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Ayman Salah-el-deen Amer

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**CYTOANALYSIS OF PANCREATIC B-CELLS: USING AN AVIAN  
MODEL, MAMMALIAN TISSUE CULTURE AND IMPLICATIONS  
OF ANTISENSE OLIGONUCLEOTIDES TRANSFECTION**

**By**

**Ayman Salah-el-deen Amer**

**Dissertation submitted to  
The Graduate College  
Of**

**Marshall University**

**In partial fulfillment of the requirements  
For the degree of**

**Doctor of Philosophy**

**In**

**Biomedical Sciences**

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2004**

## ABSTRACT

Calbindin-D<sub>28k</sub> (CaBP<sub>28k</sub>) is a vitamin D-dependent calcium-binding protein that may alter intracellular calcium ion levels, [Ca<sup>2+</sup>]<sub>i</sub>. This dissertation describes experiments done to gain an understanding of the potential role of CaBP<sub>28k</sub> in pancreatic B-cells in control of insulin secretion. The localization of CaBP<sub>28k</sub> and insulin in chicken pancreas are shown in Chapter 1. CaBP<sub>28k</sub> expression was found to be highest in ventral and dorsal lobes and lowest in splenic lobe. Insulin concentrations were distributed similarly among these lobes. Confocal microscopic studies demonstrated colocalization of insulin and CaBP<sub>28k</sub> in B-cells. These findings suggest a possible role for CaBP<sub>28k</sub> in chicken B-cells that could contribute to type 2 diabetes-like characteristics of chickens.

Experiments done in Chapter 2 tested the effects of changing levels of glucose in pancreatic islets *in vitro* from transgenically derived CaBP<sub>28k</sub>-knockout (KO) and wildtype (WT) mice. CaBP<sub>28k</sub>-KO islets were exposed to increasing glucose concentrations from 2.8 mM to 30 mM, levels that mimic transition from fasting to hyperglycemic states. KO islets showed significantly greater elevations in [Ca<sup>2+</sup>]<sub>i</sub> as compared to WT. These experiments provide evidence that levels of CaBP<sub>28k</sub> could play a role in controlling Ca<sup>2+</sup>-mediated, glucose-induced insulin secretion in B-cells.

In chapter 3 the effects of reduction of CaBP<sub>28k</sub> levels on genomic and nongenomic factors using CaBP<sub>28k</sub>-antisense oligonucleotides (AS-ON)

transfection in a cultured pancreatic B-cell line (RIN1046-38 cells) are described. Complete inhibition of CaBP<sub>28k</sub> expression in transfection assays was achieved using 200 nM phosphorothioate-AS-ON (PS-AS-ON) as well as 20 nM propyne-AS-ON (PY-AS-ON). In addition, cDNA microarray analysis showed up-regulation of both vitamin D receptor (VDR) and calbindin-D<sub>9k</sub> mRNAs in PS-AS-ON-transfected RIN cells as compared to controls. Western blotting indicated VDR overexpression and calbindin-D<sub>9k</sub> expression in AS-ON-transfected cells. *This study is the first demonstration of compensatory expression of calbindin-D<sub>9k</sub> in response to inhibition of CaBP<sub>28k</sub> in cultured B-cells.* Insulin secretory responses of PS-AS-ON-transfected cells were greater than in controls. These findings suggest that B-cells synthesize an alternative protein, calbindin-D<sub>9k</sub>, to preserve calcium regulation when expression of CaBP<sub>28k</sub> is abolished. Additional studies are required to help in understanding possible interactions of calbindin-D<sub>9k</sub>, [Ca<sup>2+</sup>]<sub>i</sub>, and VDR in the AS-ON-transfected B-cells.

## **DEDICATION**

**In memory of my loving dearest father Prof. Dr. Salah Amer**

**To my loving dearest mother**

**To my loving dearest brother Dr. Ehab & his family**

**To my wife & kids for their constant encouragement and support**

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## LIST OF ABBREVIATION/SYMBOLS

- ADP — adenosine-5'-diphosphate
- ANOVA — analysis of variance
- AS — antisense
- ATP — adenosine-5'-triphosphate
- B-cells —  $\beta$ -cells (beta-cells)
- BCIP — 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine
- BSA — bovine serum albumin
- $\beta$ TC-3 —  $\beta$ -tumor cell line-3
- $\text{Ca}^{2+}$  — ionic calcium
- $\text{CaBP}_{28\text{K}}$  — calbindin- $\text{D}_{28\text{k}}$
- $[\text{Ca}^{2+}]_i$  — concentration of intracellular free calcium ions
- $\text{CaMKII}$  —  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II
- cAMP — cyclic adenosine-5'-monophosphate
- cDNA — complementary deoxyribonucleic acid
- Carbachol — carbamylcholine chloride
- DAB — 3,3'-diaminobenzidine tetrahydrochloride
- DAG — diacylglycerol
- 1,25-(OH) $_2$ D $_3$  — 1,25-dihydroxyvitamin D $_3$
- DMSO — dimethyl sulfoxide
- EDTA — ethylenediamine tetra-acetic acid

EGTA — ethylene glycol-bis-( $\beta$ -amino ethyl ether) N, N'-tetra-acetic acid

ELISA — enzyme-linked immunosorbent assay

EPCon — endocrine pancreas consortium

ER — endoplasmic reticulum

FITC — fluorescein isothiocyanate

Fura-2 — Fura-2 acetoxy methyl ester dye

HBSS — Hank's balanced salt solution

HEPES — N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HRP — horseradish peroxidase

IgG — gamma immunoglobulin

IP<sub>3</sub> — inositol 1, 4, 5-trisphosphate

JNK — c-Jun N-terminal kinase

K<sup>+</sup><sub>ATP</sub> — ATP-sensitive K<sup>+</sup> channels

KO — knockout

NBT — nitro-blue tetrazolium chloride

NF-AT — nuclear factor of activated T cells

NF- $\kappa$ B — nuclear factor kappa B

NIDDK — National Institute of Diabetes and Digestive and Kidney Diseases

NIDDM — non-insulin-dependent diabetes mellitus

NS — nonsense

ON — oligonucleotides

PAGE — polyacrylamide gel electrophoresis

PBS — Dulbecco's phosphate-buffered saline

PKC — protein kinase C

PLC — phospholipase C

PS — phosphorothioate

PS-AS-ON — phosphorothioate antisense oligonucleotides

PY — propyne

PY-AS-ON — propyne antisense oligonucleotides

RIN — rat insulinoma cell line

RXR — retinoid X receptor

SDS — sodium dodecyl sulfate

SEM — standard error of the mean

SV40 — simian virus 40

TNF $\alpha$  — tumor necrosis factor alpha

TR — Texas Red

VDR — vitamin D receptor

VDREs — vitamin D response elements

WT — wildtype

## **INTRODUCTION**



Diabetes mellitus is one of the major global public health problems. It is a metabolic disorder for which the actual etiology remains to be identified. The number of people with diabetes worldwide was 150 million in 2002 and is expected to rise to 220 million in 2010 (Buse et al., 2003). The main pathologic findings in pancreatic islets of Langerhans in diabetes mellitus include B-cell ( $\beta$ -cell) destruction in type 1 diabetes, and somewhat reduced or normal  $\beta$ -cell numbers in type 2 diabetes (Buse et al., 2003; Eisenbarth et al., 2003). Type 2 diabetes accounts for 90% of diabetic cases worldwide (Buse et al., 2003). Genetic and environmental factors interact in the pathogenesis of type 2 diabetes (Buse et al., 2003). The disease results when glucose transport channels on the cell membrane (glucose transporters) are insensitive to the effects of insulin (or when there are too few transporters) or when the quantity of insulin produced by the  $\beta$ -cells of the pancreas is inadequate to activate the number of glucose transporters necessary to maintain normal cellular metabolism (Buse et al., 2003). Insulin insensitivity is a receptor and/or post-receptor event. Three cardinal abnormalities occur in type 2 diabetes: 1) defective insulin secretion in response to glucose from the pancreatic  $\beta$ -cells, 2) resistance to the action of insulin in peripheral tissues (e.g. muscle and fat cells), and 3) increased glucose production by the liver (Buse et al., 2003). It is well known that calcium ions,  $\text{Ca}^{2+}$ , play an important role in the glucose-induced insulin secretion from  $\beta$ -cells (Gilon et al., 1993; Jones and Persaud, 1998; Easom, 1999; Lang, 1999; Lingappa and Farey, 2000; Barg et al., 2002; Henquin et al., 2002). Recently, some clinical intervention trials have demonstrated that supplementation with

vitamin D or its metabolites leads to reduction of the high blood glucose levels in type 2 diabetic patients and to increased serum insulin concentrations in uremic patients (Zittermann, 2003; Holick, 2004). Vitamin D is known to mediate its effects through interactions with the vitamin D receptor. One group of proteins that are regulated by the VDR is calcium-binding proteins (Christakos et al., 1991; DeLuca and Zierold, 1998; Christakos et al., 2003b). The vitamin D-dependent calcium-binding proteins form a link between vitamin D, which alters intracellular calcium levels, and the generation of cellular responses. Our ultimate theme in this research project is to investigate a possible approach to increasing the insulin secretory response to glucose from cultured  $\beta$ -cells. We aim to substantiate the role of the vitamin D-dependent calcium-binding protein, calbindin-D<sub>28k</sub> (CaBP<sub>28k</sub>), in the  $\beta$ -cells as a possible challenge for improving the defective insulin secretion, e.g., as seen in type 2 diabetes. Herein, an overview of vitamin D and vitamin D-dependent factors, implicated for playing a possible role in glucose-induced insulin release from the pancreatic  $\beta$ -cells, is presented.

### ***Vitamin D***

The hormonally active metabolite of vitamin D, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>), is a secosteroid whose genomic mechanism of action is known to be similar to that of other steroid hormones (Christakos et al., 1991; DeLuca and Zierold, 1998; Brown et al., 1999). 1, 25-(OH)<sub>2</sub>D<sub>3</sub> is well known for its importance in controlling calcium and phosphorus homeostasis, and roles in bone mineralization (Christakos et al., 1991; DeLuca and Zierold, 1998; Brown et

al., 1999; Christakos et al., 2003b). 1,25-(OH)<sub>2</sub>D<sub>3</sub> maintains Ca<sup>2+</sup> and phosphorus homeostasis through direct stimulation of intestinal transport, enhancement of Ca<sup>2+</sup> and phosphate reabsorption in the kidney and mobilization of mineral from bone (Christakos et al., 1991; Brown et al., 1999). Vitamin D deficiency leads to a variety of abnormalities including rickets and osteomalacia, osteoporosis, disturbed muscle functions. Moreover, it is associated with many disorders including tuberculosis, rheumatoid arthritis, multiple sclerosis, psoriasis, systemic lupus erythematosus, insulin-dependent diabetes mellitus (type 1, IDDM), inflammatory bowel diseases, hypertension, and specific types of cancer (Boland, 1986; Billaudel et al., 1998; Brown et al., 1999; Zella et al., 2003; Zittermann, 2003; Holick, 2004). Experimental findings have illustrated that certain autoimmune diseases such as multiple sclerosis, psoriasis, and rheumatoid arthritis can be treated with vitamin D and its analogs (Morimoto and Kumahara, 1985; Brown, 1998; DeLuca and Zierold, 1998; Brown et al., 1999; Zittermann, 2003). Due to the antiproliferative differentiation-promoting properties of 1,25-(OH)<sub>2</sub>D<sub>3</sub> it also has been used in treating some types of leukemia and prostate cancer (Abe et al., 1981; Liu et al., 1996; Zittermann, 2003; Holick, 2004).

Vitamin D deficiency is reported to result in impaired glucose-mediated insulin secretion that can be reversed by vitamin D repletion (Chertow et al., 1983; Labriji-Mestaghanmi et al., 1988; Kumar et al., 1994; Bourlon et al., 1999; Zella et al., 2003). In addition, some clinical intervention trials have demonstrated

that supplementation with 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its metabolites is able to reduce blood pressure in hypertensive patients, improve blood glucose levels in type 2 diabetics, and to increase serum insulin concentrations in uremic patients (Quesada et al., 1990; Zittermann, 2003; Holick, 2004). It has been postulated that 1,25-(OH)<sub>2</sub>D<sub>3</sub>, through the vitamin D receptor (VDR)-mediated modulation of expression of Ca<sup>2+</sup>-binding proteins, controls the concentration of intracellular free calcium ions, [Ca<sup>2+</sup>]<sub>i</sub> (Christakos et al., 1979; Christakos and Norman, 1980; Mayer et al., 1981). Moreover, the immunosuppressive properties of 1,25-(OH)<sub>2</sub>D<sub>3</sub> have extended its therapeutic application to reducing the incidence of insulinitis and type 1 diabetes in both animal models and humans (Mathieu et al., 1992, 1994b; Stene et al., 2000; Hypponen et al., 2001; Zella et al., 2003; Holick, 2004) and to preventing the recurrence of autoimmune diabetes after islet transplantation (Mathieu et al., 1994a, 1995; Lemire, 1997). In addition, other experiments reported the importance of the use of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and its analogs in suppressing transplant rejection (Lemire, 1997; DeLuca and Zierold, 1998). Furthermore, a role for 1,25-(OH)<sub>2</sub>D<sub>3</sub> in reproduction was suggested by the demonstration of reduced female fertility in vitamin D-deficient rats (Halloran and DeLuca, 1980) that could be corrected only by 1,25-(OH)<sub>2</sub>D<sub>3</sub>, but not by simply raising the serum Ca<sup>2+</sup> (Kwiecek et al., 1989). The biological actions of vitamin D are mediated by the stereospecific interaction of 1,25-(OH)<sub>2</sub>D<sub>3</sub> with VDR (Christakos et al., 1991; DeLuca and Zierold, 1998; Christakos et al., 2003b).

## **VDR**

VDR is one of the members of the nuclear steroid receptor superfamily that act as ligand-activated transcription factors (Ross et al., 1992; Sone et al., 1991; Schröder et al., 1995; Brown et al., 1999). Upon ligand binding, the activated cytoplasmic VDR rapidly translocates to the nucleus along microtubules (Brown et al., 1999). A critical role for VDR translocation on 1,25-(OH)<sub>2</sub>D<sub>3</sub> transcriptional regulation was proposed by earlier studies in normal human monocytes in which the disruption of microtubular integrity prevented 1,25-(OH)<sub>2</sub>D<sub>3</sub> induction of 24-hydroxylase mRNA, a mitochondrial cytochrome P-450 enzyme which is the major catabolic enzyme of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Kamimura et al., 1995). The 24-hydroxylase enzyme is highly inducible by 1,25-(OH)<sub>2</sub>D<sub>3</sub>, providing a mechanism for attenuating the response to the vitamin D hormone and reducing 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels when they are abnormally high (Brown et al., 1999). In fact, mice lacking a functional 24-hydroxylase gene have high serum 1,25-(OH)<sub>2</sub>D<sub>3</sub> levels due to the decreased capacity to degrade it (St.-Arnaud et al., 1996; Brown et al., 1999). Moreover, vitamin D-resistant rickets was reported to occur due to a defective cytoplasmic to nuclear translocation of an otherwise normal VDR (Hewison et al., 1993). VDR mediates the signal of 1,25-(OH)<sub>2</sub>D<sub>3</sub> through binding to specific DNA motifs (vitamin D response elements, VDREs) in the promoter of target genes. It acts primarily as a heterodimer composed of one VDR subunit and one retinoid X receptor (RXR) subunit, or as a homodimer, thereby interacting with many nuclear factors including the coactivators, and the general transcription factor TFIIB, a component of the basal transcription

complex which plays a critical role in ligand-dependent transcription (Christakos et al., 1991; Blanco et al., 1995; MacDonald et al., 1995; Nishikawa et al., 1995; Schröder et al., 1995; Masuyama et al., 1997; DeLuca and Zierold, 1998; Brown et al., 1999; Christakos et al., 2003b). Both 1,25-(OH)<sub>2</sub>D<sub>3</sub> and Ca<sup>2+</sup> were previously reported to regulate the expression of VDR (Brown et al., 1995; Zineb et al., 1998; Healy et al., 2003). Nongenomic functions of VDR have been reported previously (Nemere et al., 1994; Sergeev and Rhoten, 1995; Norman et al., 1999, 2001, 2002a, b). A plasma membrane VDR was identified to mediate rapid opening of the voltage-gated Ca<sup>2+</sup> channels located in the plasma membrane and to stimulate Ca<sup>2+</sup> transport in the intestine, Ca<sup>2+</sup> mobilization in osteoblasts, and release of insulin by rat pancreatic beta-cells and RIN1046-38 cells (Nemere et al., 1994; Sergeev and Rhoten, 1995; Norman et al., 1999, 2001, 2002a, b). Moreover, earlier studies indicated that mice lacking the VDR had impaired bone formation, vitamin D-dependent rickets, hypocalcemia, alopecia, uterine hypoplasia, infertility, and growth retardation after weaning (Yoshizawa et al., 1997).

It has been reported that the VDREs were detected in both the murine calbindin-D<sub>9k</sub> and calbindin-D<sub>28k</sub> (CaBP<sub>28k</sub>) genes (Darwish and DeLuca, 1992; Gill and Christakos, 1993). Developmental studies reported that inductions of VDR mRNA in the rat kidney and duodenum were significantly correlated with the induction of mRNAs for two vitamin-D-dependent calcium-binding proteins, CaBP<sub>28k</sub> in the kidney and calbindin-D<sub>9k</sub> in the duodenum, at the same

developmental stage. These findings suggest that the induction of VDR has an important role in regulating the gene expression of both CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> (Huang et al., 1989; Christakos et al., 1991). Moreover, in the vitamin D-deficient adult rat, repletion of vitamin D led to the up-regulation of VDR and CaBP<sub>28k</sub> in the kidney, and calbindin-D<sub>9k</sub> in the intestine (Christakos et al., 1991). Furthermore, VDR induces CaBP<sub>28k</sub> gene transcription in the chicken intestine (Spencer et al., 1976; Christakos and Norman, 1980; Siebert et al., 1982), but in the chicken kidney the levels of CaBP<sub>28k</sub> were less stringently regulated by VDR (Hunziker, 1986).

### ***Calbindins***

The vitamin D-dependent Ca<sup>2+</sup>-binding proteins (calbindins) are intracellular proteins. They have high affinity Ca<sup>2+</sup>-binding activity, and play an important role in the regulation of [Ca<sup>2+</sup>]<sub>i</sub> (Christakos et al., 1989; Nelson et al., 2002), and are sometimes referred to as Ca<sup>2+</sup>-receptor proteins (Zimmer et al., 1997). The calbindin family includes calmodulin, parvalbumin, troponin C, S100 proteins, calbindin-D<sub>9k</sub>, and calbindin-D<sub>28k</sub> (Rhoten et al., 1985; Christakos et al., 1989; Heinzmann and Hunziker, 1991; Zimmer et al., 1997). Calmodulin, a ubiquitous Ca<sup>2+</sup>-binding protein present in all eukaryotic cells, has four Ca<sup>2+</sup>-binding sites that bind both Ca<sup>2+</sup> and magnesium ions competitively and is involved in the triggering of several cellular processes (Rhoten et al., 1982; Kilhoffer et al., 1983; Hammes et al., 1994). Calmodulin acts as a Ca<sup>2+</sup>-signaling protein since it regulates the activity of many endogenous target enzymes in a

Ca<sup>2+</sup>-dependent manner including the cyclic nucleotide phosphodiesterase, calmodulin-dependent protein kinases, and calcium adenosine triphosphatase (Ca<sup>2+</sup>-ATPase) (Cheung et al., 1978). The Ca<sup>2+</sup>/calmodulin-dependent kinases play a role in linking changes in [Ca<sup>2+</sup>]<sub>i</sub> to the distal events in exocytosis and insulin release from the pancreatic islet β-cells (Rhoten et al., 1982; Hammes et al., 1994; Easom, 1999; Tabuchi et al., 2000; Yamamoto et al., 2003). Parvalbumin is expressed in many tissues including the central nervous system, such as hippocampus and amygdala, where it contributes to intracellular Ca<sup>2+</sup> buffering in neurons (Grateron et al., 2003; Felmy and Schneggenburger, 2004; Hajszan et al., 2004). Parvalbumin in fast-twitch skeletal muscle binds Ca<sup>2+</sup> and magnesium with high affinity and seems to be involved in muscle relaxation (Kilhoffer et al., 1983; Coutu et al., 2004; Michele et al., 2004). On the other hand, troponin C acts as a Ca<sup>2+</sup>-sensing switch in muscle tissue conferring Ca<sup>2+</sup>-sensitivity to the acto-myosin interaction, and exhibits both triggering and relaxing structural sites (Kilhoffer et al., 1983; Landesberg et al., 2004; Matsumoto et al., 2004; Tikunova and Davis, 2004). S-100 protein was first described as being unique to the nervous system, but it has been identified subsequently in a variety of cell types of neuroectodermal (i.e., melanocytes, glial and Schwann cells) and non-neuroectodermal origin (i.e., adipocytes, pancreatic islets of Langerhans, parathyroid and anterior pituitary glands) (Zabel et al., 1986; Laszik et al., 1989; Watanabe and Hashimoto, 1993; Atanassova, 2001; Park and Min, 2003; Sandelin et al., 2004). Moreover, S-100 protein was detected in the core of all



types of  $\beta$ -cell insulin secretory granules in the chicken pancreas and it was thought to be involved in their maturation (Takayanagi and Watanabe, 1996).

### ***Calbindin-D<sub>9k</sub>***

Calbindin-D<sub>9k</sub> was detected in the rat duodenum, kidneys, placenta, pancreas, and mineralized tissues (bone, cartilage, and teeth) (Marche et al., 1980; Rhoten et al., 1982; Schreiner et al., 1983; Thomasset et al., 1982; Balmain et al., 1986; Warembourg et al., 1986a, b; Balmain, 1991; Bourlon et al., 1996). The calbindin-D<sub>9k</sub> gene expression is characteristic of mammals only (Thomasset, 1997). It is expressed in both mouse and rat kidneys (Thomasset et al., 1982; Delorme et al., 1983; Rhoten et al., 1985); however, it is less active in the rat kidney where CaBP<sub>28k</sub> is highly expressed and predominates in Ca<sup>2+</sup> regulation (Schreiner et al., 1983; Bindels et al., 1991; Thomasset, 1997). Calbindin-D<sub>9k</sub> binds two Ca<sup>2+</sup> ions (Juffer and Vogel, 2000). Several physiological functions are postulated for calbindin-D<sub>9k</sub> that range from a Ca<sup>2+</sup>-dependent regulatory function analogous to that of calmodulin, to a role as a Ca<sup>2+</sup>-shuttle (carrier protein) that facilitates transcellular Ca<sup>2+</sup> diffusion in the Ca<sup>2+</sup>-transporting cells (Bronner et al., 1986; Bronner, 1987, 1988; Wasserman and Fullmer, 1995; Bronner, 2003a, b).

### ***CaBP<sub>28k</sub>***

CaBP<sub>28k</sub> is predominantly a cytosolic protein that was initially detected in the chicken duodenum (Wasserman and Taylor, 1966). It is also found in many

other tissues, including kidneys, brain, bones, parathyroid glands, and pancreatic islets (Buffa et al., 1989; Christakos et al., 1989; Pochet et al., 1989; Rhoten and Christakos, 1990; Bouhtiauy et al., 1994), and is evolutionarily highly conserved in various vertebrates (Rhoten et al., 1985, 1986; Parmentier et al., 1987). CaBP<sub>28k</sub> is known to have at least four high affinity Ca<sup>2+</sup>-binding sites (Hunziker, 1986). It was previously reported to act as a Ca<sup>2+</sup>-buffering protein in different cell types (Gross and Kumar, 1990). It prevents [Ca<sup>2+</sup>]<sub>i</sub> from reaching toxic levels in the brain (Mattson et al., 1991, 1995; Guo et al., 1998) and protects against cytokine-induced apoptosis in cultured β-cell lines (Rabinovitch et al., 2001; Riachy et al., 2002; Christakos et al., 2003a). Cytokines are produced by the immune system cells that infiltrate pancreatic islets and mediate islet β-cell destruction in autoimmune (type 1) diabetes mellitus (Rabinovitch et al., 2001). Moreover, Dowd et al. (1992) showed that stable overexpression of CaBP<sub>28k</sub> in lymphocytes decreased apoptosis, and this reduction was correlated directly with the increased relative amounts of CaBP<sub>28k</sub> in these cells. Also, overexpression of CaBP<sub>28k</sub> in lymphocytes increased cell survival in the presence of a calcium ionophore that resulted in a greatly increased cytoplasmic influx of Ca<sup>2+</sup> (Dowd et al., 1992). The kinetics of Ca<sup>2+</sup>-binding by CaBP<sub>28k</sub> may be important in its effect on Ca<sup>2+</sup>-mediated apoptosis. Chard et al. (1993) determined that the Ca<sup>2+</sup>-binding kinetics of CaBP<sub>28k</sub> were fast enough (seconds to minutes) to reduce the global increase in [Ca<sup>2+</sup>]<sub>i</sub> mediating the potentiation of neurotransmitter release. CaBP<sub>28K</sub> also functions as a Ca<sup>2+</sup>-carrier that regulates cellular Ca<sup>2+</sup> transport in

the intestine and kidneys (Bredderman and Wasserman, 1974; Feher, 1983; Borke et al., 1989; Bronner, 1989; Christakos et al., 1989).

