients and therefore insulin release from the pancreatic \(\beta\) cell.

**DISCUSSION**

In this study we found that calbindin acts as a modulator of depolarization-induced calcium transients in the pancreatic \(\beta\) cell and that calbindin has a role in controlling depolarization-induced insulin release via regulation of \([\text{Ca}^{2+}]_i\). The data show that in islets isolated from calbindin-D 28k KO mice, in addition to an increase in \([\text{Ca}^{2+}]_i\), in response to KCl, there is also an increase in sustained insulin release when compared with WT islets. Although both the peak and plateau \([\text{Ca}^{2+}]_i\), in response to KCl are significantly greater in the islets from KO mice, only the sustained phase of KCl-induced insulin release (and not peak insulin release) is significantly greater in islets from KO mice when compared with WT islets (Figs. 2 and 3). The most likely explanation of this finding is that 45 mM KCl increases \([\text{Ca}^{2+}]_i\) in WT islets to a level that is sufficient to maximally induce first phase insulin release. The greater rise in peak \([\text{Ca}^{2+}]_i\), observed in the islets from the KO mice cannot result in a further increase in the first phase insulin release rate, although the sustained phase of insulin release (which may not be maximally induced in the WT islets) is significantly increased. Thus the role of calbindin may be as a modulator of the sustained phase of insulin release via regulation of \([\text{Ca}^{2+}]_i\). A role for calbindin in modulation of insulin release via regulation of \([\text{Ca}^{2+}]_i\), was also noted in the studies using calbindin overexpressing \(\beta\) cells. In response to \(K^+\) stimulation both the \([\text{Ca}^{2+}]_i\), peak as well as insulin release are decreased or inhibited compared with \(\beta\) cells not expressing calbindin (Figs. 5, 6, 8, and 9). Thus the changes noted in insulin release from cells overexpressing calbindin and islets from KO mice as well as the presence of calbindin in the normal \(\beta\) cell strongly suggest that calbindin plays a role in normal insulin secretory physiology. Reduction of \([\text{Ca}^{2+}]_i\), transients evoked by voltage depolarization has previously been observed to be a function of calbindin in neurons (24, 36, 37). It was suggested that impaired motor coordination, which is the phenotype of the calbindin KO mice, may be the result of abnormal cerebellar activity because of altered depolarization-induced calcium transients in the Pur-
kininje cells (24). Calbindin was also reported to play a role in the control of hypothalamic neuroendocrine neuronal firing patterns. Calbindin was introduced into rat supraoptic neurons using the whole cell patch clamp method. Calbindin suppressed Ca\textsuperscript{2+}-dependent depolarization after-potentials, and it was suggested that calbindin, by regulating depolarization-induced potentials, may be involved in the control of hormone secretion from hypothalamic neuroendocrine neurons (38). Thus calbindin appears to act similarly in \(\beta\) cells and neurons. Functional and phenotypic similarities have previously been reported between neurons and pancreatic \(\beta\) cells. For example \(\beta\) cells, similar to neurons, express proteins and amino acids specialized for neurotransmission such as glutamate receptors, \(\gamma\)-aminobutyric acid and synapsin I (39–43). Glutamate has been shown to induce depolarization in islets and currents evoked by glutamate in islets show properties very similar to currents induced in neurons (39). It is of interest that in previous studies in neurons, calbindin has been reported to reduce [Ca\textsuperscript{2+}], in response to glutamate (8). Thus it is possible that calbindin may similarly modulate the rise [Ca\textsuperscript{2+}], in response not only to K\textsuperscript{+} but also to glutamate in the \(\beta\) cell. In our study we focused on the effect of calbindin on modulation of calcium transients in islets and \(\beta\) cell lines induced by depolarizing concentrations of KCl. Not much is known, however, about the ability of calbindin to modulate calcium influx in the \(\beta\) cell in response to other secretagogues. In one study using RIN1046-38 cells, induction of calbindin was shown to attenuate the [Ca\textsuperscript{2+}], response to the secretagogues glucose and KCl as well as to the calcium ionophore ionomycin and to thapsigargin (which releases Ca\textsuperscript{2+} from intracellular calcium stores) (44). These studies, combined with our findings, suggest a basic role for calbindin in controlling various calcium fluxes in the \(\beta\) cell.

In addition to calbindin, calmodulin (45) and calcyclin (46) are two other calcium-binding proteins present in the \(\beta\) cell that have been reported to play a role, through their interaction with Ca\textsuperscript{2+}, in modulating the insulin secretory response. Calcyclin, unlike calbindin, which acts as a Ca\textsuperscript{2+} buffer, was reported to enhance insulin release by a mechanism involving Ca\textsuperscript{2+}-induced exocytosis (46). Studies using transgenic mice with the calcium-binding protein calmodulin overexpressed in the pancreatic \(\beta\) cells show that these mice (referred to as CaM mice) have decreased plasma insulin levels, leading to increased serum glucose levels and early onset of diabetes (47). Impairment in the metabolism of glucose and the subsequent generation of ATP was reported to be the underlying mechanism involved in the defective insulin secretion in the calmodulin transgenic mouse (48). Further studies were done on another mouse transgenic line, referred to as CaM-8. CaM-8 expresses in its \(\beta\) cells a mutant form of calmodulin that is functionally similar to calbindin because it binds calcium with high affinity, and, unlike calmodulin but similar to calbindin, it does not activate effector proteins such as protein phosphatases and kinases (49, 50). The CaM-8 transgenic mice also display defective insulin secretion. However the mechanism responsible for the underlying defect in insulin secretion is different than the mechanism reported for the CaM mice. The primary defect in the CaM-8 mice is not a defect in glucose utilization but rather a reduction in Ca\textsuperscript{2+} current flowing through voltage-gated calcium channels resulting in a reduction in the rise in [Ca\textsuperscript{2+}] (50). CaM-8 mice showed a marked attenuation in the elevation in [Ca\textsuperscript{2+}], observed in response to depolarizing concentrations of KCl (50), similar to the attenuation observed in our studies in isolated islets and in \(\beta\) cells overexpressing calbindin. Further, patch clamp measurements using \(\beta\) cells from CaM-8 mice revealed a significant decline in the peak amplitude of voltage-gated Ca\textsuperscript{2+} channel currents.

The Ca\textsuperscript{2+} channels affected by the CaM-8 mutation are the L-type Ca\textsuperscript{2+} channels because dihydropyridines blocked these currents (50). It is of interest that previous patch clamp studies using GH3 pituitary cells stably transfected with calbindin found that calbindin reduces Ca\textsuperscript{2+} influx through voltage-dependent L-type Ca\textsuperscript{2+} channels (51). In addition, immunohistochemical studies mapping calbindin in brain noted the similarity between the distribution of calbindin immunoreactivity and the distribution of L-type calcium channels mapped using autoradiography (52). Further studies are needed to determine (as suggested by our studies with KCl and the similarities observed between calbindin and CaM-8) whether calbindin can act not only as a buffer protein but also as a protein that can affect other Ca\textsuperscript{2+}-regulating proteins such as Ca\textsuperscript{2+} channels not only in GH3 cells but also in \(\beta\) cells. In the future, it will be of interest to examine whether calbindin can modulate Ca\textsuperscript{2+} channel activity by a direct binding mechanism or whether calbindin can affect other proteins involved in regulating calcium channels.

In addition to depolarizing concentrations of potassium, calbindin overexpression was also found to suppress insulin secretion in response to the phorbol ester TPA, which is known to activate certain forms of protein kinase C. TPA can stimulate insulin secretion in the presence or absence of basal glucose (35, 53, 54). It has been suggested that phorbol ester-stimulated insulin secretion is linked with membrane depolarization and an increase in [Ca\textsuperscript{2+}], similar to the effect of KCl. The Ca\textsuperscript{2+} channels responsible for the increased [Ca\textsuperscript{2+}], signal in response to TPA have been reported to be the L-type Ca\textsuperscript{2+} channels (35, 55, 56). It has been suggested that the role for PKC is to maintain the phosphorylation state of the voltage-gated L-type Ca\textsuperscript{2+} channel, thus enabling the appropriate function of this channel (55). The resulting effect of TPA is similar but not identical to the effect of KCl because less depolarization is observed in response to TPA and additional actions of TPA have been suggested (35, 55). Thus calbindin may be a modulator of insulin secretion in response to both KCl and TPA because both secretagogues act, at least in part, by a similar mechanism. Calbindin, in response to both secretagogues, may act by reducing Ca\textsuperscript{2+} influx through voltage dependent calcium channels.

Although this study clearly establishes for the first time a role for calbindin in the modulation of depolarization-stimulated insulin release (in response to KCl or TPA), the exact role of calbindin in response to other secretagogues (including glucose, a variety of neuropeptides, and other transmitter substances that can combine to activate Ca\textsuperscript{2+} oscillations in the \(\beta\) cell) remains to be determined. Further studies in \(\beta\)HC cells, which have been reported to preserve the major characteristics of glucose metabolism of native \(\beta\) cells better than other murine \(\beta\) cell lines (22, 57, 58), will be of interest to examine the consequences of calbindin overexpression on glucose-dependent functions in the \(\beta\) cells. It is possible that calbindin has a “fine tuning” modulatory role on glucose-dependent insulin release that may not be obvious unless the effect is amplified (for example by activation of protein kinase A).

It is also likely that calbindin can have functions in the \(\beta\) cell in addition to modulation of insulin release. Previous studies by Bourlon et al. (59) indicated the presence of calbindin in \(\alpha\) as well as \(\beta\) cells of the rat pancreatic islet and suggested an additional role for islet calbindin in glucagon secretion. In this study correlations were made between levels of calbindin as measured by densitometry of immunocytochemically stained sections of pancreas and glucagon secretion. Additional studies using more sensitive methods of quantitation as well as studies with islets from calbindin KO mice may provide additional
insight with regard to the interesting possibility of a relationship between calbindin and glucagon secretion. In the nervous system the proposed role of calbindin, similar to its role in the β cell, is to buffer calcium. In the nervous system buffering of calcium by calbindin results in protection against calcium-mediated toxicity. Thus in the β cell, similar to the neuron, in response to depolarization-induced increases in [Ca\(^{2+}\)], calbindin may buffer the rise in [Ca\(^{2+}\)], to prevent calcium-mediated β cell death. Further studies are needed to examine other potential functions of calbindin in the β cell.

The role of calcium targets in the β cell has not been well understood. Although further studies are needed to determine additional mechanisms and multiple consequences of calbindin in the β cell, our findings, using islets from KO mice and β cells lines, are important because they define a role for calbindin in the β cell in calcium regulation and modulation of insulin release.

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