1,25-(OH)<sub>2</sub>D<sub>3</sub> is known to regulate the levels of chicken intestinal CaBP<sub>28k</sub> from undetectable in vitamin D-deficient chickens up to 1 - 3% of the cytoplasmic protein in the intestinal cell in vitamin D-replete chickens (Christakos et al., 1979). The interaction of 1,25-(OH)<sub>2</sub>D<sub>3</sub> with VDR and the association of VDR with the VDRE that is located in the CaBP<sub>28k</sub> gene results in expression of CaBP<sub>28k</sub> (Christakos et al., 1991; Gill and Christakos, 1993). Kadowaki and Norman (1984) and Hall and Norman (1991) have achieved reductions in chicken pancreatic CaBP<sub>28k</sub> content using a diet that has normal calcium and phosphorus content, but is vitamin D-deficient. Moreover, the use of pharmacological doses of 1,25-(OH)<sub>2</sub>D<sub>3</sub> was reported to induce an increase in the rat renal CaBP<sub>28k</sub> concentrations (Hemmingsen et al., 1998). Also, a direct influence of [Ca<sup>2+</sup>]<sub>i</sub> in increasing the cytosolic concentrations of CaBP<sub>28k</sub> have been shown (Norman et al., 1981; Hall and Norman, 1990). Hemmingsen et al. (2002) found that the concentration of rat renal CaBP<sub>28k</sub> was significantly increased upon increase of the plasma level of 1,25-(OH)<sub>2</sub>D<sub>3</sub>. In contrast, there was no reduction in renal CaBP<sub>28k</sub> after a 50% decrease in plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub>.

Furthermore, recent studies with mammalian pancreatic islets suggested that CaBP<sub>28k</sub> may modulate insulin secretion from the β-cell. Sooy et al. (1999) reported that β-cells from CaBP<sub>28k</sub>-null-mutant (KO, knockout) mice, with

complete ablation of CaBP<sub>28k</sub>, had a significant increase (3.5 fold) in their insulin release compared to controls (WT, wildtype) when depolarized with 45 mM KCl. In contrast, in murine  $\beta$ -cell lines stably transfected and overexpressing CaBP<sub>28k</sub> ( $\beta$ HC-13 CaBP40 and  $\beta$ TC-3) there was a marked attenuation of insulin secretion when depolarized with 45 mM KCl compared to control  $\beta$ -cells. In addition, RIN (rat insulinoma) cells with elevated levels of CaBP<sub>28k</sub> have increased cytoplasmic Ca<sup>2+</sup>-buffering capacity (Rhoten and Sergeev, 1994; Reddy et al., 1997).

### **[Ca<sup>2+</sup>]<sub>i</sub>**

It is well known that the [Ca<sup>2+</sup>]<sub>i</sub> regulates many cellular functions, such as gene expression, cell signal transduction, cell proliferation, and differentiation (Allbritton et al., 1992; Sergeev and Rhoten, 1995; Airaksinen et al., 1997; Sergeev and Rhoten, 1998; Putney, 1999; Sooy et al., 1999). The [Ca<sup>2+</sup>]<sub>i</sub> of unstimulated cells is maintained between less than 100 nM and 200 nM (Hodgkin and Keynes, 1957; Schanne et al., 1979; Carafoli, 1987; Nicotera and Orrenius, 1992). There is a concentration difference of about four orders of magnitude between the extracellular Ca<sup>2+</sup> level (approximately 1.3 mM) and the cytosolic Ca<sup>2+</sup> concentration [which is < 100 – 200 nM] (Frankenhaeuser and Hodgkin, 1957; Hodgkin and Keynes, 1957; Rasmussen, 1970; Schanne et al., 1979; Nicotera and Orrenius, 1992). This electrochemical driving force is balanced primarily by active Ca<sup>2+</sup> extrusion through the plasma membrane (Ca<sup>2+</sup>-ATPase pumps), by the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger, by the coordinated activity of Ca<sup>2+</sup>-sequestering systems located in the mitochondrial, endoplasmic reticular and

nuclear membranes, and by the buffering action of calbindins (Borle, 1967; Rasmussen, 1970; Schanne et al., 1979; Carafoli, 1987; Carafoli and Longoni, 1987; Nicotera and Orrenius, 1992; Chard et al., 1993; Carafoli, 1994).

Transient elevations in the  $[Ca^{2+}]_i$  in  $\beta$ -cells are necessary for  $\beta$ -cell functions and insulin secretion, but prolonged increases in  $[Ca^{2+}]_i$  lead to deleterious conditions and even  $\beta$ -cell death (Farber, 1990; Pralong et al., 1990, Gilon et al., 1993; Bertuzzi et al., 1999). The sustained increase in  $[Ca^{2+}]_i$  due to excessive entry of extracellular  $Ca^{2+}$  and/or mobilization of  $Ca^{2+}$  from intracellular stores constitute a major signal transduction pathway of apoptosis (Farber, 1990; Dowd, 1995; Thompson, 1995; McConkey and Orrenius, 1996, 1997). Sustained increases in the  $[Ca^{2+}]_i$  result in activation of  $Ca^{2+}$ -dependent proteases, such as the calpains which belong to the superfamily of cysteine proteases that affect apoptosis (Thompson, 1995; Peter et al., 1997). The buffering of  $[Ca^{2+}]_i$  by  $CaBP_{28k}$  has been shown with the direct introduction of  $CaBP_{28k}$  into cultured dorsal root ganglion cells (Chard et al., 1993). In these cells,  $CaBP_{28k}$  caused a marked decrease in the rate of rise in  $[Ca^{2+}]_i$  and a significant reduction in the maximum levels of  $[Ca^{2+}]_i$  in response to depolarization. As a result of transfection of the  $CaBP_{28k}$  gene into a pituitary cell line (GH<sub>3</sub>), a faster inactivation of voltage-dependent L- and T-type  $Ca^{2+}$  currents was observed (Lledo et al., 1992).

Several research studies have indicated an important role of  $\text{Ca}^{2+}$  in the regulation of gene expression in different cells (Drummond et al., 1987; Bading et al., 1993; Berridge, 1997a, b; Dolmetsch et al., 1997; Hardingham et al., 1997; Kuo et al., 1997; Perez-Terzic et al., 1997), and that the depletion of the intracellular  $\text{Ca}^{2+}$  stores results in inhibition of protein synthesis (Takuma et al., 1984; Brostrom et al., 1986; Greber and Gerace, 1995). In addition, it has been reported that the presence and activity of some common eukaryotic nuclear transcriptional factors (such as c-Jun N-terminal kinase, JNK; nuclear factor of activated T cells, NF-AT; and nuclear factor kappa B,  $\text{NF-}\kappa\text{B}$ ) were dependent on the intracellular  $\text{Ca}^{2+}$  regulation in immune and neural cells (Ho et al., 1994; Herdegen et al., 1997; Lezoualc'h and Behl, 1998). These nuclear transcription factors are involved in the regulation of gene expression that is critical for a large number of cellular processes including immune and inflammatory responses, cellular growth, development, and apoptosis (Barish, 1998). Gick and Bancroft (1985) reported that the addition of ethylene glycol-bis-( $\beta$ -amino ethyl ether) N, N'-tetra-acetic acid (EGTA), a  $\text{Ca}^{2+}$ -chelator, to the culture media of primary cultures of rat pituitary cells inhibited the basal and hormonally stimulated synthesis of both prolactin and growth hormone. Also, it was demonstrated that subculture of the rat pituitary tumor cell line GH<sub>3</sub> in a serum-free  $\text{Ca}^{2+}$ -free media caused the relative synthesis of prolactin to decline below 0.1% of the total protein synthesis of these cells (Gautvik and Tashjian, 1973; White et al., 1981), while the addition of  $\text{CaCl}_2$  to this culture medium produced a dose-dependent increase in prolactin synthesis (White et al., 1981, 1989; Delidow et al., 1992).

Nonetheless, White (1985) observed that calmodulin mediates the effects of  $\text{Ca}^{2+}$  on prolactin gene expression. Moreover, Preston et al. (1990) and Delidow et al. (1992) postulated that there is transcriptional and posttranscriptional regulation of the rat prolactin gene by  $\text{Ca}^{2+}$ . It was also demonstrated that  $1,25\text{-(OH)}_2\text{D}_3$ , through its interaction with VDR, affects the production of prolactin hormone in  $\text{GH}_3$  cells in a  $\text{Ca}^{2+}$ -dependent manner (Haug and Gautvik, 1985; Mortensen et al., 1993; Haug et al., 1987).

$\text{Ca}^{2+}$  was also reported to link cellular electrical activity to the transcription, translation and the insertion of different channels and receptors in the plasma membrane (Rosen et al., 1995; Berridge, 1997a, b; Barish, 1998). It is also of interest that dietary  $\text{Ca}^{2+}$  was shown to control expression of the rat intestinal calbindin- $\text{D}_{9\text{k}}$  gene at both the transcriptional and posttranscriptional levels (Freund and Bronner, 1975; Bronner et al., 1986). The effect of  $\text{Ca}^{2+}$  on calbindin- $\text{D}_{9\text{k}}$  gene expression has been confirmed by *in vitro* studies that showed increasing the  $\text{Ca}^{2+}$  concentration in the medium of fetal rat intestinal organ cultures causes an increase in the production of calbindin- $\text{D}_{9\text{k}}$  mRNA in the presence and absence of  $1,25\text{-(OH)}_2\text{D}_3$  (Bréhier and Thomasset, 1990). Furthermore, the expression of  $\text{CaBP}_{28\text{k}}$  in the chicken intestine and kidneys was reported to be regulated by dietary  $\text{Ca}^{2+}$  (Norman et al., 1981; Bar et al., 1990; Hall and Norman, 1990).

It is well established that  $\text{Ca}^{2+}$  plays an important role in the glucose-induced secretion of insulin (Gilon et al., 1993; Jones and Persaud, 1998; Easom, 1999; Lang, 1999; Lingappa and Farey, 2000; Barg et al., 2002; Henquin et al., 2002). In the  $\beta$ -cell, glucose is the principal factor that evokes the rise in  $[\text{Ca}^{2+}]_i$  and oscillations of  $[\text{Ca}^{2+}]_i$  that initiate (Gilon and Henquin, 1992; Leech et al., 1994) and entrain synchronous pulses in insulin secretion (Gilon et al., 1993). In addition, the multifunctional  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II (CaMKII) was reported to play an important role in glucose-induced insulin secretion (Schulman and Lou, 1989; Easom et al., 1997; Easom, 1999; Tabuchi et al., 2000; Yamamoto et al., 2003) and  $\text{Ca}^{2+}$ -evoked neural exocytosis (Matteoli et al., 1992; Maletic-Savatic et al., 1995, 1996). It was also reported that  $\text{Ca}^{2+}$  omission (Grotsky and Bennett, 1966; Milner and Hales, 1967) or the addition of blockers of the L-type voltage-dependent  $\text{Ca}^{2+}$ -channels abolishes glucose-stimulated insulin secretion (Devis et al., 1975).

### ***The relationships between diabetes and $[\text{Ca}^{2+}]_i$***

Characteristics of human diabetics and animal models of diabetes indicate that both types 1 and 2 diabetes mellitus may have altered  $[\text{Ca}^{2+}]_i$  regulation as an underlying cause (Draznin, 1988; Levy et al., 1994; Pick et al., 1998).  $\text{Ca}^{2+}$ -mediated apoptosis could lead to the development of type 1 diabetes mellitus (insulin-dependent diabetes) (Ankarcrona et al., 1994; Kaneto et al., 1995; Hoorens et al., 1996; Loweth et al., 1996; Kurrer et al., 1997; O'Brien et al., 1997; Rabinovitch et al., 2001). There is experimental evidence that an elevated  $[\text{Ca}^{2+}]_i$



and apoptosis are important elements in the process of immune system-mediated  $\beta$ -cell death in type 1 diabetes. Juntti-Berggren et al. (1993) found that serum from patients with type 1 diabetes led to increased  $\text{Ca}^{2+}$  influx through L-type  $\text{Ca}^{2+}$  channels and that the resulting increase in  $[\text{Ca}^{2+}]_i$ -initiated apoptosis in the cultured  $\beta$ -cell line RINm5F. It has been suggested that an abnormally elevated level of  $[\text{Ca}^{2+}]_i$  might be part of the signaling cascade of events occurring in the immune system-mediated  $\beta$ -cell death in type 1 diabetes (Casteels et al., 1997; Kurrer et al., 1997), including the nonobese diabetic (NOD) mouse (O'Brien et al., 1997). In support of these findings, Caraher and Newsholme (1996) reported that an L-type  $\text{Ca}^{2+}$  channel antagonist, nifedipine, markedly reduced immune system-mediated death of  $\beta$ -cell line G1-CRI cells.

In the streptozotocin-induced (STZ) diabetic rat and diabetic rat pregnancy, the circulating  $1,25\text{-(OH)}_2\text{D}_3$  level was significantly lowered. This was associated with low placental  $\text{Ca}^{2+}$  and calbindin- $\text{D}_{9k}$  content (Schneider et al., 1977; Husain et al., 1994; Verhaeghe et al., 1999; Hamilton et al., 2000), and maternal hypercalciuria and hyperphosphaturia, but normocalcemia (Nyomba et al., 1985; Anwana and Garland, 1990; Birdsey et al., 1995; Ward et al., 2001), compared to normal control rats. In addition, these diabetic rats showed low fetal tibial  $\text{Ca}^{2+}$  content (Uriu-Hare et al., 1985; Mimouni et al., 1988; Verhaeghe et al., 1990, 1999; Ward et al., 2001) and reduced serum osteocalcin (a biochemical marker of osteoblast function and bone formation) (Verhaeghe et al., 1990; Ward et al., 2001), whereas the levels of urinary deoxypyridinoline cross links (the

biochemical marker for bone resorption) were not significantly altered (Ward et al., 2001). It is of interest that the diabetic maternal duodenal calbindin-D<sub>9k</sub> content was significantly lowered which, in turn, led to reduced duodenal Ca<sup>2+</sup> absorption (Verhaeghe et al., 1990; Stone et al., 1990, 1991; Verhaeghe et al., 1999; Ohara, 2000; Ward et al., 2001). The duodenal VDR was up-regulated, compared to normal control rats. The diabetic rats had markedly increased food and Ca<sup>2+</sup> intake, so that their net Ca<sup>2+</sup> balance remained positive despite a 13-fold increase in urinary Ca<sup>2+</sup> excretion and a decrease in active duodenal Ca<sup>2+</sup> absorption (Nyomba et al., 1989; Ohara, 2000). It was suggested that the low circulating level of 1,25-(OH)<sub>2</sub>D<sub>3</sub> prevented the amplification of 1,25-(OH)<sub>2</sub>D<sub>3</sub>'s action, as evidenced by the reduction in the calbindin-D<sub>9k</sub> level, despite the up-regulation of duodenal VDR as compared to that of controls (Stone et al., 1990, 1991). All of the above mentioned parameters were normalized in the diabetic rats when they were treated early with either exogenous insulin or given subcutaneous injections of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Nyomba et al., 1985; Stone et al., 1990; Husain et al., 1994; Orihuela et al., 1999; Verhaeghe et al., 1999; Ohara, 2000). Placental calbindin-D<sub>9k</sub> expression was the exception, because it is distinctly regulated by estrogen and progesterone during pregnancy and not by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in that specific tissue (Glazier et al., 1995; Verhaeghe et al., 1999; An et al., 2004). Delayed insulin treatment of type 1 diabetes mellitus only partly rectifies the hypercalciuria indicating that some irreversible changes occurred in the kidney due to the prolonged insulin insufficiency that leads to a defect in the renal ability to conserve Ca<sup>2+</sup> (Hoskins and Scott, 1984). However, the VDR

levels in the kidneys were not changed and renal CaBP<sub>28k</sub> was normal in these diabetic rats (Stone et al., 1990, 1991; Ward et al., 2001). Moreover, in humans hypercalciuria (Raskin et al., 1978; Malone et al., 1986; Hough, 1987; Malone et al., 1987; Harangi et al., 1989; Kodama et al., 1992; Gunczler et al., 1996), hyperphosphaturia (Malone et al., 1986), normocalcemia, hypomagnesemia, and osteopenia (reduced bone mass) were often found accompanying type 1 diabetes mellitus (Selby, 1988; Hough, 1987; Al-Qadreh et al., 1996). The altered regulation of  $[Ca^{2+}]_i$  has also been linked to the occurrence of other diabetic complications, such as micro- and macro-vascular disease (Levy et al., 1994). It has been suggested that the glomerular hyperfiltration associated with diabetes delivers an increased load of  $Ca^{2+}$  to renal tubules, which may itself cause an increased rate of excretion of  $Ca^{2+}$ . Also the fractional  $Ca^{2+}$  reabsorption is significantly lower in diabetic than in control rats, indicating that an actual tubular defect may be present in the diabetic rats (Ward et al., 2001). Serum levels of 1,25-(OH)<sub>2</sub>D<sub>3</sub> but not 25-(OH)D<sub>3</sub> were significantly reduced during STZ diabetes, indicating that the fall in 1,25-(OH)<sub>2</sub>D<sub>3</sub> was not due to a lack of substrate (Ward et al., 2001). Together, these data indicate that the reduced bone formation and mineralization could account for the hypercalciuria with normocalcemia and explain, at least in part, the progressive osteopenia seen in diabetic patients (Ward et al., 2001). In addition or alternatively, osmotic diuresis and increased dietary calcium and carbohydrate intake secondary to hyperphagia (Anwana and Garland, 1990) can account for the hypercalciuria (Ward et al., 2001). However, control of osmotic diuresis with insulin therapy, and hyperphagia with paired

feeding only partly corrects the hypercalciuria (Guruprakash et al., 1988), which indicates that changes in  $1,25\text{-(OH)}_2\text{D}_3$  are specific to the diabetic condition (Ward et al., 2001).

On the other hand, another group of researchers reported that the serum  $\text{Ca}^{2+}$  levels were markedly reduced in untreated streptozotocin-induced diabetic fetuses and pregnant rats (Verhaeghe et al., 1986; Ohara, 2000). Infants born to those mothers had an increased incidence of hypocalcemia (Tsang et al., 1972; Mimouni et al., 1986; Verhaeghe et al., 1986; Mimouni et al., 1990), in addition to a decreased bone mineral content that was associated with retarded skeletal development as compared with normal infants (Verhaeghe et al., 1988; Lapillone et al., 1997). Further studies using a  $\text{Ca}^{2+}$ -deprived diet will help in understanding the contribution of the gastrointestinal tract towards diabetic hypercalciuria.

The selection of an experimental animal model for studying the physiological factors that might be associated with non-insulin-dependent diabetes mellitus (NIDDM, type 2 diabetes) could be appropriately done by using the chicken. The low  $\beta/\alpha$  cell ratio in the chicken mimics that seen in type 2 diabetes (Rahier et al., 1983), and the chicken exhibits higher basal blood glucose levels, lower insulin output in response to glucose stimulation (King and Hazelwood, 1976; Rideau et al., 1986; Rideau and Simon, 1989, 1992; Ruffier et al., 1996), and shows a marked resistance to exogenous insulin injections compared to mammals (Naber and Hazelwood, 1977; Hazelwood, 1984).

However, the mechanisms underlying the type 2 diabetes-like characteristics of the chicken are not clear. The expression of CaBP<sub>28k</sub> has been detected before in the whole chicken pancreas and was reported to be higher than that found in the rodent pancreas. But the pattern of CaBP<sub>28k</sub> expression in the individual pancreatic lobes and whether there is any spatial relationships with insulin in the  $\beta$ -cells have not been studied before. Earlier research studies on the CaBP<sub>28k</sub>-KO, as compared to that of the WT mice islets, were done using KCl as a secretagogue. But until our recent publication (Parkash et al., 2002) there was no previous research work reported using glucose, the major physiological stimulant of  $\beta$ -cell insulin secretion, as a secretagogue to investigate the postulated role of CaBP<sub>28k</sub> in the pancreatic  $\beta$ -cells. Moreover, we have not found any published studies on the effects of *in vitro* CaBP<sub>28k</sub>-ablation on the insulin secretory responses of cultured  $\beta$ -cell lines. In addition, the study of the genomic effects of *in vitro* CaBP<sub>28k</sub>-ablation in the cultured  $\beta$ -cell lines was not done before. Thus, our studies on RIN1046-38 cells using CaBP<sub>28k</sub>-antisense oligonucleotides transfection were the first.

This dissertation has three main goals. The first is to gain an understanding of the expression pattern of CaBP<sub>28k</sub> in the chicken pancreas and to explore its spatial relationships in the  $\beta$ -cell. The second goal is to identify the influence of CaBP<sub>28k</sub>-ablation on the dynamics of  $[Ca^{2+}]_i$  in the  $\beta$ -cells of mice. The third goal is an attempt to elucidate the genomic and non-genomic effects of *in vitro* CaBP<sub>28k</sub>-ablation in a cultured  $\beta$ -cell line. The work here will be presented

as three separate chapters. Experiments will first be described using the chicken as an avian model to investigate the expression of CaBP<sub>28k</sub> and insulin in the pancreas. In chapter two, our studies will examine the change in  $[Ca^{2+}]_i$  responses of the CaBP<sub>28k</sub>-KO mice pancreatic islets to glucose stimulation, as compared to the wildtype islets, that we have published (Parkash et al., 2002). Finally, the third chapter will highlight the effects on the gene expression profile and insulin secretory responses from a cultured rat insulinoma cell line (RIN1046-38 cells) of using antisense oligonucleotides transfection to induce CaBP<sub>28k</sub>-ablation *in vitro*.

## **CHAPTER 1**

# **STUDIES ON THE EXPRESSION OF CALBINDIN-D<sub>28K</sub> AND INSULIN IN PANCREATIC $\beta$ -CELLS USING AN AVIAN MODEL**

## SUMMARY

In chickens, the pancreatic  $\beta/\alpha$  cell ratio is lower and the glucose level in the blood is higher than in mammals. In addition, resistance to insulin is present. Thus, in a number of ways the chicken simulates type 2 (non-insulin-dependent) diabetes mellitus (NIDDM) and can serve as a useful model for study. Calbindin- $D_{28k}$  (CaBP $_{28K}$ ) is a member of a family of high affinity calcium-binding proteins that regulate free intracellular calcium ion concentration and may play a regulatory role in pancreatic  $\beta$ -cells. We examined the occurrence of CaBP $_{28K}$  and insulin in chicken pancreatic lobes by Western blotting, immunohistochemistry, and confocal microscopy. Insulin concentration of chicken pancreatic lobes was measured by enzyme-linked immunosorbent assay (ELISA). Western blot analysis revealed the highest levels of expression of CaBP $_{28K}$  in ventral and dorsal lobes, while the lowest expression was in the splenic lobe. Similarly, the ELISA assays showed the highest insulin concentration to be in dorsal and ventral lobes, while the lowest concentration was in the splenic lobe. Immunohistochemistry detected a greater intensity of reaction product for localization of CaBP $_{28K}$  than insulin in pancreatic islets. Confocal microscopy studies demonstrated localization of CaBP $_{28K}$  to  $\beta$ -cells of chicken pancreas with a significant positive correlation with insulin localization ( $r = 0.87$ ;  $p < 0.001$ ). The strong colocalization of CaBP $_{28K}$  with insulin in chicken  $\beta$ -cells suggests that CaBP $_{28K}$  plays a physiological role in these cells and may contribute to the NIDDM-like characteristics of the chicken.



## INTRODUCTION

Chickens normally have a relatively high insulin-release threshold and low insulin output in response to glucose stimulation (King and Hazelwood, 1976; Rideau et al., 1986; Rideau and Simon, 1989, 1992; Ruffier et al., 1996). Several previous studies have found that relative to mammals, the chicken exhibits higher basal blood glucose levels and shows a marked resistance to exogenous insulin (Naber and Hazelwood, 1977; Hazelwood, 1984). In birds, glucagon plays a dominant role, as compared with insulin, in maintaining glucose homeostasis (Epple and Brinn, 1987; Hazelwood, 1973, 1984). Therefore, birds have higher levels of plasma glucagon and glucose than are found in mammals (Epple and Brinn, 1987). Moreover, the chicken pancreas concentration of somatostatin is approximately 20-fold higher than in the rat pancreas (Weir et al., 1976). Somatostatin can potently inhibit both insulin and glucagon release (Guilleman and Gerich, 1976; Koerker et al., 1974). In the chicken the  $\beta/\alpha$  cell ratio is decreased, compared to mammals, suggesting that these birds are in a catabolic mode (Hazelwood, 1984; Epple and Brinn, 1987; Rawdon, 1998).

The low  $\beta/\alpha$  cell ratio in the chicken mimics that seen in non-insulin-dependent diabetes mellitus (NIDDM, type 2) (Rahier et al., 1983). On the other hand, chickens are more resistant to the diabetogenic action of  $\beta$ -cytotoxic agents such as streptozotocin, to naturally occurring insulin-dependent diabetes mellitus (IDDM, type 1) and to the induction of diabetes *in vivo* (Langslow et al.,

1970; Meglasson and Hazelwood, 1982). A recent study hypothesized that the hyperglycemia and insulin resistance observed in chickens are associated with a possible deficiency of the insulin-responsive glucose transporter, GLUT4 (Seki et al., 2003). Thus, the hyperglycemia and insulin resistance found in the chickens could be due to all of the factors mentioned above interacting together. Since the  $\beta/\alpha$  cell ratio is low in the chicken pancreas, it is important to identify and characterize the different areas of the pancreas in order to elaborate their structure/function relationships. If there are areas having a greater abundance of  $\beta$ -cells, then this may make them a good model for transplantation studies.

The chicken pancreas is composed of four principal lobes: dorsal, ventral, third, and splenic (Mikami and Ono, 1962; Mikami et al., 1986). At the cellular level, the chicken pancreas has at least four types of endocrine cells: alpha ( $\alpha$ ), beta ( $\beta$ ), delta ( $\delta$ ), and PP-cells (Rawdon and Andrew, 1979; Roth et al., 1982). The hormones produced and secreted by these cells are:  $\alpha$  or A, glucagon;  $\beta$  or B, insulin;  $\delta$  or D, somatostatin; and F or PP, pancreatic polypeptide (Larsson et al., 1974; Alumets et al., 1978; Rawdon and Andrew, 1979; Andrew and Rawdon, 1980; Tomita et al., 1985). Chicken pancreatic islets are of three classes:  $\beta$ -cell-rich,  $\beta$ -cell-poor, and mixed-type islets, based on their cellular composition (Kobayashi and Fujita, 1969; Watanabe et al., 1975; Mikami et al., 1986). The  $\beta$ -cell-rich islets contain predominantly  $\beta$ -cells ( $\beta$ -islets), in addition to  $\delta$ -cells and PP-cells. The  $\beta$ -cell-poor islets contain predominantly  $\alpha$ -cells ( $\alpha$ -islets), in

addition to  $\delta$ -cells and a small number of  $\beta$ -cells (Mikami and Ono, 1962; Weir et al., 1976; Ruffier et al., 1998).

Human diabetics and animal models of diabetes indicate that both type 1 and 2 diabetes mellitus may have altered regulation of the concentration of intracellular free calcium ions,  $[Ca^{2+}]_i$ , as an underlying cause or contributing factor (Draznin, 1988; Levy et al., 1994; Pick et al., 1998). Previous studies indicate that calcium-mediated apoptosis could lead to the development of insulin-dependent diabetes mellitus (IDDM, type 1) (Ankarcrona et al., 1994; Kaneto et al., 1995; Hoorens et al., 1996; Loweth et al., 1996; Kurrer et al., 1997; O'Brien et al., 1997). CaBP<sub>28K</sub> is a calcium-binding protein with a relative molecular mass of 28,000 Daltons and is vitamin D-dependent in some cells and organs, e.g., kidney (Sergeev et al., 1998). It belongs to a family of high affinity calcium-binding proteins that play important regulatory roles in cells, including the regulation of  $[Ca^{2+}]_i$ . Other members of this diverse family include calmodulin, calbindin-D<sub>9k</sub>, parvalbumin, and S-100 proteins (Christakos et al., 1989; Heizmann and Hunziker, 1991). CaBP<sub>28K</sub> is predominantly a cytosolic protein that was initially detected in the chick duodenum (Wasserman and Taylor, 1966). It is also found in many other tissues, including kidneys, brain, bones, parathyroid glands, and pancreatic islets (Buffa et al., 1989; Christakos et al., 1989; Pochet et al., 1989; Rhoten and Christakos, 1990), and is evolutionarily highly conserved in various vertebrates (Rhoten et al., 1985, 1986; Parmentier et al., 1987). CaBP<sub>28K</sub> has been shown to have a variety of roles in different organs. It acts as

a  $\text{Ca}^{2+}$  carrier in the intestine and kidneys (Bredderman and Wasserman, 1974; Feher, 1983; Bronner, 1989; Christakos et al., 1989), while it has a protective function against  $\text{Ca}^{2+}$  toxicity in the brain (Mattson et al., 1991, 1995; Guo et al., 1998). Recent studies with mammalian pancreatic islets suggest that  $\text{CaBP}_{28\text{K}}$  modulates insulin secretion (Sooy et al., 1999), and protects  $\beta$ -cells against cytokine-induced apoptosis (Rabinovitch et al., 2001; Riachy et al., 2002; Christakos et al., 2003a). Other studies done on  $\beta$ -cell lines demonstrated a large increase in cytoplasmic  $\text{Ca}^{2+}$  buffering capacity in  $\beta$ -cells with elevated levels of  $\text{CaBP}_{28\text{K}}$  (Rhoten and Sergeev, 1994; Reddy et al., 1997). Islets of rodents have a very low  $\text{CaBP}_{28\text{K}}$  content compared to chicken islets, and are less resistant to injury and to the diabetogenic action of  $\beta$ -cytotoxins (Buffa et al., 1989; Pochet et al., 1989; Malaisse et al., 1990). In addition to the quantity of  $\text{CaBP}_{28\text{K}}$  in the pancreatic islets, the intracellular localization of  $\text{CaBP}_{28\text{K}}$  in the  $\beta$ -cells may be important, as it is in the absorptive cells of the intestine. In the intestinal absorptive cell cytoplasm,  $\text{CaBP}_{28\text{K}}$  was localized inside small vesicles and lysosome-like structures, and some  $\text{CaBP}_{28\text{K}}$  was also associated with filamentous elements (microtubules and tubulin) (Nemere et al., 1991).  $\text{CaBP}_{28\text{K}}$  localization is important for its function as a  $\text{Ca}^{2+}$  carrier across the intestinal epithelial cell from the luminal brush border to the basolateral membrane. Both  $\text{CaBP}_{28\text{K}}$  and tubulin were found to undergo dramatic changes in their cellular localization as a consequence of intestinal calcium transport (Nemere et al., 1991). The cellular localization of  $\text{CaBP}_{28\text{K}}$  in the chicken pancreatic  $\beta$ -cells has not been determined. It is also not known if  $\text{CaBP}_{28\text{K}}$  and insulin are spatially

related in the cytosol or if there is a greater predominance of CaBP<sub>28K</sub> expression in specific chicken pancreatic lobes.

This study using an avian model explores the expression of CaBP<sub>28K</sub> and insulin in different pancreatic lobes and investigates the spatial relationships of insulin and CaBP<sub>28K</sub> in the  $\beta$ -cell.

## **MATERIALS AND METHODS**

### ***Animals***

Thirty-nine White Leghorn cockerels were obtained on the day of hatching and raised on normal avian diet. Animals of 2 – 3 weeks of age were included in this study. The animal body weights were 104 – 230 grams. Individual lobes of the chicken pancreas were studied by Western blotting to determine CaBP<sub>28k</sub> expression or by ELISA to measure insulin levels. Histological sections were dually labeled to study the expression of both proteins. We conducted the experiments in accordance with the accepted standards of humane animal care as defined by the Institutional Animal Care and Use Committee of the Joan C. Edwards School of Medicine at Marshall University, WV, USA.

### ***Materials***

Monoclonal mouse anti-CaBP<sub>28K</sub>, goat anti-guinea pig IgG-FITC conjugate, goat anti-rabbit IgG-peroxidase conjugate, goat anti-mouse biotin conjugate, goat anti-guinea pig biotin conjugate, normal goat serum, bovine serum albumin, protease inhibitor cocktail, Tween 20<sup>®</sup>, 3,3'-diaminobenzidine tetrahydrochloride (DAB), and acrylamide were purchased from Sigma Chemical Company (St. Louis, MO). Goat anti-mouse IgG-Texas Red conjugate was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). SlowFade<sup>™</sup> Antifade kit was ordered from Molecular Probes, Inc. (Eugene, OR). Guinea pig anti-porcine insulin IgG, and rabbit anti-CaBP<sub>28K</sub> were obtained from

Chemicon International, Inc. (Temecula, CA). Recombinant rat CaBP<sub>28K</sub> purified protein was purchased from Swant<sup>®</sup> Swiss Antibodies Company (Bellinzona, Switzerland). Streptavidin-horseradish peroxidase (HRP) was from Amersham International PLC. (Buckinghamshire, UK). Bradford protein reagent and low-range prestained SDS-PAGE standards were from Bio-Rad Laboratories, Inc. (Hercules, CA). NitroBind<sup>™</sup> 0.45 μm unsupported pure nitrocellulose transfer membranes were purchased from GE Osmonics (Minnetonka, MN). SuperSignal<sup>®</sup> West Pico Chemiluminescent substrate for detection of HRP was from Pierce Biotechnology Inc. (Rockford, IL). Medical X-ray film (Super RX) was from Fujifilm Medical Systems, Inc. (Stamford, CT). All chemicals and reagents were of analytical grade.

### ***Western blotting***

Chickens were sacrificed and their pancreata were dissected according to their morphological lobation (dorsal, ventral, third, and splenic), frozen quickly on dry ice, and stored at -80°C until used. Each lobe was individually homogenized in protein lysis buffer [1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM ethylenediamine tetra-acetic acid (EDTA), and 1% protease inhibitor cocktail]. Then, burst sonication was done (< 10 seconds), and samples were centrifuged at 14,000 × *g* for 20 min. at 4°C. The supernatant solution was obtained and total protein estimated using the Bradford method (Bradford, 1976). Equal amounts of total protein from each pancreatic lobe were loaded per lane in

12% SDS polyacrylamide gels. CaBP<sub>28K</sub> protein (Swant<sup>®</sup>) was used as a standard. Low-range prestained SDS-PAGE standards (Bio-Rad) were also used. SDS polyacrylamide gel electrophoresis (PAGE) was performed. After protein transfer, nitrocellulose sheets were incubated in Tris-buffered saline with Tween 20<sup>®</sup> and 5% non-fat dry milk to block non-specific binding sites (Spinola and Cannon, 1985). The nitrocellulose sheets were incubated with specific primary antibodies against CaBP<sub>28K</sub>, and then subsequently washed several times in Tris-buffered saline with Tween 20<sup>®</sup>. Peroxidase-conjugated secondary antibodies were then applied (Towbin et al., 1979). Protein bands were visualized using a Pierce chemiluminescent kit. X-ray autoradiograms were scanned and their images were saved. The bands representing different pancreatic lobes were quantitated for their relative densities using the gel analysis densitometry tool of NIH image analysis software – Image J, version 1.31c [a public domain image processing and analysis program using optical density standards developed by Wayne Rasband at the Research Services Branch (RSB) of the National Institutes of Health (NIH) <http://rsb.info.nih.gov/ij/> (Humphries et al., 1997; Melrose et al., 2001; Sage and Unser, 2001)].

### ***Enzyme-linked immunosorbent assays***

Chicken pancreatic lobes were extirpated and individually cut into small pieces and insulin was extracted by incubation overnight in acidic ethanol (pH 1.7) at -20°C. The extracts were centrifuged, the supernatants collected, and the minces extracted again in acidic ethanol (pH 1.7) at -20°C overnight. Extracts



were centrifuged and the supernatants collected, then added to the previous ones as described by Kimmel et al. (1968) and Rhoten (1983). Chicken insulin standards (Eli Lilly & Company, Indianapolis, IN) were used to build a standard curve (0 – 200 pg), and the insulin concentration of pancreatic extracts was measured using a competitive enzyme-linked immunosorbent assay (ELISA) protocol modified in our laboratory from that of Kekow et al. (1988), and Webster et al. (1990).

Falcon 35-3915 pro-bind™ 96-well polystyrene microtiter assay plates (Becton Dickinson and Company, Franklin Lakes, NJ) were coated overnight at 4°C with rabbit anti-guinea pig IgG (Sigma Chemical Company, St. Louis, MO) in carbonate-bicarbonate coating buffer. After washes in phosphate incubation buffer, guinea pig anti-insulin (607/22) [a gift from the late Dr. Peter Wright, Indianapolis, IN] prepared in phosphate incubation buffer was added to the wells and incubation continued for three hours at 37°C. The plates were then washed, and the standards, quality controls, and samples were added to appropriate wells in triplicate and incubated overnight at 4°C. Peroxidase-labeled insulin (Sigma Chemical Company, St. Louis, MO) was then added to the wells, and the plates were incubated for 4 hours at 4°C. After washes, substrate solution containing O-phenylenediamine dichloride (Sigma Chemical Company, St. Louis, MO) as a chromagen was added to the wells. The plates were incubated at room temperature for one hour. Then, stop solution containing 1M H<sub>2</sub>SO<sub>4</sub> was added to the wells and after one hour at room temperature, the absorbance at 490 nm of

the wells was read using  $\mu$ Quant™ microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT) and the data were interpreted using KCjunior™ data analysis software, version 1.41.4 (Bio-Tek Instruments, Inc., Winooski, VT).

### ***Immunohistochemistry and light microscopy***

Chicken pancreata were fixed in freshly prepared 4% paraformaldehyde in Dulbecco's phosphate-buffered saline (PBS) [pH 7.2]. Six-micron sections of paraffin-embedded lobes were prepared for both light microscopic immunohistochemistry and confocal microscopy immunofluorescence studies. Sections for immunohistochemistry were treated with 3% hydrogen peroxide in PBS to suppress pancreatic endogenous peroxidase activity, then, incubated in 3% normal goat serum – 2% bovine serum albumin in Tris-buffered saline to block non-specific binding sites. Immunolabeling was done using specific primary antibodies against insulin and CaBP<sub>28K</sub>. After several washes in 3% normal goat serum – 2% bovine serum albumin in Tris-buffered saline, biotinylated secondary antibodies were applied. A streptavidin-biotin horseradish peroxidase protocol was used, and immunoreactions were visualized using DAB substrate medium as a chromagen. Quantitative immunohistochemical image analysis was done using the densitometry tool of the NIH analysis software – Image J, version 1.31c (Mize, 1994; Acarin et al., 1997; Wu et al., 1997; Bisland et al., 1999).

### ***Laser scanning confocal microscopy***

Six-micron chicken pancreatic sections were blocked with 3% normal goat serum – 2% bovine serum albumin in Tris-buffered saline, then labeled with specific primary antibodies against insulin and CaBP<sub>28K</sub>. The pancreatic sections were subsequently washed several times with 3% normal goat serum – 2% bovine serum albumin in Tris-buffered saline. Fluorescence-labeled secondary antibodies tagged with fluorescein isothiocyanate (FITC) (for insulin) and Texas Red (for CaBP<sub>28K</sub>) were then added. After several washes in 3% normal goat serum – 2% bovine serum albumin in Tris-buffered saline, the sections were then treated with SlowFade™ Antifade kit. Confocal images were acquired using a Bio-Rad MRC 1024 Laser Scanning Confocal Microscope (Bio-Rad, Microscopy Division, Hemel Hempstead Herts, England). Confocal image acquisitions were carried out using the built-in Bio-Rad Laser Sharp 3.2 software. Optical sections were taken every 0.3 micron. Confocal image processing was done using Confocal Assistant software, version 4.02 (Build 101) [Todd Clark Brelje], and the insulin and CaBP<sub>28K</sub> pixel intensities in the images were evaluated using the image correlation analysis tool of the NIH software – Image J software, version 1.31c (Brown et al., 1998; Amin et al., 2000; Sims et al., 2003).

### ***Statistical analysis***

Data are presented as the mean  $\pm$  standard error of the mean (SEM), percentages, correlation coefficient ( $r$ ). The data were analyzed for significance with the unpaired Student's  $t$  test (one-tailed) with a significance level of 0.05,

using the statistical software Sigma Stat, version 2.03 from SPSS Inc. (Chicago, IL).

## RESULTS

### ***Morphology of the chicken pancreas***

The avian pancreas is a compound organ nestled between the limbs of the U-shaped loop of the duodenum (Figure 1). It is composed of four lobes of unequal sizes: dorsal, ventral, third, and splenic (Mikami and Ono, 1962; Weir et al., 1976; Tomita et al., 1985; Mikami et al., 1986). A graphical representation of the difference in the wet mass of the pancreatic lobes is shown in Figure 2. We found some variation in the mass of the pancreatic lobes. The range of wet masses was as follows: dorsal 403.04 – 510.90 mg, ventral 203.62 – 287.62 mg, third 114.49 – 143.77 mg, and splenic 80.99 – 123.92 mg. Mean wet masses in mg ( $\pm$  SEM) were  $438.02 \pm 74.24$  for the dorsal lobe,  $347.55 \pm 36.00$  for the ventral lobe,  $124.12 \pm 15.79$  for the third lobe, and  $107.95 \pm 6.85$  for the splenic lobe. We also found that the third lobe has the largest number of pancreatic ducts (data not shown).

### ***Western blot analysis***

Immunoblotting revealed consistent variations in the content of CaBP<sub>28K</sub> in different lobes of the chicken pancreas. A single immunoreactive band of about 28,000 Daltons was visualized in the different pancreatic lobe homogenates (Figure 3). Densitometry analysis of the Western blots showed that both the ventral and dorsal lobes had a relative higher CaBP<sub>28K</sub> expression than that of the splenic lobe of chicken pancreas (Figure 4). Taken together, the dorsal and

ventral lobes had 78% of the total CaBP<sub>28K</sub> expressed in the chicken pancreas (Figure 5).

### ***Insulin ELISA***

We measured the insulin concentration in the extracts taken from the different lobes of the chicken pancreas by using ELISA. The different pancreatic lobes had varying levels of insulin. The insulin concentrations of the dorsal and ventral lobes were significantly higher than the third and splenic lobes of chicken pancreas (Table 1 and Figure 6). The insulin concentration in the whole chicken pancreas ranged from 10.59 to 15.41 ng/mg wet mass (n = 5) [Tables 2A and 2B]. We estimated the mean insulin concentration of the whole chicken pancreas to be  $12.37 \pm 0.96$  ng/mg wet mass (n = 5).

The range of relative percentages of insulin content in the different pancreatic lobes was as follows: dorsal (45.9 – 72.4%), ventral (19.4 – 43.7%), third (4.0 – 9.4%), and splenic (0.2 – 6.2%) as shown in Tables 2A and 2B. The variations in insulin content among the different lobes of chicken pancreas are shown in Figure 7.

### ***Light microscopy immunohistochemistry***

We localized CaBP<sub>28K</sub> immunoreactive material in the chicken pancreas using a specific monoclonal antiserum against CaBP<sub>28K</sub> (Figure 8b). Cells immunopositive for CaBP<sub>28K</sub> (as indicated by the presence of the reaction

product for peroxidase) were observed in all four lobes, but with varying intensity of reaction product in the different lobes. CaBP<sub>28K</sub> staining was most intense in the ventral and dorsal lobes, while other pancreatic lobes showed less immunoreactivity. Our immunohistochemical data showed reaction products positive for the presence of CaBP<sub>28K</sub> in the islets of Langerhans, while the acinar tissue of the chicken pancreas did not show any immunoreactivity (Figure 8b). Controls for antibody specificity consisted of absorption of the CaBP<sub>28K</sub> mouse monoclonal antibody with an excess of recombinant rat CaBP<sub>28K</sub> purified protein and were negative (Figure 8a).

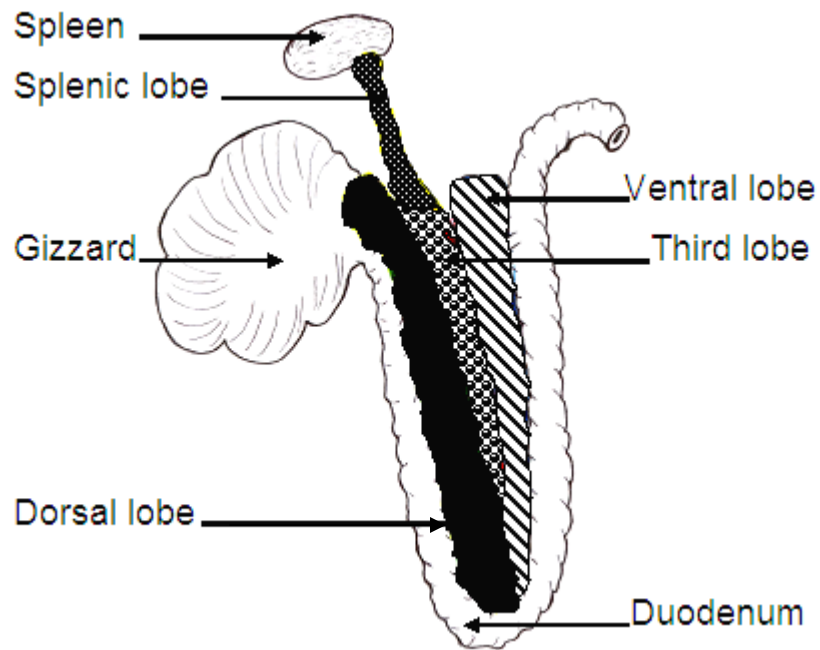
We also examined the localization and distribution of insulin immunoreactivity in the chicken pancreas. We found that the most intense labeling for insulin was in the ventral and dorsal lobes (Figure 8c), while other pancreatic lobes showed less immunoreactivity. Moreover, our quantitative immunohistochemical analysis showed that the intensity of labeling for CaBP<sub>28K</sub> in the ventral lobe pancreatic islets was higher than that for insulin, but the difference was not statistically significant (Figure 9).

### ***Confocal microscopy immunofluorescence***

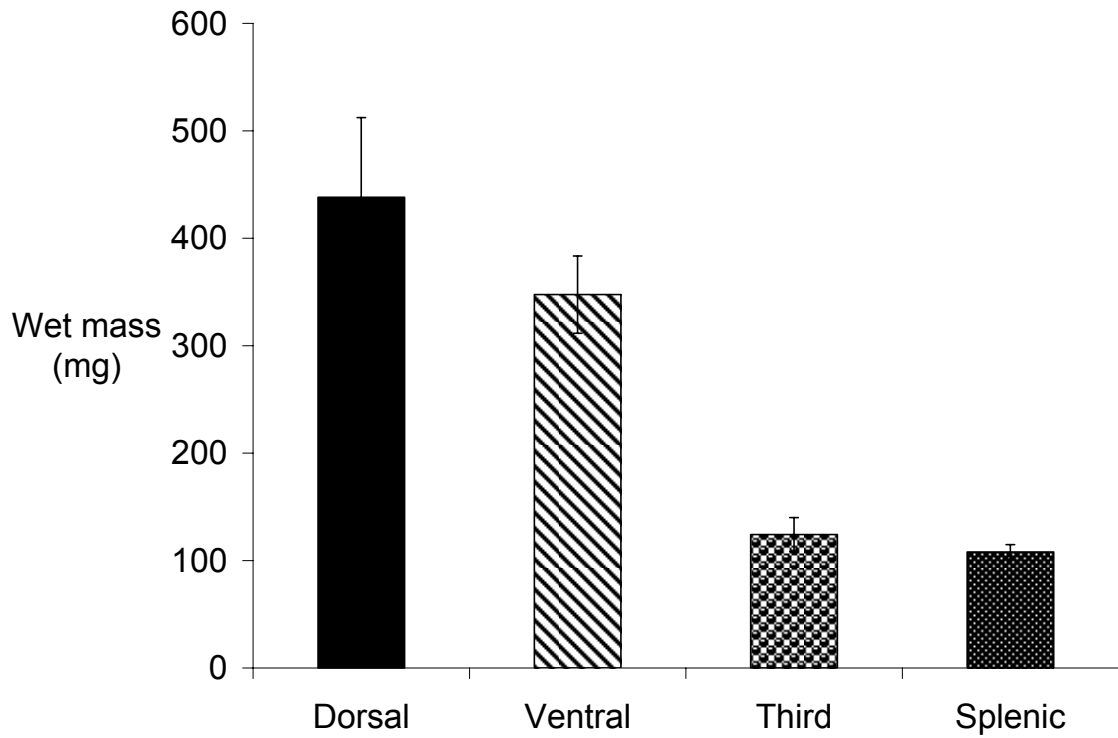
To determine a more exact localization of CaBP<sub>28K</sub> in chicken pancreatic islets, laser scanning confocal microscopy was used. Fluorescence-labeled immunopositive cells for both CaBP<sub>28K</sub> and insulin were observed in all four lobes, but with varying intensity of fluorescence. The fluorescent intensity for both

CaBP<sub>28K</sub> and insulin was highest in the ventral and dorsal lobes, while other pancreatic lobes showed less immunofluorescence. In Figure 10, CaBP<sub>28K</sub> immunoreactivity is displayed in red (secondary antibodies conjugated with Texas Red), a; while insulin immunoreactivity is displayed in green (secondary antibodies conjugated with FITC), b; in an islet of the ventral lobe of chicken pancreas. The merged image (Figure 10c) indicates substantial areas of colocalization (green + red = yellow). We further examined the islets from the ventral lobe of the chicken pancreas for a relationship between insulin and CaBP<sub>28K</sub>. Insulin and CaBP<sub>28K</sub> pixel intensities in the images were studied by using the image correlation analysis tool of Image J software, and the resulting data were statistically analyzed and found to be positively correlated ( $r = 0.87$ ) (Figure 11). This positive correlation between insulin and CaBP<sub>28K</sub> pixel intensities was found in most of the ventral lobe pancreatic islets and was determined to be highly significant ( $p < 0.001$ ) as seen in the correlation plot (Figure 11).





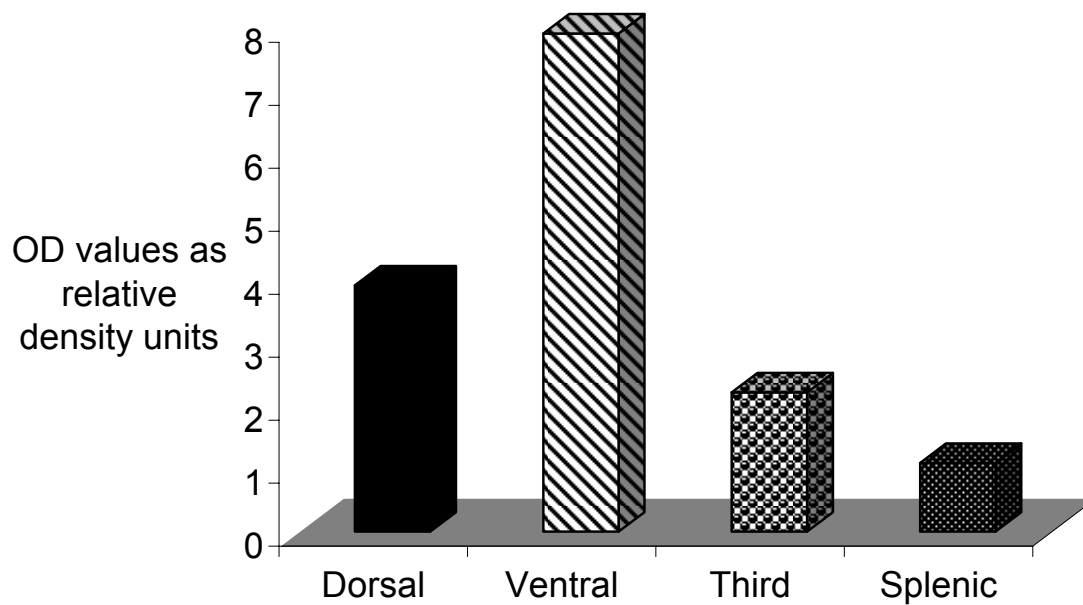
**Figure 1.** Schematic dorsal view showing the morphology of the chicken pancreas (modified from Mikami and Ono, 1962). The chicken pancreas is formed of four different lobes: dorsal, ventral, third, and splenic. The dorsal lobe is the largest in size followed by the ventral, then the third, while the splenic lobe is the smallest.



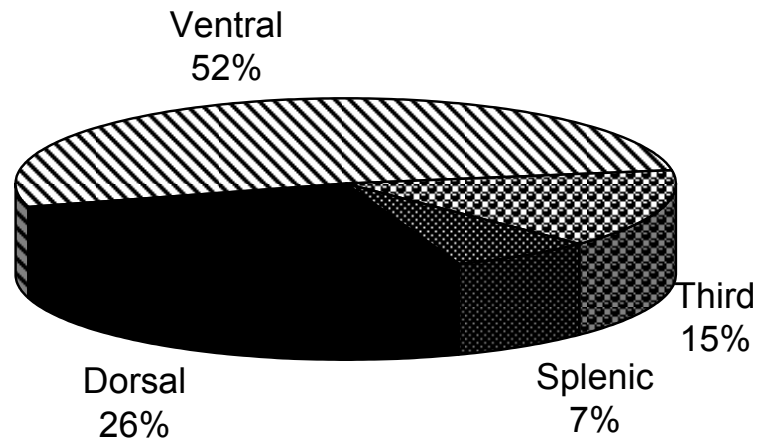
**Figure 2.** Differences in the wet mass (mg) of the lobes of the chicken pancreas. The bars reflect the mean  $\pm$  SEM values for the lobes of chickens that are included in ELISA assays in Figure 6.



**Figure 3.** Western blot analysis showing CaBP<sub>28K</sub> expression in the different lobes of chicken pancreas (left panel). Proteins in 600  $\mu$ g of cytoplasmic extracts from different lobes of chicken pancreas were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. The sheets were probed with a polyclonal rabbit anti-CaBP<sub>28K</sub> antibody. 1.5  $\mu$ g of recombinant CaBP<sub>28K</sub> pure protein (Swant<sup>®</sup>) was used as a standard (right panel). CaBP<sub>28K</sub> protein was visualized as an approximately 28 kDa single immunoreactive band using the chemiluminescent peroxidase method. [a representative experiment is shown].



**Figure 4.** Differential expression of CaBP<sub>28K</sub> in the chicken pancreas. Graphical representation of densitometry analysis of the Western blots data showed that each of the ventral and dorsal lobes had a relative higher CaBP<sub>28K</sub> expression than that of the splenic lobe of chicken pancreas [representative experiment].



**Figure 5.** Relative percentage of CaBP<sub>28K</sub> expression in the different lobes of chicken pancreas. Graphical representation of the relative percentage of CaBP<sub>28K</sub> expression was done based on Western blot densitometry data [representative experiment]. Together, the dorsal and ventral lobes represent 78% of the CaBP<sub>28K</sub> expression in the chicken pancreas.

**Table 1.** Insulin concentration in the different lobes of the chicken pancreas.

Lobes	Wet mass (mg) [mean $\pm$ SEM]	Insulin concentration (ng/mg wet mass) [mean $\pm$ SEM]	Number of samples
Dorsal	438.02 $\pm$ 74.24	14.45 $\pm$ 2.59 <sup>a,b,c</sup>	8
Ventral	347.55 $\pm$ 36.00	8.49 $\pm$ 2.40 <sup>a,d,e</sup>	14
Third	124.12 $\pm$ 15.79	5.99 $\pm$ 0.96 <sup>b,d,f</sup>	8
Splenic	107.95 $\pm$ 6.85	4.58 $\pm$ 1.43 <sup>c,e,f</sup>	6

Comparisons between insulin concentrations of different pancreatic lobes were carried out by unpaired Student's *t* test (one-tailed):

<sup>a</sup> Dorsal v Ventral,  $p = 0.06$

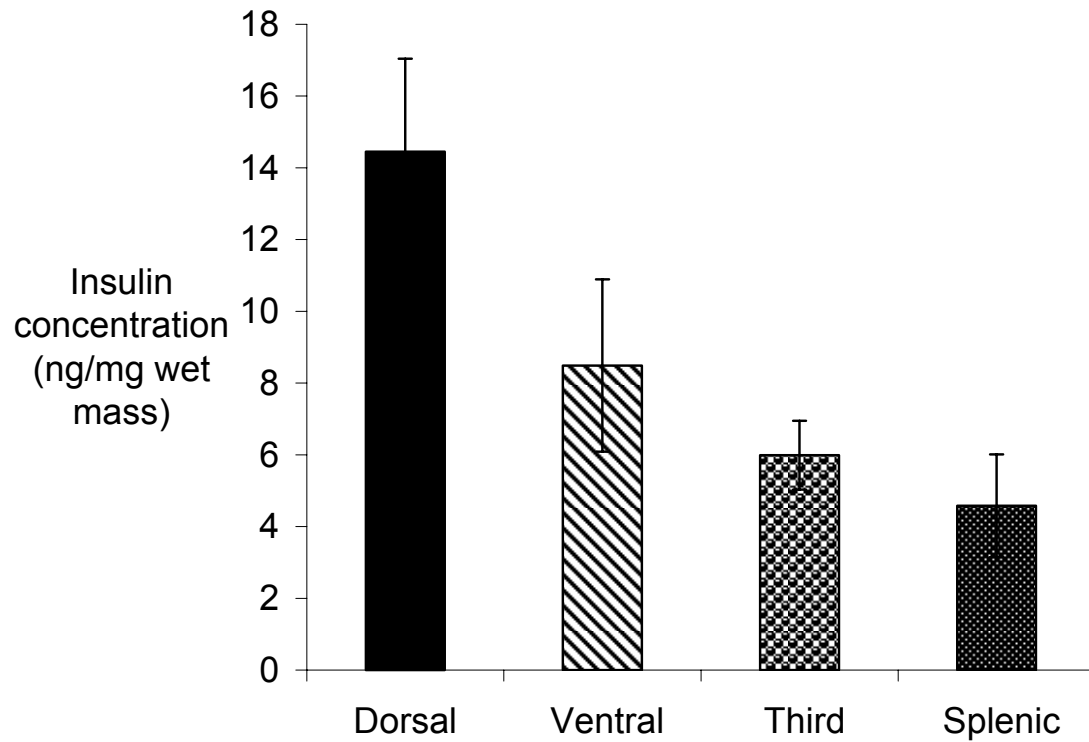
<sup>b</sup> Dorsal v Third,  $p < 0.05$

<sup>c</sup> Dorsal v Splenic,  $p < 0.005$

<sup>d</sup> Ventral v Third,  $p = 0.09$

<sup>e</sup> Ventral v Splenic,  $p < 0.05$

<sup>f</sup> Third v Splenic,  $p = 0.21$



**Figure 6.** Insulin concentration in the different lobes of chicken pancreas. Graphical representation of the insulin concentration as measured by ELISA (mean values  $\pm$  SEM). The highest insulin concentration is in the dorsal lobe and the lowest insulin concentration is in the splenic lobe of chicken pancreas.

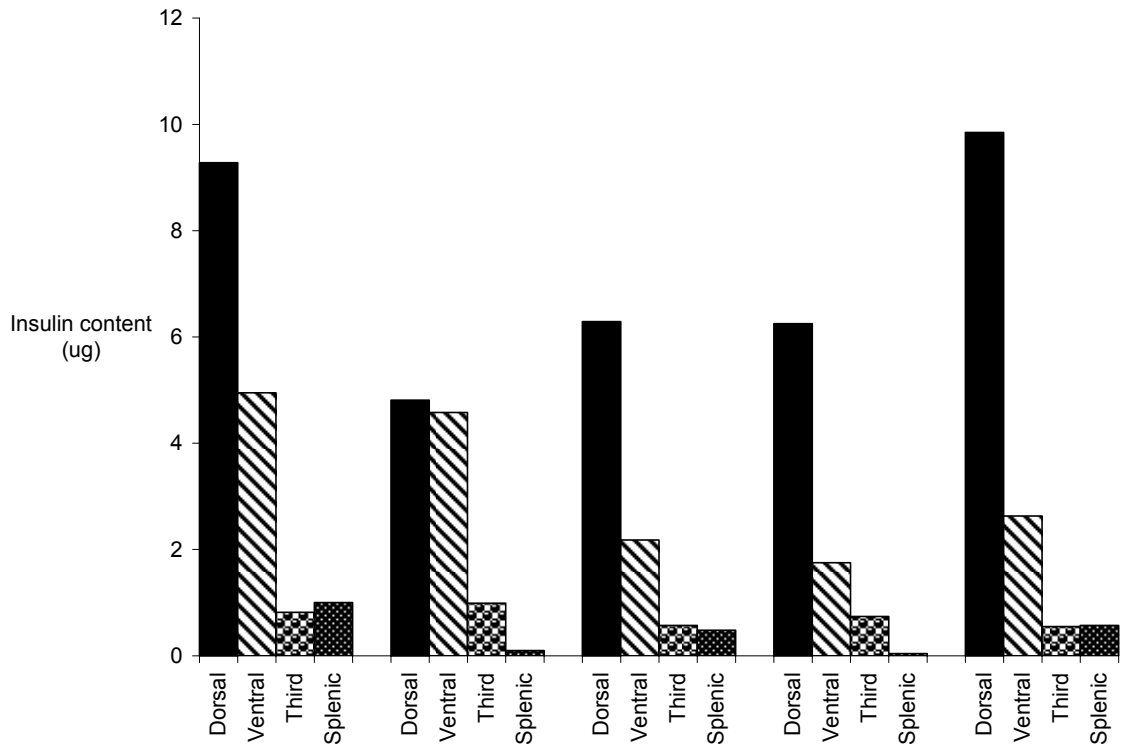
**Table 2A.** Insulin content in the lobes of individual chicken pancreases.

Chicken I.D.	Lobes	Lobe weight (mg)	Insulin conc. (ng/mg wet mass)	Total insulin content ( $\mu\text{g}$ )
1	Dorsal	500.23	18.56	9.28 (57.8%)
	Ventral	287.62	17.20	4.95 (30.9%)
	Third	130.05	6.31	0.82 (5.1%)
	Splenic	123.92	8.05	1.00 (6.2%)
	Whole pancreas	1041.82	15.41	16.05
2	Dorsal	403.04	11.94	4.81 (45.9%)
	Ventral	286.63	15.99	4.58 (43.7%)
	Third	143.77	6.86	0.99 (9.4%)
	Splenic	98.32	1.00	0.10 (1.0%)
	Whole pancreas	931.76	11.25	10.48
3	Dorsal	420.00	14.98	6.29 (66.1%)
	Ventral	238.30	9.15	2.18 (22.9%)
	Third	117.68	4.87	0.57 (6.0%)
	Splenic	108.27	4.38	0.47 (5.0%)
	Whole pancreas	884.25	10.77	9.52

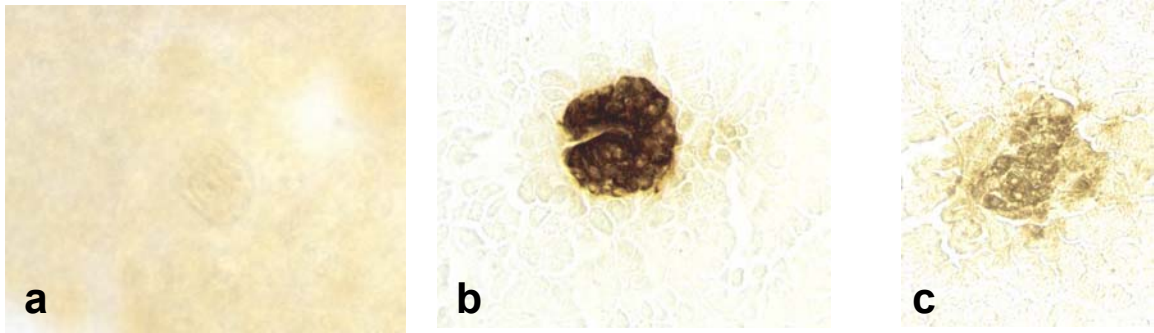


**Table 2B.** Insulin content in the lobes of individual chicken pancreases.

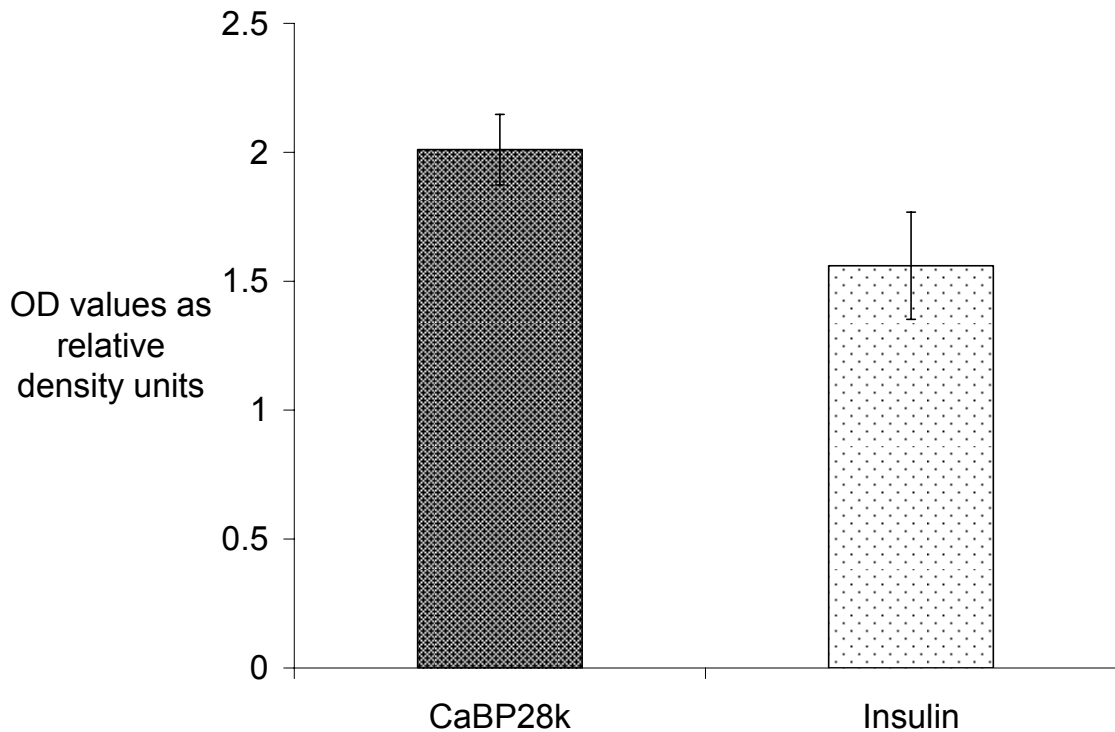
Chicken I.D.	Lobes	Lobe weight (mg)	Insulin conc. (ng/mg wet mass)	Total insulin content ( $\mu\text{g}$ )
4	Dorsal	419.89	14.89	6.25 (71.4%)
	Ventral	203.62	8.59	1.75 (20.0%)
	Third	122.99	6.01	0.74 (8.4%)
	Splenic	80.99	0.22	0.02 (0.2%)
	Whole pancreas	827.49	10.59	8.76
5	Dorsal	510.90	19.27	9.85 (72.4%)
	Ventral	246.64	10.66	2.63 (19.4%)
	Third	114.49	4.78	0.55 (4.0%)
	Splenic	110.09	5.19	0.57 (4.2%)
	Whole pancreas	982.12	13.84	13.60



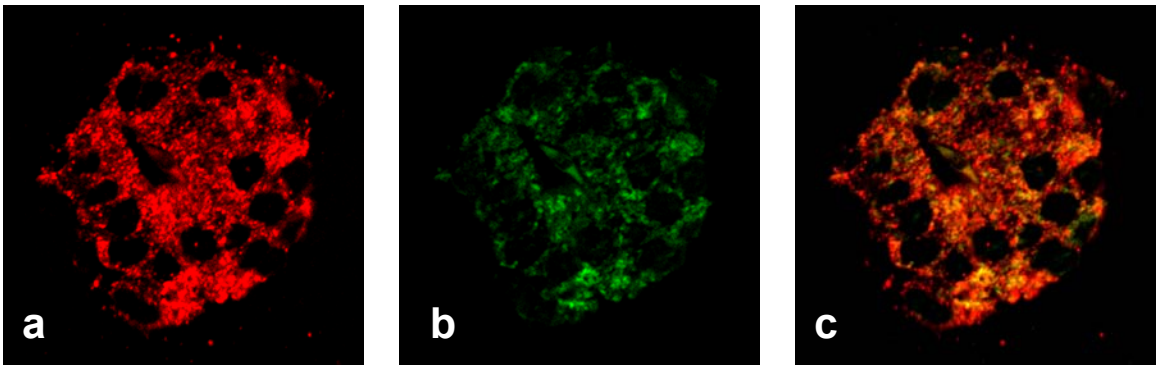
**Figure 7.** Variability of the insulin content in the different lobes of chicken pancreas. This graphical representation reflects the insulin ELISA results for five chickens that had a complete data set. The actual insulin content values are presented in micrograms ( $\mu\text{g}$ ) for each lobe of the chicken pancreas. The dorsal lobes show the highest insulin content, while the splenic lobes show the lowest.



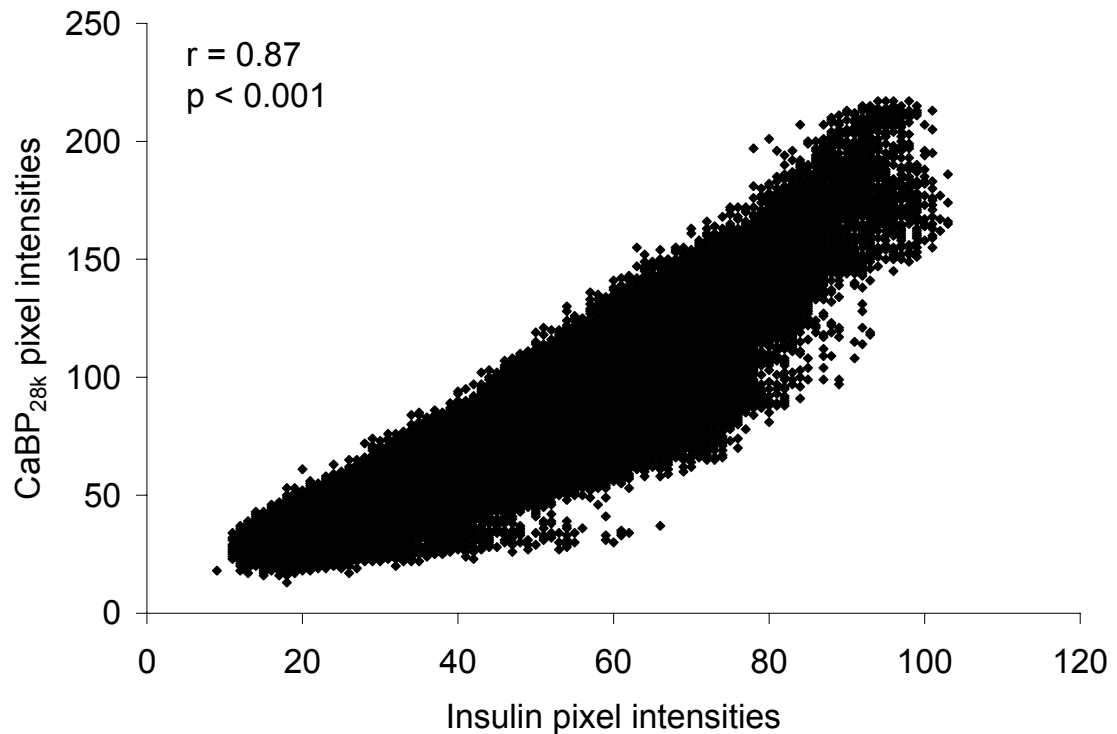
**Figure 8.** Photomicrographs of islets from the ventral lobe of chicken pancreas show immunohistochemical localization of CaBP<sub>28K</sub> and insulin [ $\times 400$ ]. Six micron paraffin sections of the ventral lobe of chicken pancreas were stained immunohistochemically with anti-CaBP<sub>28K</sub> serum (b), and anti-insulin serum (c). Controls for antibody specificity consisted of absorption of the CaBP<sub>28K</sub> mouse monoclonal antibody with an excess of recombinant rat CaBP<sub>28K</sub> purified protein and were negative (a). The pancreatic islets show immunopositive reactions (brown color) to anti-CaBP<sub>28K</sub> serum (b) and anti-insulin serum (c), while no staining for either protein was found in the surrounding exocrine pancreatic tissue. The intensity of reaction product in the islets of the ventral lobes was higher for CaBP<sub>28K</sub> (b) than for insulin (c) (see Figure 9).



**Figure 9.** Quantitative immunohistochemical analysis of the relative CaBP<sub>28K</sub> and insulin expressions in the ventral lobe islets. Based on image densitometry analysis of immunohistochemistry images of the islets from ventral lobes of the chicken pancreas, levels of CaBP<sub>28K</sub> expression were higher than for insulin. These quantitative immunohistochemical differences between CaBP<sub>28K</sub> and insulin relative density units were statistically insignificant in the analyzed pancreatic sections ( $p = 0.25$ ,  $n = 6$ ).



**Figure 10.** Confocal microscopy pseudo-color images of an islet from the ventral lobe of chicken pancreas double-labeled for CaBP<sub>28K</sub> and insulin [ $\times 600$ ]. Sections of pancreas were labeled with antibodies against CaBP<sub>28K</sub> [Texas Red conjugated] and insulin [FITC conjugated]. Immunopositive staining is seen for CaBP<sub>28K</sub> (red color) (a), and insulin (green color) (b). Sites of colocalization exhibit a yellow color (red + green = yellow) in the merged image (c). The positive reaction for both proteins was present only in the islets of Langerhans, with no immunoreactivity in the surrounding acinar tissue. The nuclei and unlabeled areas appear black.



**Figure 11.** Correlation plot of localizations of CaBP<sub>28K</sub> and insulin in islets from the ventral lobe of chicken pancreas. Based on confocal microscopy image data, the pixel intensities of CaBP<sub>28K</sub> and insulin immunopositive areas were statistically evaluated in the islets from all analyzed sections (n = 9) of the ventral lobes of chicken pancreas. The correlation coefficient,  $r = 0.87$ , indicates a strongly positive correlation between the expression of CaBP<sub>28K</sub> and insulin in these  $\beta$ -cell-rich islets. This relationship is highly significant ( $p < 0.001$ , using two factor ANOVA) in the islets from the ventral lobe.

## DISCUSSION

Since its initial discovery in the avian intestine by Wasserman and Taylor (1966), CaBP<sub>28K</sub> has been reported to occur in a variety of species and tissues (see below). Although CaBP<sub>28K</sub> has been described as occurring in the chicken pancreas (Roth et al., 1982), we found no studies reporting its prevalence in the four lobes and its potential colocalization with insulin in the chicken pancreas. CaBP<sub>28K</sub> does bind free intracellular calcium ions, which are an important component of insulin secretion, and Ca<sup>2+</sup> as a second messenger encodes information about the magnitude, frequency, and spatial organization of concentration changes. The functional role of CaBP<sub>28K</sub> in the  $\beta$ -cells of the chicken pancreas has not been determined. In the present study, we determined the levels of expression of CaBP<sub>28K</sub> and insulin in the different lobes of the pancreas. We also show the spatial relationship between insulin and CaBP<sub>28K</sub> in the chicken pancreas.

Our ELISA data demonstrated that both the dorsal and ventral lobes of the chicken pancreas have higher insulin concentrations than the splenic lobe. Our results are in accordance with previous findings that reported the ventral and dorsal lobes contain most of the insulin in the chicken pancreas due to their high content of  $\beta$ -cell-rich islets (Mikami and Ono, 1962; Weir et al., 1976; Hazelwood, 1984). The splenic lobe of the chicken pancreas is considered a minor source of insulin, but a major source of glucagon ( $\alpha$ -islets), while the third lobe contains

both  $\beta$ -islets and  $\alpha$ -islets (Mikami and Ono, 1962; Weir et al., 1976; Roth et al., 1982). The yield of insulin from the chicken pancreas is reported to be about one-tenth that from mammalian pancreas (Kimmel et al., 1968; Langslow et al., 1973; Weir et al., 1976). We report here the insulin concentration of the whole chicken pancreas to be about  $12.37 \pm 0.96$  ng/mg wet mass, which is close to the findings of Kimmel et al. (1968): using acidic ethanol extraction, gel filtration, ion exchange paper chromatography, desalting of insulin fraction then crystallization to isolate chicken insulin, they recorded the insulin concentration of the whole chicken pancreas as 10 – 20 ng/mg wet mass. Our results showed that the mean insulin concentration of the lobes of the chicken pancreas ranged from a low of about 4.5 ng/mg wet mass in the splenic lobe to a high of more than 14 ng/mg wet mass in the dorsal lobe. Previous studies estimated the insulin concentration in the adult human pancreas to be 140 ng/mg wet mass (Sutherland et al., 1976), in the normal male mouse pancreas  $147.9 \pm 12.9$  ng/mg wet mass (Tasaka et al., 1985), and in the bovine pancreas 100 – 150 ng/mg wet mass (Kimmel et al., 1968). Thus, our results confirm that the chicken pancreas has a concentration of insulin about  $1/10^{\text{th}}$  that of mammals, including humans. Furthermore, we found a wide range of variability of insulin content of the different lobes of the chicken pancreas as shown in Tables 2A and 2B. For example, the relative insulin content of the dorsal lobe presented a range from 45.9% to 72.4% of the total insulin content of the pancreas, while that of the ventral lobe was from 19.4% to 43.7% (Tables 2A and 2B).



Using Western blot assays, we found that both the ventral and dorsal lobes of chicken pancreas showed a higher expression of CaBP<sub>28K</sub> than both the third and the splenic lobes. This pattern of CaBP<sub>28K</sub> expression is in accordance with our ELISA data for insulin concentrations in those lobes, suggesting an underlying mechanism affecting expression of both proteins.

Our light microscopic immunohistochemical observations were carried out to compare the localization of CaBP<sub>28K</sub> with insulin in the different lobes of chicken pancreas. We detected the immunoreactive material for both CaBP<sub>28K</sub> and insulin exclusively localized in the islets. Both proteins showed more intense immunoreactions in the islets of dorsal and ventral lobes than other lobes. Previous observations reported that  $\beta$ -cell-rich islets were more numerous in the ventral and dorsal lobes than in other lobes of the chicken pancreas (Kobayashi and Fujita, 1969; Watanabe et al., 1975; Mikami et al., 1986). Our immunohistochemical data confirms and extends previous observations that the distribution of CaBP<sub>28K</sub> immunoreactive material is similar to the insulin immunoreactivity in the  $\beta$ -cells of chicken pancreatic islets (Roth et al., 1982; Buffa et al., 1989). Our densitometric analysis of immunohistochemical images indicates even a higher expression of CaBP<sub>28K</sub> than insulin in the  $\beta$ -cell-rich islets. Previous investigations also found higher levels of CaBP<sub>28K</sub> than insulin in the chicken pancreas.

Christakos et al. (1979) reported by radioimmunoassay (RIA) the concentration of CaBP<sub>28K</sub> in the whole chicken pancreas to be 1.5 µg/mg protein. By ELISA we have found CaBP<sub>28K</sub> concentration in the whole chicken pancreas of  $1.06 \pm 0.2$  µg/mg protein (Sergeev and Rhoten, unpublished data). Contrast these values for CaBP<sub>28K</sub> with the estimated insulin concentration in the whole chicken pancreas of  $12.37 \pm 0.96$  ng/mg wet mass in the present study or 10 – 20 ng/mg wet mass of Kimmel et al. (1968). Thus, there is strong evidence of a higher level of CaBP<sub>28K</sub> than insulin in the chicken pancreas. This differs from the situation in mammals where levels of CaBP<sub>28K</sub> are very low and the protein is not localized to β-cells (Pochet et al., 1987).

We examined the cellular distribution of CaBP<sub>28K</sub> and insulin immunoreactivity in the chicken pancreas by confocal microscopy. We found that the acinar tissue of the chicken pancreas did not show any immunoreactivity for either CaBP<sub>28K</sub> or insulin. We also found that both CaBP<sub>28K</sub> and insulin were localized in the β-cells with no marked nuclear immunoreactivity for either protein in the pancreatic islet cells. We report here, for the first time, colocalization and a strong positive correlation between insulin and CaBP<sub>28K</sub> pixel intensities in the islets from the ventral lobe of the chicken pancreas. These findings highlight a possible role for CaBP<sub>28K</sub> affecting the physiology of insulin inside the β-cells of chicken pancreas.

CaBP<sub>28K</sub> is known for its role in regulating  $[Ca^{2+}]_i$  in many cells, including pancreatic  $\beta$ -cells (Rhoten and Sergeev, 1994; Reddy et al., 1997), and it may alter insulin release from mammalian pancreatic  $\beta$ -cells (Sooy et al., 1999; Parkash et al., 2002). The concentration of free intracellular calcium ions is known to play an important role in glucose-induced insulin release from pancreatic  $\beta$ -cells (Devis et al., 1975; Herchuelz and Malaisse, 1980; Malaisse et al., 1980; Wollheim and Sharp, 1981; Hellman and Gylfe, 1984; Hoenig and Sharp, 1986; Mislser et al., 1992; Gerber and Sudhof, 2002; Donelan et al., 2002; Barg, 2003; Patel, 2003). However, when CaBP<sub>28K</sub> was investigated in mammals by immunohistochemistry and Western blotting it was found in a higher concentration in the non- $\beta$ -cells than in  $\beta$ -cells of the pancreatic islets (Pochet et al., 1987). Nonetheless, the experimental findings by Sooy et al. (1999) and our research data (Parkash et al., 2002) suggested a regulatory role for CaBP<sub>28K</sub> in the control of insulin release in mammals. Isolated pancreatic islets from CaBP<sub>28K</sub> knockout (null mutant) mice and  $\beta$ -cell lines overexpressing CaBP<sub>28K</sub> were used in those studies. Their results showed a significant increase in  $[Ca^{2+}]_i$  and insulin release from the KO mice compared to controls when  $\beta$ -cells were either depolarized with a high concentration of  $K^+$  (Sooy et al., 1999) or exposed to a high concentration of glucose (Parkash et al., 2002). In contrast, there was a marked attenuation in  $[Ca^{2+}]_i$  and insulin secretion from the CaBP<sub>28K</sub> overexpressing cell lines when depolarized with  $K^+$  or exposed to high glucose. Therefore, all of these findings are consistent with CaBP<sub>28k</sub> having an indirect

role at the least, and perhaps a direct role, in regulating hormone release from islet cells.

Human  $\beta$ -cells, like chicken  $\beta$ -cells, contain CaBP<sub>28K</sub> (Pochet et al., 1989; Johnson et al., 1994) and are more resistant to the diabetogenic action of streptozotocin, a  $\beta$ -cytotoxin, than rat or mouse  $\beta$ -cells (Langslow et al., 1970; Meglasson and Hazelwood, 1982; Eizirik et al., 1993, 1994). Our findings of a strong colocalization and significant positive correlation of insulin and CaBP<sub>28K</sub> indicate their close spatial relationship in chicken  $\beta$ -cells. A previous study showed that S-100 protein, a family member related to CaBP<sub>28K</sub>, was colocalized to the core of insulin secretory granules of chicken  $\beta$ -cells (Takayanagi and Watanabe, 1996). Both our previous observations (Rhoten and Sergeev, 1994; Sooy et al., 1999; Parkash et al., 2002) and our current findings support the hypothesis that CaBP<sub>28K</sub> acts as a modulator of insulin release via the regulation of intracellular calcium ion concentration. Moreover, the strong colocalization of insulin and CaBP<sub>28K</sub> suggests an important physiological role for CaBP<sub>28K</sub> in chicken  $\beta$ -cells that may contribute to the relatively high insulin-release threshold and low insulin output in response to insulin secretagogues (NIDDM-like characteristics) of the chicken.

## **CHAPTER 2**

### **GLUCOSE-INDUCED CHANGES IN INTRACELLULAR CALCIUM ION OF CALBINDIN-D<sub>28K</sub> KNOCKOUT COMPARED TO WILDTYPE MICE PANCREATIC ISLETS: *IN VITRO* STUDIES**

## SUMMARY

*In vitro* studies on glucose-stimulated changes in intracellular free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) were done on pancreatic islets of Langerhans from calbindin- $\text{D}_{28\text{k}}$  knockout (CaBP $_{28\text{k}}$ -KO) and wild-type (WT) mice (C57BL6). The dynamics of  $[\text{Ca}^{2+}]_i$  were determined in pancreatic islets in a microincubation chamber on a Nikon Diaphot inverted fluorescence microscope equipped for fluorescent digital ratiometric imaging. Upon increasing the glucose concentration from 2.8 mM to 30 mM in the incubation medium, levels that mimic the transition from fasting to hyperglycemic states, the pancreatic islets of CaBP $_{28\text{k}}$ -KO mice exhibited a significantly greater increase in  $[\text{Ca}^{2+}]_i$  (mean increase in  $[\text{Ca}^{2+}]_i$ , i.e.,  $\Delta [\text{Ca}^{2+}]_i$ , was 296 nM) compared with WT mice ( $\Delta [\text{Ca}^{2+}]_i = 97$  nM,  $p < 0.001$ ). Qualitative differences in the kinetics of the  $[\text{Ca}^{2+}]_i$  were also noted between CaBP $_{28\text{k}}$ -KO and WT islets. The data demonstrate that the levels of calbindin- $\text{D}_{28\text{k}}$  (CaBP $_{28\text{k}}$ ) in  $\beta$ -cells affected the changes in  $[\text{Ca}^{2+}]_i$  in response to glucose. It is well known that the  $[\text{Ca}^{2+}]_i$  in pancreatic  $\beta$ -cells controls the exocytosis of insulin granules. Therefore, these results indicate a significant effect of CaBP $_{28\text{k}}$  in regulating  $[\text{Ca}^{2+}]_i$  in mammalian islets and suggest an important role for CaBP $_{28\text{k}}$  in controlling glucose-induced insulin secretion from the pancreatic  $\beta$ -cell.

## INTRODUCTION

Several previous studies showed that the regulation of  $[Ca^{2+}]_i$  is very crucial for controlling various cellular functions, such as gene expression, cell proliferation, and cell differentiation (Sergeev and Rhoten, 1995; Airaksinen et al., 1997; Sergeev and Rhoten, 1998; Putney, 1999; Sooy et al., 1999). CaBP<sub>28k</sub> belongs to a family of high affinity  $Ca^{2+}$ -binding regulatory proteins that have been shown to play important roles in modulating  $[Ca^{2+}]_i$  in various cell types and thus affecting several biochemical events within the cell including depolarization-induced insulin secretion by  $\beta$ -cells (Sooy et al., 1999). Other high affinity  $Ca^{2+}$ -binding proteins include calmodulin, troponin C, calbindin-D<sub>9k</sub>, parvalbumin, and S100 proteins (Rhoten et al., 1985; Christakos et al., 1989; Heinzmann and Hunziker, 1991; Zimmer et al., 1997). CaBP<sub>28k</sub> is comprised of 261 amino acid residues with a molecular weight of 28 kDa and mainly appears to be a cytoplasmic protein (Rhoten et al., 1985; Christakos et al., 1989; Heinzmann and Hunziker, 1991). CaBP<sub>28k</sub> has at least four high affinity calcium ion binding sites (Hunziker, 1986). The genomic structure of CaBP<sub>28k</sub>, which is well conserved phylogenetically, consists of 11 exons and 10 introns. In humans, the CaBP<sub>28k</sub> gene is located on chromosome 8 (Minghetti et al., 1988; Wilson et al., 1988; Varghese et al., 1989). It was suggested that CaBP<sub>28k</sub> protected against cellular degradation. Rabinovitch et al. (2001) showed that CaBP<sub>28k</sub> can protect  $\beta$ -cells from cytokine-mediated destruction by inhibiting free radical formation. Yenari et al. (2001) found that overexpression of CaBP<sub>28k</sub> in Sprague-Dawley rats led to

neuroprotection against focal stroke. Bellido et al. (2000) reported that the transient expression of rat CaBP<sub>28k</sub> cDNA in MC3T3-E1 osteoblastic cells inhibited tumor necrosis factor alpha (TNF $\alpha$ )-induced apoptosis and caspase-3 activity. In addition, it caused increased calcium buffering, thus showing the anti-apoptotic properties of CaBP<sub>28k</sub>. In HEK 293 cells stably transfected with human recombinant CaBP<sub>28k</sub>, Rintoul et al. (2001) showed that the presence of CaBP<sub>28k</sub> significantly reduced the ionophore 4-Br-A23187-induced rise in [Ca<sup>2+</sup>]<sub>i</sub>. Rat insulinoma cells (RIN1046-38 cells) with elevated levels of CaBP<sub>28k</sub> have increased cytoplasmic Ca<sup>2+</sup> buffering capacity (Rhoten and Sergeev, 1994; Reddy et al., 1997). In summary, several investigators have reported important functional roles for CaBP<sub>28k</sub> in the regulation of [Ca<sup>2+</sup>]<sub>i</sub> in many tissues and organs including pancreatic islet  $\beta$ -cells.

The  $\beta$ -cells are unique in their responses to physiological changes in glucose concentrations, converting metabolic energy into electrical activity (Ishihara et al., 1993). Glucose metabolism via the cytosolic glycolysis pathway and the mitochondrial oxidation of the glycolytic products in  $\beta$ -cells increases the ATP to ADP ratio resulting in the closure of ATP-sensitive K<sup>+</sup> (K<sup>+</sup><sub>ATP</sub>) channels (Prentki and Matschinsky, 1987; Theler et al., 1992; Leech et al., 1994; Aguilar-Bryan et al., 1998; Seino, 1999; Antunes et al., 2000; Schofl et al., 2000; Schuit et al., 2001). This closure of K<sup>+</sup><sub>ATP</sub> channels leads to membrane depolarization that causes activation of L-type voltage-dependent Ca<sup>2+</sup>-channels and an influx of Ca<sup>2+</sup> into the cytoplasm down the electrochemical gradient of Ca<sup>2+</sup> across the



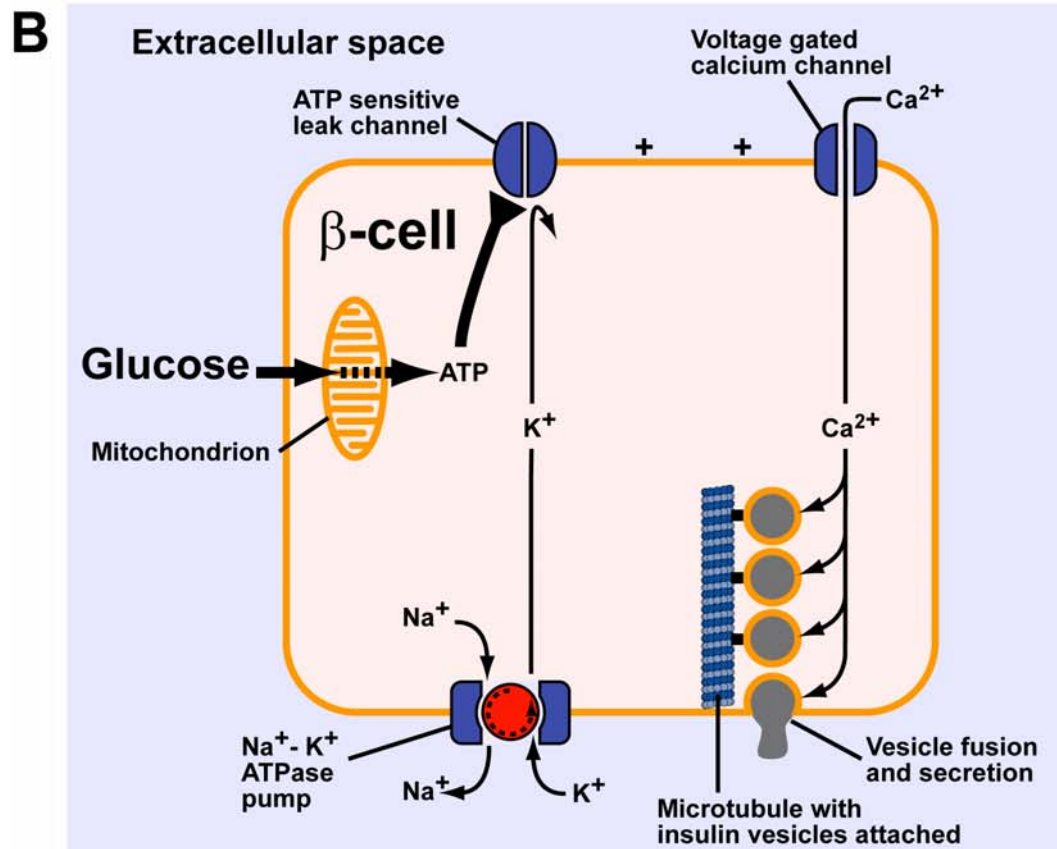
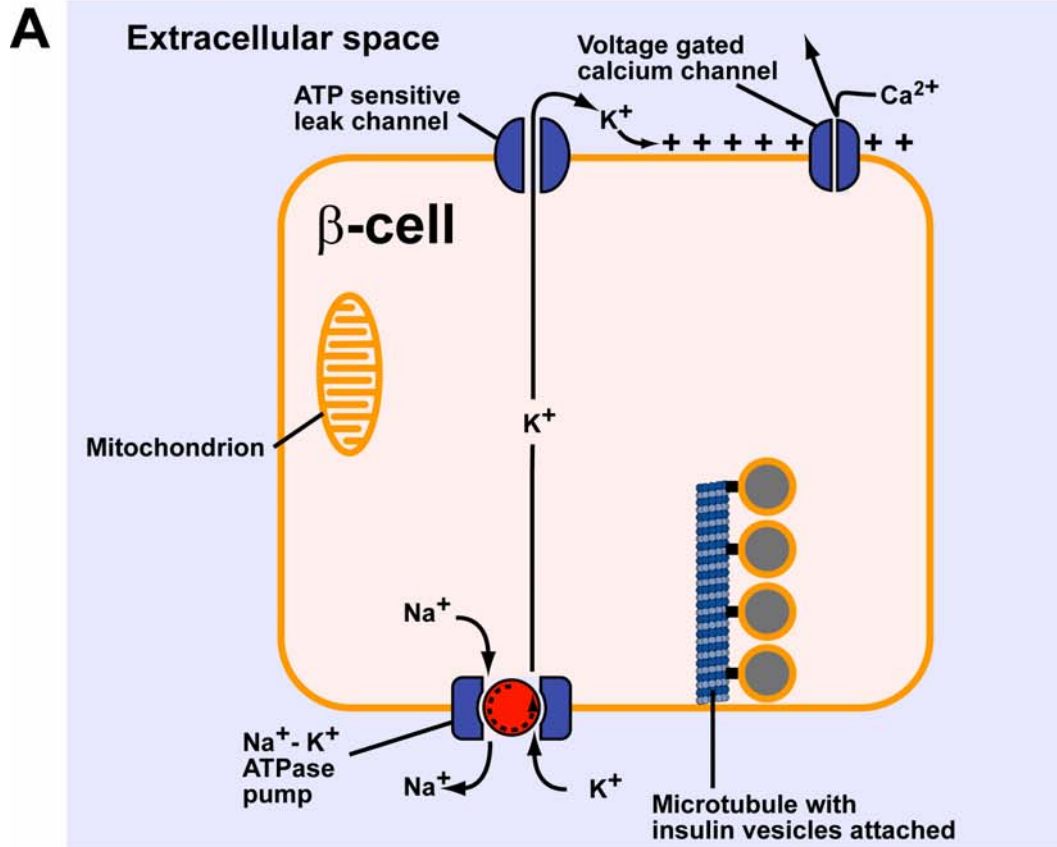
plasma membrane (Roe et al., 1996 and Figure 12). The increase in  $[Ca^{2+}]_i$  of  $\beta$ -cells triggers exocytosis of insulin granules by promoting the fusion of the secretory vesicles with the cell membrane (Jones and Persaud, 1998; Easom, 1999; Lang, 1999 and Figure 12). Zaitsev et al. (1997) have suggested that the defective metabolism of glucose in islets from non-obese diabetic rats results in a delayed response to an increase in  $[Ca^{2+}]_i$ .

In an earlier study, Sooy et al. (1999) found that, following  $K^+$ -depolarization (45 mM KCl),  $[Ca^{2+}]_i$  was significantly greater in isolated pancreatic islets of CaBP<sub>28k</sub> null-mutant (knockout, KO) mice compared with wildtype islets. Conversely,  $\beta$ TC-3 and  $\beta$ HC-13 cells overexpressing rat CaBP<sub>28k</sub> showed markedly reduced  $[Ca^{2+}]_i$  responses to  $K^+$ . It was also shown that CaBP<sub>28k</sub> played a regulatory role in depolarization-induced insulin secretion via control of  $[Ca^{2+}]_i$ . However, the exact mechanism(s) of action of CaBP<sub>28k</sub> in the regulation of  $[Ca^{2+}]_i$  and thereby signal transduction events is not yet clear.

The study presented here is an attempt to understand the interaction(s) of CaBP<sub>28k</sub> in the insulin-secreting  $\beta$ -cells. In this study we explored the effect of glucose, the major nutrient regulator of insulin secretion, on intracellular  $Ca^{2+}$  dynamics in CaBP<sub>28k</sub>-KO and WT pancreatic islets.

**Figure 12.** Schematic diagram of the role of  $[Ca^{2+}]_i$  in glucose-induced insulin secretion from the pancreatic  $\beta$ -cells (modified from Lingappa and Farey, 2000).

(A) In the resting condition, potassium ion ( $K^+$ ) efflux from the  $\beta$ -cell, through the ATP-sensitive  $K^+$ -leak channels, leads to polarization of the cell membrane that prevents  $Ca^{2+}$  entry by closing the voltage-gated calcium channels. (B) When glucose is taken up by the  $\beta$ -cell, its metabolism raises the cytosolic concentration of ATP that in turn closes ATP-sensitive  $K^+$ -leak channels. The prevention of  $K^+$  efflux leads to its accumulation in the  $\beta$ -cell that causes depolarization of the cell membrane and opening of the voltage-gated calcium channels allowing entry of  $Ca^{2+}$  into the  $\beta$ -cell. The increased  $[Ca^{2+}]_i$  stimulates movement of insulin vesicles over the microtubules, insulin vesicle fusion with the cell membrane, and release of insulin from the  $\beta$ -cell [exocytosis].



## **MATERIALS AND METHODS**

### ***Animals***

The mice used in this study (WT and CaBP<sub>28k</sub>-KO mice) were kindly provided by Dr. Sylvia Christakos, Department of Biochemistry and Molecular Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ. The experiments, utilizing WT mice (C57BL6) and CaBP<sub>28k</sub>-KO mice, were conducted in accord with the accepted standards of humane animal care as defined by the Institutional Animal Care and Use Committee of the Joan C. Edwards School of Medicine at Marshall University, WV, USA.

### ***Materials***

Collagenase type V, ethylene glycol-bis-( $\beta$ -amino ethyl ether) N, N'-tetraacetic acid (EGTA), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), bovine serum albumin (BSA), ionomycin, dimethyl sulfoxide (DMSO), the primary antibody against CaBP<sub>28K</sub> (monoclonal mouse anti-CaBP<sub>28K</sub>), the secondary antibody goat anti-mouse IgG alkaline phosphatase conjugate, Sigma Fast™ 5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine/nitro-blue tetrazolium chloride (BCIP/NBT) buffered substrate tablets, Tween 20®, and MnCl<sub>2</sub> were obtained from Sigma Chemical Company (St. Louis, MO). Protran® pure nitrocellulose transfer membranes were ordered from Schleicher & Schuell Bioscience (Keene, NH). Cell-Tak was purchased from Becton-Dickinson Labware (Bedford, MA). Hank's balanced salt solution (HBSS) was purchased

from Gibco BRL<sup>®</sup> Life Technologies (Grand Island, NY). Fura-2 acetoxy methyl ester dye (Fura-2), and Pluronic F-127 were obtained from Molecular Probes Inc. (Eugene, OR). All other chemicals and reagents were of analytical grade.

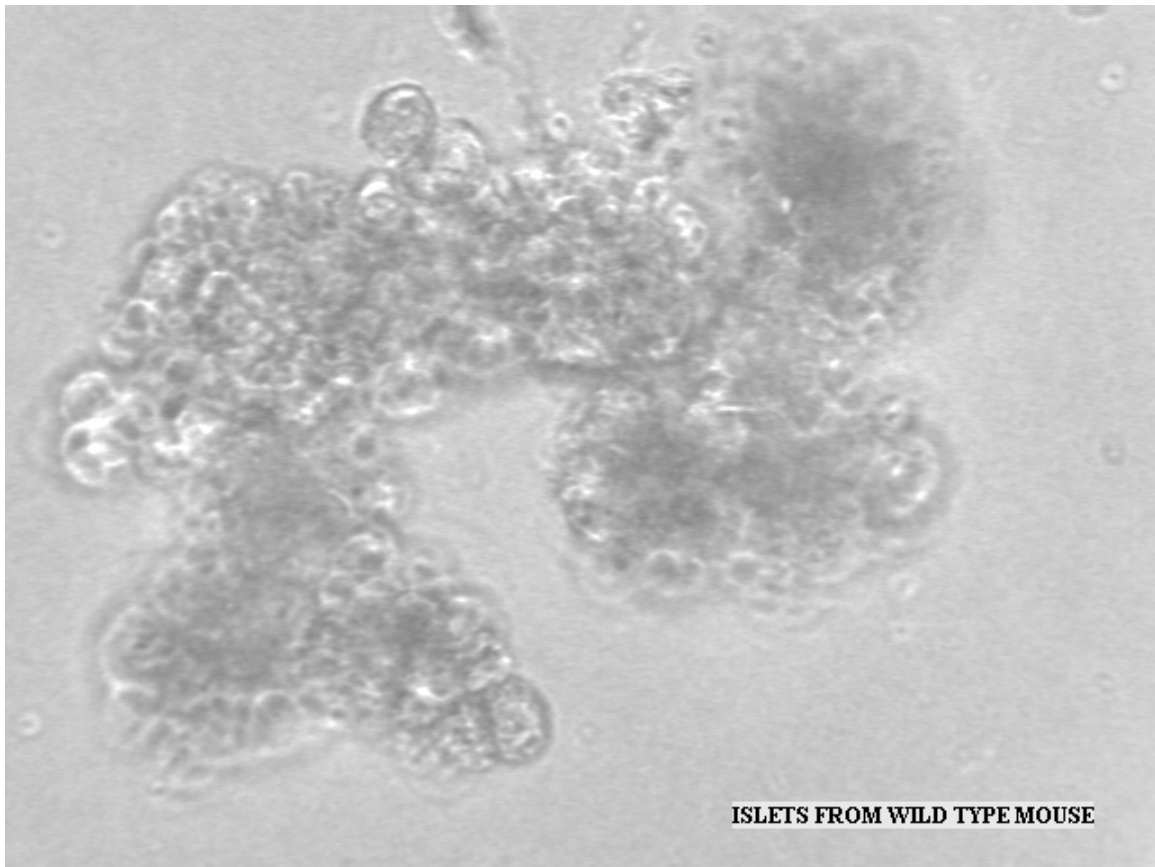
### ***Isolation and preparation of murine pancreatic islets***

A protocol of multiple injections of HBSS into the pancreas *in situ* was applied and modified to achieve a maximal yield of viable pancreatic islets of Langerhans. The mice were fasted overnight. After using anesthesia (ketamine 100 µg/g body mass, and xylazine 25 µg/g body mass), HBSS was injected into the pancreas to inflate the tissue. The islets were dispersed from the surrounding pancreas. The distended pancreas was transferred to a petri dish and under a dissecting microscope fat, lymph nodes and extraneous fibrous tissues were removed. The pancreas was then chopped into small pieces. This mince was transferred to a tissue culture tube and allowed to sediment. The amount of type V collagenase (w/v) required to digest the pancreas was calculated and added to the tissue culture tube. The tube with the minced pancreas was placed in a reciprocating wrist action shaker Model 75 (Burrell Corporation, Pittsburgh, PA) in a water bath (Fisher Scientific Company, Pittsburgh, PA) at 37°C for 9 – 11 min. The digestion end point was the appearance of white sandy spots on the tube wall. The reaction was stopped by adding excess cold HBSS and vortex mixing. Then a series of centrifugations were carried out, discarding the supernatant, and resuspending the pellets in cold HBSS and vortex mixing again [repeated five times at centrifugation speeds of 1000, 500, 250, 187.5, 125 and

62.5 × g]. After the last centrifugation, the sediment was resuspended in HBSS and poured into a petri dish for handpicking of islets using a thin, glass looped-rod under a dissecting microscope as previously described (Clements and Rhoten, 1976).

### ***Fura-2 dye loading***

Isolated islets of WT and CaBP<sub>28k</sub>-KO mice were loaded *in vitro* with 5 μM Fura-2 in a modified Krebs-Ringer solution with 0.5% DMSO and 0.05% (w/v) Pluronic F-127 (a surfactant that aids in dispersing the Fura-2 dye in the aqueous loading medium) for 45 min. at 37°C. After three washes with HBSS, the islets were further incubated in HBSS for 15 min. at 37°C. The modified Krebs-Ringer medium was composed of: 118 mM NaCl, 4.8 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 5 mM NaHCO<sub>3</sub>, 5.5 mM glucose, and 0.1% w/v BSA, pH 7.40. The pancreatic islets were then suspended in 100 μl of HBSS and placed on a Cell-Tak coated, 25 mm. circular microscope cover glass (Fisherbrand®) [Fisher Scientific Company, Pittsburgh, PA]. The islets were allowed to settle and adhere to the surface of the cover glass for 15 min. at room temperature (Figure 13). Then, 1 ml of the modified Krebs-Ringer medium was added and the glucose concentration adjusted so that the final concentration of glucose in the basal incubation medium was 2.8 mM.



**Figure 13.** Phase contrast light microscopy live image of murine islets of Langerhans. The islets, prepared from a wildtype (WT) mouse, are seen as aggregates in culture incubation medium (modified Krebs-Ringer medium containing 2.8 mM glucose). These aggregates of WT mouse pancreatic islets were used in the experiments shown in Figures 14 and 15. The phase contrast live image acquisition was facilitated using MetaMorph Imaging System software version 4.6r5 (Universal Imaging Corporation, Westchester, PA).

### ***[Ca<sup>2+</sup>]<sub>i</sub> measurements and digital video fluorescence imaging***

The Fura-2 loaded pancreatic islets, attached to the cover glass, were transferred into a Narishige microincubation chamber that was placed on the stage of a Nikon Diaphot TMD inverted fluorescent microscope (Nikon Corporation, Tokyo, Japan). The ratio fluorescence microscopy experiments were carried out with the incubation medium temperature controlled at 37°C. The pancreatic islets were then successively excited at 340 nm & 380 nm, and the fluorescence emitted at 510 nm was intensified by a DAGE-MTI GenII Sys image intensifier and captured by a DAGE-MTI CCD72 video camera (DAGE-MTI, Inc., Michigan City, IN). Metafluor Imaging System software version 4.1.7 (Universal Imaging Corporation, Westchester, PA) was used for image acquisition and analysis. All images were corrected for the background emission. The [Ca<sup>2+</sup>]<sub>i</sub> was calculated according to the equation used by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_D \beta (R - R_{min}) / (R_{max} - R)$$

Where:

**K<sub>D</sub>** is the dissociation constant for Fura-2 binding to Ca<sup>2+</sup> and is = 224 nM.

**β** is the ratio of fluorescence of Ca<sup>2+</sup>-free Fura-2 to the fluorescence of Ca<sup>2+</sup>-saturated Fura-2 at 380 nm excitation.

**R** is the ratio of Fura-2 fluorescent intensity with 340 nm excitation to the Fura-2 fluorescent intensity with 380 nm excitation.

**R<sub>min</sub>** is the fluorescent ratio when Fura-2 is Ca<sup>2+</sup>-free.

**R<sub>max</sub>** is the fluorescent ratio when Fura-2 is Ca<sup>2+</sup>-saturated.



### **Western blotting**

CaBP<sub>28k</sub>-KO and WT mice were sacrificed and their kidneys were used for Western blotting. We selected the kidneys for Western blotting because kidneys of WT mice contain much higher levels of CaBP<sub>28k</sub> than do pancreata. After homogenization in protein lysis buffer, the samples were centrifuged at 14,000 × *g* for 20 min. at 4°C. The supernatant was obtained and total protein estimated using the Bradford method (Bradford, 1976). Equal amounts of total protein from CaBP<sub>28k</sub>-KO and WT kidneys were loaded per lane in 12% SDS polyacrylamide gels. The standard molecular weight markers used were of 193, 86.8, 47.8, 33.3, 28.6, and 20.7 kDa. SDS polyacrylamide gel electrophoresis (PAGE) was performed. After protein transfer, nitrocellulose membranes were blocked in Tris-buffered saline with 3% BSA and then incubated with the primary antibody against CaBP<sub>28k</sub> (monoclonal mouse anti-CaBP<sub>28K</sub>). After washes, alkaline phosphatase-conjugated secondary antibody was applied. The specific protein bands were visualized using Sigma Fast™ BCIP/NBT as a precipitating substrate for the detection of alkaline phosphatase activity (Blake et al., 1984).

### **Statistical analysis**

Results are presented as the mean ± standard error of the mean (SEM). The differences in the [Ca<sup>2+</sup>]<sub>i</sub> between the WT and KO mice islets were analyzed for statistical significance using the unpaired Student's *t* test (one-tailed) with a significance level of 0.05 (SigmaStat software, version 2.03 from SPSS Inc., Chicago, IL).

## RESULTS

### ***Glucose-induced changes in $[Ca^{2+}]_i$ in WT pancreatic islets***

An immediate increase in  $[Ca^{2+}]_i$  occurred in pancreatic islets prepared from WT mice upon increasing glucose concentration of the incubation medium to 30 mM (Figure 14). The peak in  $[Ca^{2+}]_i$  was observed 1 - 2 min. after glucose addition (Figure 15). This peak in  $[Ca^{2+}]_i$  was followed by a decline and then a slow rise in  $[Ca^{2+}]_i$  to a plateau seen 20 - 25 min. after the addition of 30 mM glucose (Figure 15). The maximum increase in  $[Ca^{2+}]_i$  in WT islets in response to high glucose ranged between 68 nM to 120 nM (mean increase in  $[Ca^{2+}]_i$ ,  $\Delta [Ca^{2+}]_i$ , was =  $96.7 \pm 8.4$  nM, n = 3) [Table 3]. The initial rate of increase in  $[Ca^{2+}]_i$ , the rate of decline in  $[Ca^{2+}]_i$  after attaining the peak, and the rate of slow rise to a plateau value, as well as the magnitude of changes in  $[Ca^{2+}]_i$  varied from islet to islet (Figure 15). The basal values of  $[Ca^{2+}]_i$  in islets obtained in the presence of 2.8 mM glucose remained constant (0 - 5 min., Figure 15), thus showing that the observed increase in  $[Ca^{2+}]_i$  upon addition of 30 mM glucose is due to the physiological substrate.

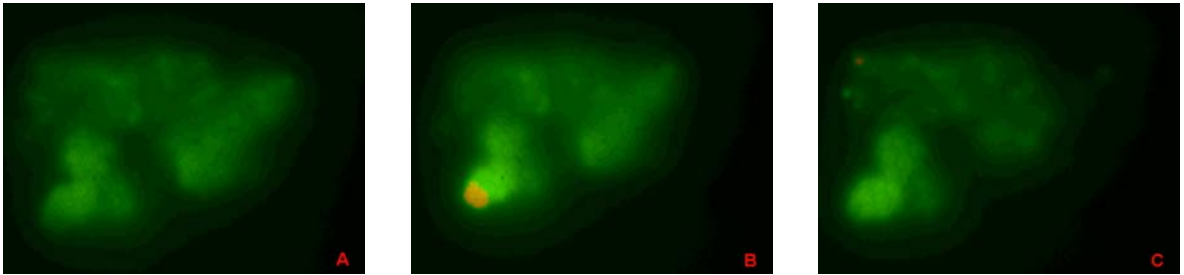
### ***Glucose-induced changes in $[Ca^{2+}]_i$ in CaBP<sub>28k</sub>-KO pancreatic islets***

When glucose concentration was increased in the incubation medium to 30 mM, pancreatic islets prepared from CaBP<sub>28k</sub>-KO mice showed a much greater increase in  $[Ca^{2+}]_i$  (approximately 3 times) as compared to that in WT mice islets (Figures 15, 17 and 19). The time course of this increase in  $[Ca^{2+}]_i$

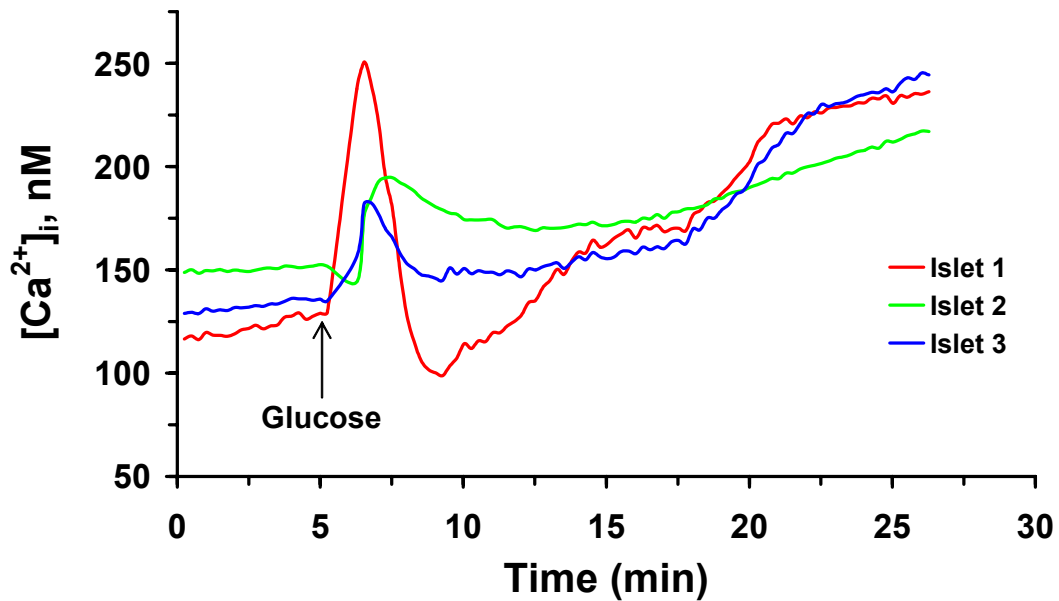
was similar to that in WT islets (compare Figures 15 and 17, note differences in scales for  $[Ca^{2+}]_i$ ). The glucose-induced increase in  $[Ca^{2+}]_i$  was observed about 1 min. after high glucose addition (Figures 16 and 17). The increase in  $[Ca^{2+}]_i$  upon the addition of high glucose to the CaBP<sub>28k</sub>-KO islets ranged from 200 to 366 nM during the 20 - 25 min. recording period ( $\Delta [Ca^{2+}]_i = 296.3 \pm 21.2$  nM, n = 3) [Table 3 and Figure 19] and was significantly greater ( $p < 0.001$ ) when compared to that in WT islets (Table 3 and Figure 19). The digital video images showing the effects of adding high glucose to the incubation medium on a single pancreatic islet taken from a CaBP<sub>28k</sub>-KO mouse are presented in Figure 18.

### ***Western blotting***

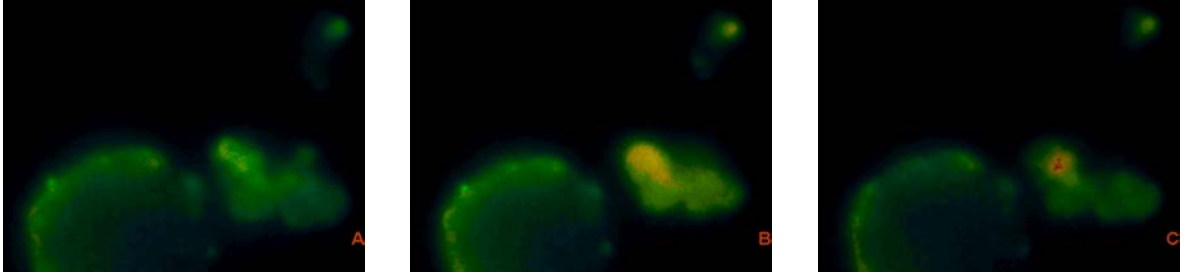
CaBP<sub>28k</sub>-KO and WT mice kidney homogenates were analyzed to confirm the absence of CaBP<sub>28k</sub> in KO mice as shown in Figure 20.



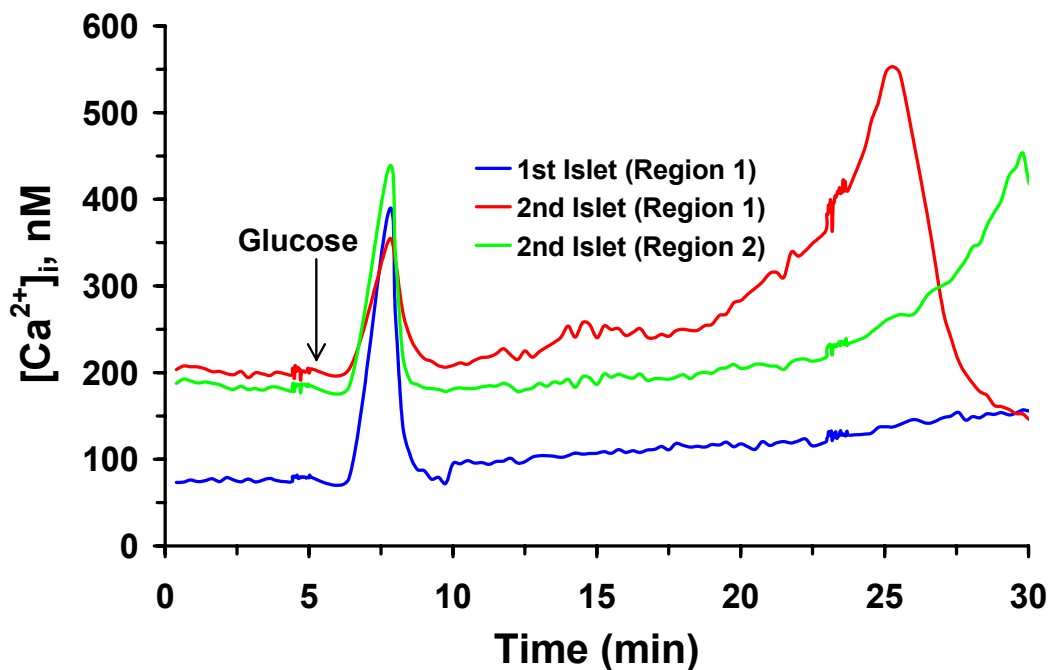
**Figure 14.** Fura-2 ratio fluorescence images show aggregates of WT mouse pancreatic islets of Langerhans incubated in a modified Krebs-Ringer medium containing 2.8 mM glucose (A); then after 1 min. (B), and after 20 min. (C) of addition of 30 mM glucose. Note the change in color intensity from green (A) to yellow (B and C) that indicates increase in  $[Ca^{2+}]_i$



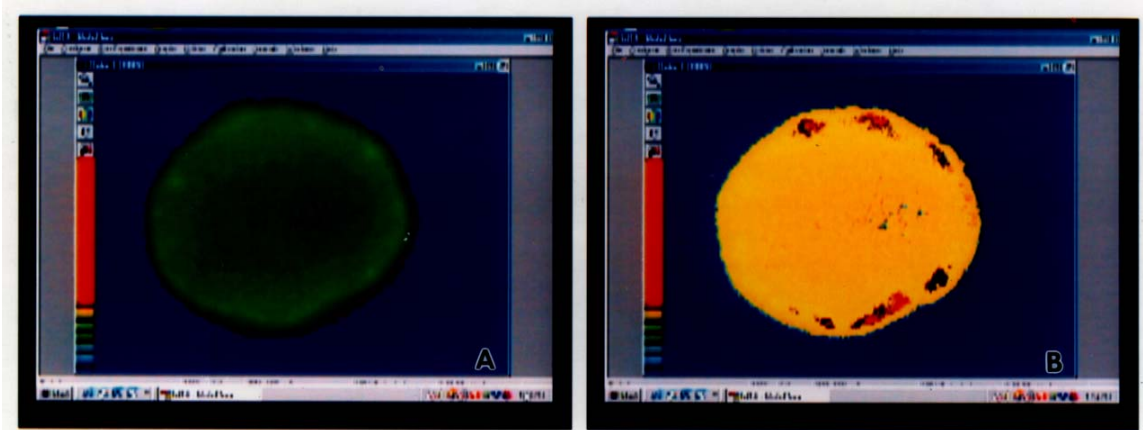
**Figure 15.** Kinetics of the glucose-induced increase in  $[Ca^{2+}]_i$  in WT islets. The WT islets incubated in a modified Krebs-Ringer medium containing 2.8 mM glucose were exposed to 30 mM glucose (at the time shown by arrow) and the time course of changes in  $[Ca^{2+}]_i$  was measured using Fura-2 ratio fluorescent microscopy.



**Figure 16.** Fura-2 ratio fluorescence images show aggregates of CaBP<sub>28k</sub>-KO mouse pancreatic islets of Langerhans incubated in a modified Krebs-Ringer medium containing 2.8 mM glucose (A); then after 1 min. (B), and after 20 min. (C) of addition of 30 mM glucose. Note the change in color intensity from green (A) to yellow-red (B and C) that indicates an increase in  $[Ca^{2+}]_i$ .



**Figure 17.** Kinetics of the glucose-induced increase in  $[Ca^{2+}]_i$  in  $CaBP_{28k}$ -KO islets. The  $CaBP_{28k}$ -KO islets were incubated in a modified Krebs-Ringer medium containing 2.8 mM glucose and were exposed to 30 mM glucose (at time shown by arrow). The time course of changes in  $[Ca^{2+}]_i$  was measured using Fura-2 ratio fluorescent microscopy.



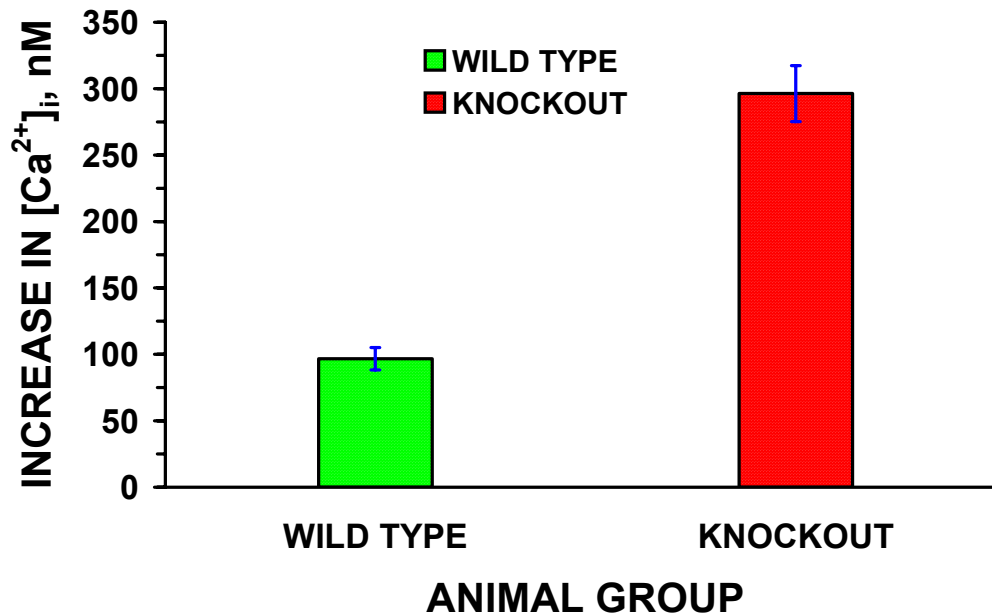
**Figure 18.** Ratio fluorescent images illustrating the effects of adding high glucose to the incubation medium on a single pancreatic islet taken from a CaBP<sub>28k</sub>-KO mouse. The pancreatic islet was loaded with Fura-2 dye and incubated in a modified Krebs-Ringer medium containing 2.8 mM glucose (A, left panel), and 20 min. after the addition of 30 mM glucose (B, right panel). The pancreatic islet of Langerhans was dually excited at 340 nm & 380 nm, and the fluorescence emission was measured at 510 nm. The digital video images were acquired using MetaFluor Imaging System software version 4.1.7 (Universal Imaging Corporation, Westchester, PA). The change in color intensity from green (A, left panel) to yellow-red 20 min. after addition of 30 mM glucose (B, right panel) indicates a very marked increase in  $[Ca^{2+}]_i$ .



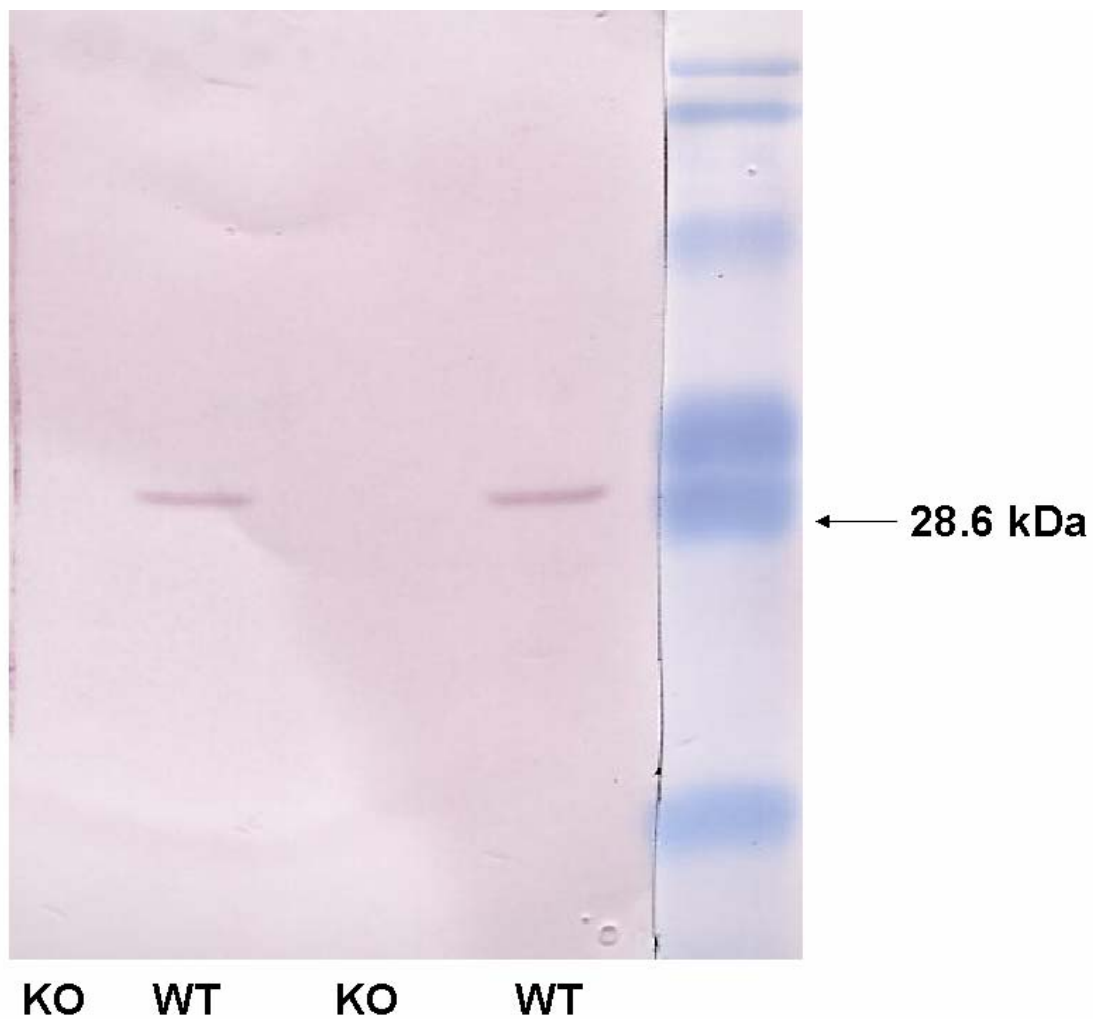
**Table 3.** Comparison of  $\Delta[\text{Ca}^{2+}]_i$  for CaBP<sub>28k</sub>-KO versus WT mice pancreatic islets in response to glucose stimulation.

Pancreatic islets	$[\text{Ca}^{2+}]_i$ , nM	$[\text{Ca}^{2+}]_i$ , nM	$\Delta[\text{Ca}^{2+}]_i$ , nM <sup>a</sup>
	2.8 mM glucose	30 mM glucose	
<b>A. <u>WT mice</u></b>			
Islet 1	117	236	119
Islet 2	149	217	68
Islet 3	129	244	115
<b>B. <u>CaBP<sub>28k</sub>-KO mice</u></b>			
Islet 1 (Region 1)	78	384	306
Islet 2 (Region 1)	197	553	356
Islet 2 (Region 2)	183	469	286

<sup>a</sup> indicates nanomolar increase in the  $[\text{Ca}^{2+}]_i$  upon increasing glucose concentration in the incubation medium from 2.8 mM to 30 mM ( $p < 0.001$ )



**Figure 19.** Bar graph showing the relative glucose responsiveness in WT and KO mice islets presented as the mean increase in  $[Ca^{2+}]_i \pm$  SEM during the 25 min. of 30 mM glucose incubation (from experiments presented in Figures 15 and 17 and Table 3). The mean increase in  $[Ca^{2+}]_i$  in KO islets was about three times more than that in WT islets ( $p < 0.001$ ).



**Figure 20.** CaBP<sub>28k</sub>-KO mice do not express CaBP<sub>28k</sub>. Western blot of kidney homogenates from WT (2<sup>nd</sup> and 4<sup>th</sup> lanes from the left) and KO mice (1<sup>st</sup> and 3<sup>rd</sup> lanes from the left) carried out using monoclonal mouse anti-CaBP<sub>28k</sub> antibody shows the absence of CaBP<sub>28k</sub> in KO mice. The standard molecular weight markers are shown in the rightmost lane with molecular mass (from top to bottom) of 139, 86.8, 47.8, 33.3, 28.6, and 20.7 kDa.

## DISCUSSION

In order to study the role of CaBP<sub>28k</sub> in glucose-induced changes in intracellular Ca<sup>2+</sup> in β-cells, we used a transgenic knockout (CaBP<sub>28k</sub>-KO, null mutant) mouse model devoid of the CaBP<sub>28k</sub> gene (Airaksinen et al., 1997). Our results show that the [Ca<sup>2+</sup>]<sub>i</sub> responses to glucose in CaBP<sub>28k</sub>-KO pancreatic islets were at least 3 times greater (p < 0.001) than that of WT pancreatic islets (in the presence of normal levels of CaBP<sub>28k</sub> protein). A previous study showed that membrane depolarization induced by addition of 45 mM KCl to the islets caused an approximately 3.5 times greater increase in the [Ca<sup>2+</sup>]<sub>i</sub> in CaBP<sub>28k</sub>-KO pancreatic islets compared to pancreatic islets prepared from WT mice (Sooy et al., 1999). Therefore, our data using glucose as a physiological secretagogue support the previous observation of a several fold greater increase in [Ca<sup>2+</sup>]<sub>i</sub> in CaBP<sub>28k</sub>-KO islets. The increase in [Ca<sup>2+</sup>]<sub>i</sub> upon addition of glucose in the present study or KCl (Sooy et al., 1999) was transient in nature thereby emphasizing depolarization-induced influx of Ca<sup>2+</sup> across the cell membrane.

As is evident in our results, the presence of 30 mM glucose caused a plateau in [Ca<sup>2+</sup>]<sub>i</sub> in WT mouse islets, and a second peak in [Ca<sup>2+</sup>]<sub>i</sub> in CaBP<sub>28k</sub>-KO mice islets after about 20 - 25 min. The presence or absence of a plateau in [Ca<sup>2+</sup>]<sub>i</sub> has been reported in several studies involving islets derived from mouse and rat (Martin et al., 1995; Bertuzzi et al., 1999; Antunes et al., 2000), and could be correlated in the present study to the maintenance of the membrane

depolarization of  $\beta$ -cells by the action of the glucose, in addition to closure of  $K^+_{ATP}$  channels. The maintenance of membrane depolarization in  $\beta$ -cells is also dependent upon other mechanisms including a  $Na^+/Ca^{2+}$  exchanger and activation of  $Ca^{2+}$ -dependent  $K^+$  channels, with the outward  $K^+$  current contributing to rhythmic firing of action potentials in pancreatic  $\beta$ -cells (Van Eylen et al., 1998; Gopel et al., 1999). In studies involving Purkinje neurons from  $CaBP_{28k}$ -KO mice, Airaksinen et al. (1997) have shown much larger amplitude of synaptically evoked calcium ion transients as compared to WT mice. Their result correlates well with the larger magnitude of the glucose-induced rise in  $[Ca^{2+}]_i$  in  $CaBP_{28k}$ -KO islets compared to WT islets described above. These observations based on the removal of  $CaBP_{28k}$  from such diverse cell-types as neurons on one hand and pancreatic islet  $\beta$ -cells on the other hand from  $CaBP_{28k}$ -KO mice, support a role for  $CaBP_{28k}$  in the regulation of cellular calcium and  $Ca^{2+}$ -dependent processes (Airaksinen et al., 1997; Sooy et al., 1999).

In our earlier study on  $\beta$ HC-13  $CaBP_{40}$  cells, we showed that the overexpression of  $CaBP_{28k}$  caused an almost complete absence of the typical glucose-induced rise in  $[Ca^{2+}]_i$  compared to control  $\beta$ HC-13 cells that had a very substantial increase in  $[Ca^{2+}]_i$  of  $550 \pm 19$  nM (Parkash et al., 2002). The absence of a glucose-induced rise in  $[Ca^{2+}]_i$  responses by  $\beta$ HC-13  $CaBP_{40}$  cells supports further a role for  $CaBP_{28k}$  in modulating  $Ca^{2+}$ -dependent processes. One mechanism of action of  $CaBP_{28k}$  in  $\beta$ HC-13  $CaBP_{40}$  cells may be intracellular calcium buffering. An earlier study with RIN cells demonstrated a

large increase in cytoplasmic  $\text{Ca}^{2+}$  buffering capacity in RIN cells with levels of  $\text{CaBP}_{28\text{K}}$  about 3-fold higher than in control cells (Rhoten and Sergeev, 1994). In cells with elevated  $\text{CaBP}_{28\text{K}}$  the  $[\text{Ca}^{2+}]_i$  responses to glucose,  $\text{K}^+$ -depolarization, a calcium ionophore (ionomycin), and the  $\text{Ca}^{2+}$  channel agonist Bay K 8644 were altered, but basal  $[\text{Ca}^{2+}]_i$  was unchanged (Parkash et al., 2002).  $[\text{Ca}^{2+}]_i$  peak values were reduced and the increase in  $[\text{Ca}^{2+}]_i$  in response to mobilization of  $\text{Ca}^{2+}$  from intracellular stores was also attenuated. A high concentration of ionomycin (5  $\mu\text{M}$ ) overcame the buffering effect of  $\text{CaBP}_{28\text{K}}$  and produced a dramatic rise in  $[\text{Ca}^{2+}]_i$  (Parkash et al., 2002). The finding of buffering of  $\text{Ca}^{2+}$  by moderately elevated levels of  $\text{CaBP}_{28\text{K}}$  in RIN cells was confirmed in RIN cells transfected with rat  $\text{CaBP}_{28\text{K}}$  and markedly overexpressing (6 - 35 fold increase) this protein (Reddy et al., 1997). Evidence of  $\text{CaBP}_{28\text{K}}$  as a mediator of other rapid  $\text{Ca}^{2+}$  responses include translocation of  $\text{Ca}^{2+}$  in intestine (Nemere et al., 1986, 1991), activation of  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ -ATPase (Morgan et al., 1986),  $\text{Ca}^{2+}$ -ATPase and phosphodiesterase (Reisner et al., 1992), and altered  $\text{Ca}^{2+}$  fluxes in luminal vesicles of distal convoluted tubules (Bouhtiauy et al., 1994). Yet, further experiments are certainly warranted to improve our understanding of the roles of  $\text{CaBP}_{28\text{K}}$  in the pancreatic islet  $\beta$ -cells.

## **CHAPTER 3**

# **EFFECTS OF INHIBITION OF CALBINDIN-D<sub>28K</sub> GENE EXPRESSION BY ANTISENSE OLIGONUCLEOTIDE TRANSFECTION IN CULTURED RAT INSULINOMA CELLS**

## SUMMARY

The effects of antisense oligonucleotides (AS-ON) to calbindin-D<sub>28K</sub> (CaBP<sub>28k</sub>) on gene and protein expression were examined *in vitro* in cultured pancreatic  $\beta$ -cells. Rat insulinoma cells (RIN1046-38), a pancreatic  $\beta$ -cell line, were seeded at  $\sim 1 - 3 \times 10^6$  cells/25 cm<sup>2</sup> in tissue culture flasks and cultivated in RPMI1640 medium. Results of the application of AS-ON targeted against CaBP<sub>28k</sub> were compared with control nonsense oligonucleotides (NS-ON). ON were delivered using Lipofectamine 2000 reagent and uptake was confirmed by fluorescence microscopy imaging of fluorescein-labeled AS-ON. Western blots showed that 200 nM phosphorothioate AS-ON (PS-AS-ON) produced complete inhibition of CaBP<sub>28k</sub> protein. This result was further confirmed using the more potent propyne AS-ON (PY-AS-ON) at 20 nM. mRNA levels in cells treated with NS-ON and AS-ON were assessed by cDNA microarray analysis in 200 nM PS-AS-ON transfected cells. Expression of the vitamin D receptor (VDR) and calbindin-D<sub>9k</sub> mRNAs were up-regulated. Western blotting further demonstrated that the VDR protein was up-regulated and calbindin-D<sub>9k</sub> protein was expressed in the 200 mM PS-AS-ON transfected cells. These results are notable as the first demonstration of a compensatory calbindin-D<sub>9k</sub> expression by AS-ON to calbindin-D<sub>28k</sub>. Finally, ELISA showed a significantly greater increase in insulin release in response to secretagogues from cells transfected with PS-AS-ON compared to PS-NS-ON. Additional studies are required to understand calbindin-D<sub>9k</sub>, VDR and calcium ions in AS-ON transfected  $\beta$ -cells.



## INTRODUCTION

The vitamin D-dependent  $\text{Ca}^{2+}$ -binding proteins (calbindins) belong to a family of intracellular proteins having high affinity  $\text{Ca}^{2+}$ -binding activity (Kawasaki and Kretsinger, 1994; Nelson et al., 2002). This family includes calmodulin, troponin C, calbindin- $\text{D}_{9\text{k}}$ , parvalbumin, and S100 proteins (Rhoten et al., 1985; Christakos et al., 1989; Heinzmann and Hunziker, 1991; Zimmer et al., 1997). Calmodulin, the ubiquitous  $\text{Ca}^{2+}$ -binding protein present in all eukaryotic cells, has four  $\text{Ca}^{2+}$ -binding sites which bind both  $\text{Ca}^{2+}$  and magnesium ions competitively and is involved in the triggering of cellular processes (Rhoten et al., 1982; Kilhoffer et al., 1983; Hammes et al., 1994). Calmodulin acts as a  $\text{Ca}^{2+}$ -signaling protein since it regulates the activity of many endogenous target enzymes in a  $\text{Ca}^{2+}$ -dependent manner including the cyclic nucleotide phosphodiesterase, calmodulin-dependent protein kinases, and calcium adenosine triphosphatase ( $\text{Ca}^{2+}$ -ATPase) (Cheung et al., 1978). The  $\text{Ca}^{2+}$ /Calmodulin-dependent kinases play a role in linking changes in the concentration of intracellular free calcium ions,  $[\text{Ca}^{2+}]_i$ , to the distal events in exocytosis and insulin release (Rhoten et al., 1982; Hammes et al., 1994; Easom, 1999; Tabuchi et al., 2000; Yamamoto et al., 2003).

The hormonally active metabolite of vitamin D, 1,25-dihydroxyvitamin  $\text{D}_3$  (1,25-(OH) $_2\text{D}_3$ ), is a secosteroid whose genomic mechanism of action is similar to that of other steroid hormones and is mediated by stereospecific interaction of

1,25-(OH)<sub>2</sub>D<sub>3</sub> with a specific cellular receptor, the vitamin D receptor (VDR) (Schröder et al., 1995; Brown et al., 1999; Christakos et al., 2003b). VDR is a member of the nuclear steroid hormone receptors superfamily which functions as a ligand-activated transcription factor (Sone et al., 1991; Ross et al., 1992; Schröder et al., 1995; Brown et al., 1999). Upon ligand binding, the activated cytoplasmic VDR rapidly translocates to the nucleus along the microtubules (Brown et al., 1999). The VDR heterodimerizes with another nuclear receptor, the retinoid X receptor (RXR), and the heterodimer binds to specific DNA motifs (vitamin D response elements, VDREs) (Schröder et al., 1995; Brown et al., 1999; Christakos et al., 2003b). After interaction with the vitamin D response element (VDRE) in the promoter of target genes, transcription proceeds through the interaction of VDR with coactivators and with the transcription machinery (Schröder et al., 1995; Christakos et al., 2003b). VDR interacts with many nuclear transcription factors and coactivators including the general transcription factor TFIIB, a component of the basal transcription complex, which plays a critical role in ligand-dependent transcription (Blanco et al., 1995; MacDonald et al., 1995; Masuyama et al., 1997). Both 1,25-(OH)<sub>2</sub>D<sub>3</sub> and Ca<sup>2+</sup> were previously reported to regulate the expression of VDR (Brown et al., 1995; Zineb et al., 1998; Healy et al., 2003).

Nongenomic functions of VDR also have been previously described (see Sergeev and Rhoten, 1995; Norman et al., 1999, 2001, 2002a, b). A plasma membrane VDR was shown to mediate the rapid opening of the voltage-gated

Ca<sup>2+</sup> channels located in the plasma membrane and stimulates Ca<sup>2+</sup> transport in the intestine, Ca<sup>2+</sup> mobilization in the osteoblasts, and the release of insulin by rat pancreatic beta-cells and RIN1046-38 cells (Nemere et al., 1994; Sergeev and Rhoten, 1995; Norman et al., 1999, 2001, 2002a, b).

VDREs have been found in both the murine calbindin-D<sub>9k</sub> and CaBP<sub>28k</sub> genes (Darwish and DeLuca, 1992; Gill and Christakos, 1993). Developmental studies showed that the induction of VDR mRNA in the rat intestine is associated with the induction of calbindin-D<sub>9k</sub> mRNA in the third postnatal week, coinciding with the period of increased active duodenal transport of Ca<sup>2+</sup> (Christakos et al., 1991). It has been shown that during the development of the rat kidney, the induction of VDR mRNA in this tissue is correlated with the induction of CaBP<sub>28k</sub> mRNA between birth and one week of age, the period of rapid nephron differentiation (Christakos et al., 1991). Also, 1,25-(OH)<sub>2</sub>D<sub>3</sub> has been shown to regulate the concentrations of the chicken intestinal CaBP<sub>28k</sub> from undetectable levels in vitamin D-deficient chickens to up to 1 - 3% of the cytoplasmic protein in the intestinal cell in vitamin D-replete chickens (Christakos et al., 1979). Pharmacological doses of 1,25-(OH)<sub>2</sub>D<sub>3</sub> have been shown to increase rat renal CaBP<sub>28k</sub> concentrations (Hemmingsen et al., 1998).

CaBP<sub>28k</sub> was first discovered in the chicken intestine (Wasserman and Taylor, 1966). This molecule has at least four high affinity Ca<sup>2+</sup>-binding sites (Hunziker, 1986). CaBP<sub>28k</sub> has been shown to have a variety of roles in different

organs. It acts as a  $\text{Ca}^{2+}$  carrier in the intestine and kidneys (Bredderman and Wasserman, 1974; Feher, 1983; Bronner, 1989; Christakos et al., 1989).  $\text{CaBP}_{28\text{k}}$  can also serve as a  $\text{Ca}^{2+}$ -sensor protein: extracellular  $\text{Ca}^{2+}$  concentrations directly influence the cytosolic  $\text{CaBP}_{28\text{k}}$  concentrations and  $\text{CaBP}_{28\text{k}}$  undergoes  $\text{Ca}^{2+}$ -induced structural conformational changes resulting in the exposure of a hydrophobic surface upon the  $\text{Ca}^{2+}$  activation of the cell (Norman et al., 1981; Hall and Norman, 1990; Enomoto et al., 1992; Berggard et al., 2002). In the brain,  $\text{CaBP}_{28\text{k}}$  acts as a  $\text{Ca}^{2+}$ -buffer that prevents  $[\text{Ca}^{2+}]_i$  from reaching toxic levels (Gross and Kumar, 1990; Mattson et al., 1991, 1995; Guo et al., 1998; Blatow et al., 2003; Jackson and Redman, 2003; Venters et al., 2003). Recent studies with mammalian pancreatic islets suggest that  $\text{CaBP}_{28\text{k}}$  modulates the secretion of insulin (Sooy et al., 1999; Parkash et al., 2002), and protects the  $\beta$ -cells against the cytokine-induced apoptosis (Rabinovitch et al., 2001; Riachy et al., 2002; Christakos et al., 2003a). RIN1046-38 cells are known to normally express  $\text{CaBP}_{28\text{k}}$  in relatively high concentrations ( $0.8 \pm 0.2 \mu\text{g}/\text{mg}$  protein) (Lee et al., 1994). Furthermore, RIN1046-38 cells overexpressing  $\text{CaBP}_{28\text{k}}$  have demonstrated a much larger increase in their cytoplasmic  $\text{Ca}^{2+}$  buffering capacity than control RIN cells (Rhoten and Sergeev, 1994; Reddy et al., 1997).

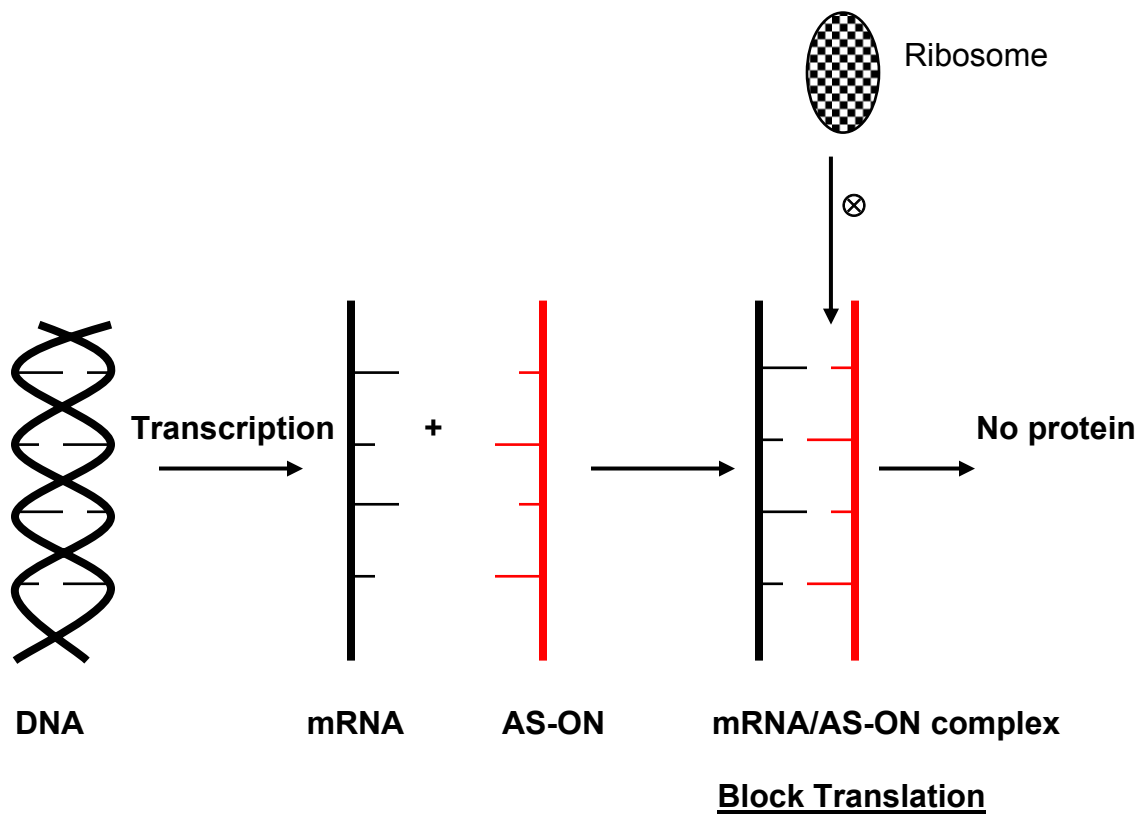
Calbindin- $\text{D}_{9\text{k}}$  was first reported by Wasserman's group to be a  $\text{Ca}^{2+}$ -binding protein in the rat intestinal mucosa that is induced by vitamin D (Kallfelz et al., 1967). It binds two  $\text{Ca}^{2+}$  ions (Juffer and Vogel, 2000). Calbindin- $\text{D}_{9\text{k}}$  is not

closely related to CaBP<sub>28k</sub>, and there is no evidence indicating that calbindin-D<sub>9k</sub> arose evolutionarily from CaBP<sub>28k</sub> (Perret et al., 1988a, b; Kretsinger and Nakayama, 1993). Antisera to calbindin-D<sub>9k</sub> are not known to cross-react with CaBP<sub>28k</sub> (Thomasset et al., 1982; Baudier et al., 1985). Calbindin-D<sub>9k</sub> gene expression is only found in mammalian species (including human, cow, pig, rat, and mouse) (Thomasset, 1997). Calbindin-D<sub>9k</sub> gene activity is controlled by 1,25-(OH)<sub>2</sub>D<sub>3</sub> at both the transcriptional and posttranscriptional levels (Thomasset et al., 1982; Perret et al., 1985; Dupret et al., 1987; Thomasset, 1997). Dietary Ca<sup>2+</sup> levels also modulate the gene expression of intestinal calbindin-D<sub>9k</sub> in the rat (Freund and Bronner, 1975; Bronner et al., 1986). Several physiological functions are postulated for calbindin-D<sub>9k</sub>, including a Ca<sup>2+</sup>-dependent regulatory function analogous to that of calmodulin and a role as a Ca<sup>2+</sup>-shuttle (carrier protein) that facilitates transcellular calcium diffusion between the two cell membranes in the Ca<sup>2+</sup>-transporting cells (Bronner et al., 1986; Bronner, 1987, 1988; Wasserman and Fullmer, 1995; Bronner, 2003a, b). The calbindin-D<sub>9k</sub> gene is expressed in both mouse and rat kidneys (Thomasset et al., 1982; Delorme et al., 1983; Schreiner et al., 1983; Rhoten et al., 1985; Bindels et al., 1991); however, it is less active in the rat kidney where CaBP<sub>28k</sub> is highly expressed and shares in Ca<sup>2+</sup> handling (Schreiner et al., 1983; Bindels et al., 1991; Thomasset, 1997).

In order to evaluate the role of CaBP<sub>28k</sub> in RIN1046-38 cells, we used antisense oligonucleotides (AS-ON) complementary to the CaBP<sub>28k</sub> messenger RNA (sense mRNA) to inhibit the translation of that specific mRNA. Antisense

agents are valuable tools to inhibit the expression of a target gene in a sequence-specific manner and may be used for functional genomics, target validation and therapeutic purposes (Kurreck, 2003). AS-ON are synthesized as short sequences of single-stranded nucleotides that pair with their complementary mRNA, thus preventing the translation machinery from synthesizing the target proteins (Kurreck, 2003) (Figure 21). AS-ON combine many desired properties such as broad applicability, direct utilization of sequence information, rapid development at low costs, high probability of success, and high specificity compared to alternative technologies for gene functionalization and target validation (Bennett and Cowser, 1999). However, all AS-ON are large polar molecules, which can not effectively cross the cell membrane (Akhtar et al., 1991). Previous research showed improvement in the cellular uptake and the transfection efficiency of AS-ON by using Lipofectamine™ 2000 reagent (a cationic lipid carrier) when compared to other reagents (Ciccarone et al., 1999; Ohki et al., 2001; Dalby et al., 2004). The AS-ON (uncomplexed with cationic lipids) that were added to the cell culture media usually enter the cells via endocytosis, then they are degraded, remain sequestered in lysosomes, or exocytosed back to the culture medium (Neckers, 1993; Wagner et al., 1993). In contrast, the AS-ON complexed with cationic lipids enter the cultured cells, are released into the cytoplasm (and may enter the nucleus) and affect the cytosolic translation machinery to result in gene-specific inhibition (Wagner et al., 1993).

In Chapter 2, we studied role of CaBP<sub>28k</sub> in buffering glucose-induced  $\Delta$  [Ca<sup>2+</sup>]<sub>i</sub> in the  $\beta$ -cells of CaBP<sub>28k</sub>-KO mice pancreatic islets. However, the genomic effects of a lack of CaBP<sub>28k</sub> were not studied before. The aim of this study is to explore the effect of CaBP<sub>28k</sub> depletion on gene expression profiles of cultured  $\beta$ -cells using the AS-ON technology. We also examine the effects of this inhibition on the expression of other calcium-binding proteins and the vitamin D receptor, and characterize the changes that occur in the induced release of insulin.



**Figure 21.** Schematic diagram illustrating the concept of applying AS-ON transfection. The AS-ON are short sequences of single-stranded nucleotides that are specifically made to be complementary to certain target sequences of the mRNA. The presence of the mRNA/AS-ON complex will prevent the ribosome from reading the segment having that complex. This will block the translation process and inhibit the expression of the specific protein.



## **MATERIALS AND METHODS**

### ***β-cells***

We selected RIN1046-38 cells for this study because they are β-cells with a relatively high expression level of CaBP<sub>28k</sub> ( $0.8 \pm 0.2 \mu\text{g}/\text{mg}$  protein, Lee et al., 1994 using radioimmunoassay). RIN1046-38 cells are a rat tumoral β-cell line generated from a transplantable x-ray irradiation-induced rat insulinoma in an inbred NEDH (New England Deaconess Hospital) rat (Gazdar et al., 1980; Philippe et al., 1987). Those rat β-cell tumors were originally maintained by serial transplantations in NEDH rats. Cells derived from them were established as continuous cell line cultures (Gazdar et al., 1980; Philippe et al., 1987). RIN1046-38 cells were obtained as a generous gift from Dr. Bruce Chertow (Department of Internal Medicine, Joan C. Edwards School of Medicine at Marshall University, Huntington, WV).

### ***Cell culture***

RIN1046-38 cells (passages 14 - 17) were included in this study. The cells were seeded at  $\sim 1 - 3 \times 10^6$  cells/25 cm<sup>2</sup> in tissue culture flasks. RIN1046-38 cells were cultivated in RPMI1640 medium supplemented with 10% FBS, penicillin 100 units/ml, and streptomycin 100 μg/ml at 37°C in humidified atmosphere of 5% CO<sub>2</sub> - 95% air.

## **Materials**

Goat anti-rabbit IgG-peroxidase conjugate antibody, goat anti-rat biotin conjugate antibody, goat anti-mouse IgG-peroxidase conjugate antibody, mouse anti-calmodulin antibody, mouse anti- $\beta$ -actin antibody, rabbit anti-guinea pig IgG, peroxidase-labeled insulin, TRI REAGENT™, O-phenylenediamine dichloride, bovine serum albumin (BSA), carbamylcholine chloride (carbachol), protease inhibitor cocktail, Tween 20®, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), dimethyl sulfoxide (DMSO), and acrylamide were purchased from Sigma Chemical Company (St. Louis, MO). D-glucose (dextrose) was obtained from National Bureau of Standards [NBS] (Gaithersburg, MD). Lipofectamine™ 2000 Transfection Reagent was ordered from Invitrogen™ Life Technologies (Carlsbad, CA). Guinea pig anti-insulin (607/22) was a gift from the late Dr. Peter Wright (Indianapolis, IN). Rat insulin standards were from Eli Lilly and Company (Indianapolis, IN). Rabbit anti-CaBP<sub>28K</sub> antibody was obtained from Chemicon International, Inc. (Temecula, CA). Rabbit anti-calbindin-D<sub>9K</sub> antibody was purchased from Swant® Swiss Antibodies Company (Bellinzona, Switzerland). Rat anti-VDR antibody was ordered from Affinity BioReagents, Inc. (Golden, CO). Streptavidin-horseradish peroxidase (HRP) was from Amersham International PLC. (Buckinghamshire, UK). Bradford protein reagent and low range prestained SDS-PAGE standards were from Bio-Rad Laboratories, Inc. (Hercules, CA). Protran® pure nitrocellulose transfer and immobilization membranes (0.45  $\mu$ m) were purchased from Schleicher and Schuell BioScience (Keene, NH). SuperSignal® West Pico Chemiluminescent substrate for detection of HRP was

from Pierce Biotechnology Inc. (Rockford, IL). Medical X-ray film (Super RX) was from Fujifilm Medical Systems, Inc. (Stamford, CT). Trypan blue stain (0.4%), RPMI1640 tissue culture medium, heat inactivated fetal bovine serum (FBS), OPTI-MEM<sup>®</sup> I reduced serum medium, Hank's balanced salts solution (HBSS), and penicillin G sodium-streptomycin sulfate antibiotics were purchased from Gibco BRL<sup>®</sup> Life Technologies (Grand Island, NY). 25 cm<sup>2</sup> polystyrene tissue culture flasks were from Corning Inc. (Corning, NY). Falcon 35-3915 pro-bind<sup>™</sup> 96-well polystyrene microtiter assay plates were from Becton Dickinson and Company (Franklin Lakes, NJ). All chemicals and reagents were of analytical grade.

### ***In vitro transfection assays***

Transfection assays with Lipofectamine<sup>™</sup> 2000 were conducted according to the manufacturer's instructions (Invitrogen<sup>™</sup> Life Technologies, Carlsbad, CA). RIN1046-38 cells were grown until they were 90 - 95% confluent on the day of transfection. Both the ON and Lipofectamine<sup>™</sup> 2000 transfection reagent (vehicle) were individually diluted in Opti-MEM<sup>®</sup> I reduced serum medium in separate microcentrifuge tubes. The ON and Lipofectamine<sup>™</sup> 2000 were then combined, mixed and incubated for 20 min. at room temperature. Then, the ON/Lipofectamine<sup>™</sup> 2000 liposome mixtures were added to the cells in the Opti-MEM<sup>®</sup> I reduced serum medium and incubated at 37°C in a CO<sub>2</sub> incubator for six hours as previously described (Lewis et al., 1996; Cheng et al., 2004). Neither serum nor antibiotics were added to the cells during the transfection period. After

the first six hours of incubation, complete growth medium containing antibiotics and serum was added to the incubation medium (without removing the ON/Lipofectamine™ 2000 mixtures), and the cells were further incubated for a total of 48 hours from the start of the transfection assay (Ciccarone et al., 1999; Hamada et al., 2003; Cheng et al., 2004; Dalby et al., 2004). The cells were then washed thrice in HBSS and studied for the effects of the transient transfection on cellular gene expression and insulin secretion.

The AS-ON were prepared as a 20-mer oligonucleotide complementary in sequence to a target segment of the rat CaBP<sub>28k</sub> mRNA to inhibit the translation process, which are selected for the transfection assays using the previously established criteria (Yamakuni et al., 1987; Hunziker and Schrickel, 1988; Nordquist et al., 1988; Wagner, 1994) (Figures 21 and 22). Scrambled oligonucleotides (Nonsense, NS-ON) were used as a control. NS-ON with the same base composition were synthesized as a sequence of nucleotides containing 17 mismatches out of 20 bases (Figure 22). Previous research showed that NS-ON with at least 4-mer mismatches are inactive and do not inhibit the process of translation (Wagner et al., 1993). We applied 6'-fluorescein-labeled-AS-ON to the RIN1046-38 cells to evaluate uptake of AS-ON by RIN cells. Cellular uptake was monitored by fluorescence microscopy (Busuttil et al., 1996; Coats et al., 1996; Lewis et al., 1996) (Figure 23). Two different types of ON were included in our study; phosphorothioate- (PS-ON) and propyne-oligonucleotides (PY-ON). All the linkages used in the synthesis of the ON (in

both PS-ON and PY-ON) were sulfur. In the PS-ON, the phosphorothioate linkages are created in place of phosphodiester bonds. These linkages increase the resistance of the ON to endogenous nucleases and have great efficacy in gene inhibition (Hoke et al., 1991; Beltinger et al., 1995). In the PY-ON the pyrimidine residues (C and T) normally created in PS-ON are replaced with C-5 propynyl-substituted pyrimidines (pdC and pdU). Specifically, C-5-(1-propynyl) uracil replaces thymine and C-5-(1-propynyl) cytosine replaces cytosine. The C-5 propynyl substitution is thought to increase the potency of AS-ON by enhancing base stacking interactions and mRNA/AS-ON binding affinity. It also increases the nuclease stability of AS-ON, and may stimulate the RNase H cleavage of the sense mRNA (Wagner et al., 1993; Raviprakash et al., 1995). All the ON were synthesized on an ABI Model 394 DNA Synthesizer in the Marshall University DNA Core Facility.

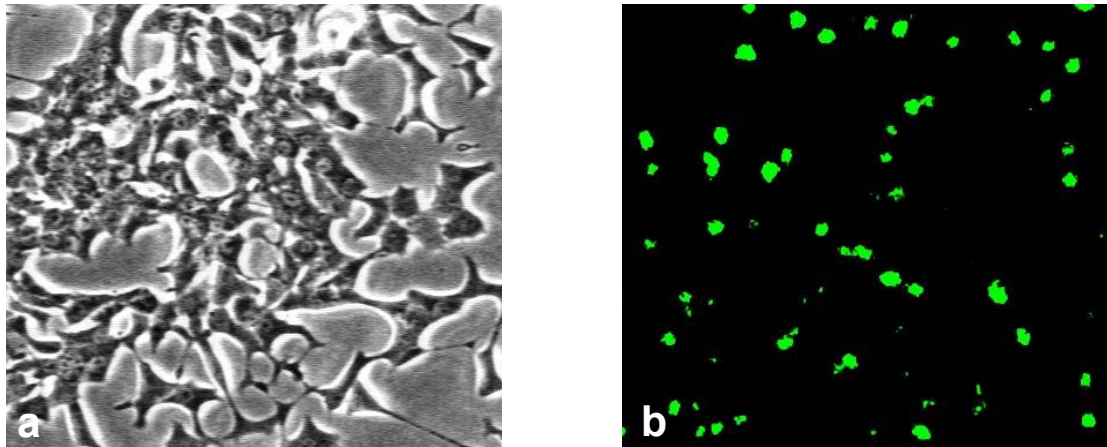
A range of PS-AS-ON concentrations from 50 to 200 nM was used to determine the most effective inhibitory concentration for CaBP<sub>28k</sub> expression. Cellular toxicity was evaluated by morphological appearance and trypan blue exclusion. Cells that stained with trypan blue were considered nonviable while those which do not stain were considered viable. Once an effective concentration of the AS-ON was determined, the control cultures were treated identically with the NS-ON.

-----3' AGG ACC UAG UUC AAG ACG UC 5'----- ← CaBP<sub>28K</sub> mRNA

5' TCC TGG ATC AAG TTC TGC AG 3' ← AS-ON

5' CGT TGT TCA AGT CCC TAG GA 3' ← NS-ON

**Figure 22.** Diagram showing the structure of the ON sequences that were used in the transfection assays and their corresponding target segment of the CaBP<sub>28K</sub> mRNA. The AS-ON sequence was synthesized to inhibit the translation process. The NS-ON, containing 17 mismatches out of 20-mer, was synthesized as a scrambled sequence of nucleotides that does not inhibit the translation process. The NS-ON was found to have no complementary hybridization sequence to any of the genes found in a search of the rat (*Rattus norvegicus*) GenBank<sup>®</sup> database ([www.ncbi.nlm.nih.gov/Genbank/index.html](http://www.ncbi.nlm.nih.gov/Genbank/index.html)). All the linkages used were sulfur. The bases used are: A = Adenine, C = Cytosine, G = Guanine, T = Thymine, and U = Uracil. ON were synthesized in two formats: phosphorothioate (PS) (as above), and propyne (PY). The PY-ON sequence has pdC and pdU (propynyl) instead of C and T. Labeling the PS-AS-ON was done by adding 6'-fluorescein acetoxy methyl ester (6'-FAM) to the 5' terminus, to help monitoring their cellular uptake using fluorescence microscopy.



**Figure 23.** *In vitro* follow-up of RIN1046-38 cells during their cellular uptake of fluorescein-labeled PS-AS-ON. Images of RIN1046-38 cells were captured from tissue culture flasks on a Nikon Diaphot inverted fluorescence microscope [ $\times 200$ ]. The image acquisition was facilitated using MetaMorph Imaging System software version 4.6r5 (Universal Imaging Corporation, Westchester, PA). (a) Phase-contrast image of RIN1046-38 cells before incubation with fluorescein-labeled PS-AS-ON. (b) Fluorescence image of RIN1046-38 cells after six hours of incubation with 50 nM fluorescein-labeled PS-AS-ON showing the uptake of AS-ON [same cells as in panel a, washed and viewed live].

### ***Western blot analysis***

Protein expression in RIN1046-38 cells was studied 48 hours post-transfection (as recommended by the Lipofectamine™ 2000 transfection protocol; Ciccarone et al., 1999; Hamada et al., 2003; Cheng et al., 2004; Dalby et al., 2004). RIN cells were washed thrice in HBSS, harvested from the tissue culture flasks, centrifuged, and the pellets were frozen quickly on dry ice, and stored at -80°C until used. The pellets were then placed into lysis buffer [1% Nonidet P-40 (NP-40), 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 2 mM EDTA, and 1% protease inhibitor cocktail]. After homogenization in protein lysis buffer, cells were disrupted by burst sonication (< 10 seconds) while cooling on ice, and samples were centrifuged at 14,000 × *g* for 20 min. at 4°C. The supernatant solution was obtained and total protein estimated using the Bradford method (Bradford, 1976). Equal amounts of total protein from each sample were loaded per lane in 12% SDS polyacrylamide gels. Low range prestained SDS-PAGE standards (Bio-Rad) were also used. The standard molecular weight marker proteins used were of 113, 93, 50.3, 35.5, 28.8, and 21.4 kDa. After protein transfer (Towbin et al., 1979), nitrocellulose sheets were incubated in Tris-buffered saline with Tween 20® and 5% non-fat dry milk to block non-specific binding sites (Spinola and Cannon, 1985). After applying the specific primary and peroxidase-conjugated secondary antibodies, the specific protein bands were visualized using SuperSignal® West Pico Chemiluminescent Substrate kit.



### ***cDNA Microarray data analysis***

RIN1046-38 cells transfected with 200nM PS-AS-ON, and those transfected with 200nM PS-NS-ON as controls, were included in this study. The cells were studied 48 hours post-transfection (as above). RIN cells were washed thrice in HBSS, harvested from the tissue culture flasks, centrifuged, and the pellets were placed into TRI REAGENT™. The pellets were homogenized immediately on ice using an Ultra-Turrax® type T25 basic S1 homogenizer (Ika® Works, Inc., Wilmington, NC). Total RNA was isolated according to the TRI REAGENT™ manufacturer's instructions (Sigma, St. Louis, MO). The method we used for total RNA isolation was single-step simultaneous isolation of RNA, DNA and proteins from the cells using extraction with an acidic guanidinium thiocyanate-phenol-chloroform mixture to get undegraded total RNA in a high yield as described before (Chomczynski and Sacchi, 1987; Chomczynski, 1993).

The PancChip is an endocrine pancreas specific microarray slide (Corning® ULTRA Gaps glass slide of 22 × 75mm, Corning Inc. Life Sciences, Acton, MA) having 48 grids (12 × 4). Each grid consists of 306 spots (18 × 17) (Kaestner et al., 2003). The Mouse PancChip5.0 represents a collection of mouse complementary DNAs (cDNAs) chosen for their expression in various stages of pancreatic development (Scearce et al., 2002). The PancChip5.0 contains 14,688 spots of cDNAs which include clones of genes, 3,139 of them are non-redundant genes, representing every assembly found to be expressed in the endocrine pancreas and insulinomas. The clones of genes were selected by

both sequence analysis of Consortium Libraries [endocrine pancreas consortium clones, EPCon Clones (Beta Cell Biology Consortium, Nashville, TN), and integrated molecular analysis of genomes and their expression consortium, IMAGE Clones (National Institute of Diabetes and Digestive and Kidney Diseases, NIDDK 3.4K set)], which were found to be expressed in the pancreas using a combination of expression analysis and database mining. On the PancChip5.0 microarray slide there are positive and negative cDNA controls that were provided in the SpotReport™ - 10 cDNA Array Validation System from Stratagene (La Jolla, CA).  $\beta$ -actin cDNA was included in the positive controls; while salmon sperm cDNA, and several hundred blank spots were included in the negative controls. The principle of microarray technology is based on the isolation of the cellular mRNA, labeling mRNA with a fluorescent dye, and hybridizing the labeled mRNA to cDNAs attached to microarray slide. After several washes, the fluorescence intensity of each spot on the microarray slide is measured using a special scanner, then image analysis is performed (Fellenberg et al., 2001; Kaestner et al., 2003).

### ***Insulin secretion and content of RIN1046-38 cells***

The RIN1046-38 cells were grown in 25 cm<sup>2</sup> tissue culture flasks. 48 hours post-transfection the cells were washed thrice with HBSS and further incubated in the basal medium for 25 min. at 37°C. The basal incubation medium was a modified Krebs-Ringer medium composed of: 118 mM NaCl, 4.8 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 10 mM HEPES, 5 mM NaHCO<sub>3</sub>, 2.8

mM glucose, and 0.1% w/v BSA, pH 7.40 (as previously described in chapter 2; Sooy et al., 1999; Parkash et al., 2002). The stimulation medium was composed of the basal incubation medium and either 0.5 mM carbamylcholine chloride (carbachol) alone, or 0.5 mM carbachol plus a final glucose concentration of 17 mM.

Carbachol is a cholinergic muscarinic agonist (acetylcholine derivative) known to activate phospholipase C (PLC), leading to the generation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). This leads to Ca<sup>2+</sup>-mobilization from intracellular stores, a rise in [Ca<sup>2+</sup>]<sub>i</sub>, signal transduction, and insulin secretion from the β-cells (Wollheim and Biden, 1986; Biden et al., 1987; Nilsson et al., 1987; Prentki and Matschinsky, 1987; Peter-Riesch et al., 1988; Wolf et al., 1989; Theler et al., 1992; Yada et al., 1992; Jones and Persaud, 1998). DAG is the endogenous activator of protein kinase C, a Ca<sup>2+</sup>- and phospholipid-dependent enzyme (Nishizuka, 1986, 1988; Wollheim et al., 1988). Carbachol is known to significantly increase membrane-associated protein kinase C (PKC) activity while simultaneously decreasing its activity in the cytosol (Nishizuka, 1986, 1988; Wollheim et al., 1988; Persaud et al., 1989). These events are associated with an increase in insulin secretion from RIN cells and rat islets of Langerhans (Wollheim and Biden, 1986; Yamatani et al., 1988; Persaud et al., 1989).

After incubating the RIN cells in the basal medium for 25 min., the medium was collected and the cells were incubated in the stimulation medium. The stimulation medium was then replaced at 25 min. intervals with a new stimulation medium to assess the time course (75 min.) of insulin secretion in response to secretagogues. Then, the cells were further incubated in the basal medium for the final 25 min. The samples of insulin secretion were stored at -80°C until analyzed. The insulin content of the RIN1046-38 cells was extracted by incubation overnight in acidic ethanol (pH 1.7) at -20°C as previously described (Rhoten, 1983).

In order to measure both the insulin secretion and insulin content of the RIN1046-38 cells, we have applied a competitive enzyme-linked immunosorbent assay (ELISA) protocol modified in our laboratory from that of Kekow et al. (1988), and Webster et al. (1990). Rat insulin was used to build a standard curve (0 – 100 pg). The absorbance of the samples loaded in the 96-well assay plates were read at 490 nm using  $\mu$ Quant™ microplate spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT) and the data were interpreted using KCjunior™ data analysis software, version 1.41.4 (Bio-Tek Instruments, Inc., Winooski, VT).

### ***Statistical Analysis***

Results are presented as the mean  $\pm$  standard error of the mean (SEM), and percentages, using Sigma Stat, version 2.03 from SPSS Inc. (Chicago, IL).

The data were analyzed for significance with the unpaired Student's *t* test (one-tailed) with a significance level of 0.05.

## RESULTS

To evaluate the effects of CaBP<sub>28k</sub> depletion on the gene expression of RIN1046-38 cells, the cells were studied 48 hours after transfection with AS-ON. This time point was chosen because previous reports showed that the effects of ON transfection on the knock-down of gene expression were detectable in this time frame (according to the Lipofectamine™ 2000 manufacturer's instructions, Invitrogen™ Life Technologies, Carlsbad, CA; Ciccarone et al., 1999; Hamada et al., 2003; Cheng et al., 2004; Dalby et al., 2004). The gene expression profiles of RIN1046-38 cells were examined in the CaBP<sub>28k</sub>-ablated cells (AS-ON transfected) and control cells (NS-ON transfected).

### ***In vitro transfection assays***

We detected the cellular uptake of 50 nM fluorescein-labeled AS-ON after six hours of incubation with the RIN1046-38 cells (Figure 23). Using the inverted fluorescence microscope, we monitored the cellular uptake of the ON. Considerable amounts of the fluorescein-labeled AS-ON were delivered into the RIN cells (Figure 23). The ON-transfected RIN cells remained viable as determined by trypan blue exclusion (images not shown).

### ***Inhibition of CaBP<sub>28k</sub> expression by AS-ON***

Western blots were conducted 48 hours after transfection to evaluate the efficiency of the ON transfection assays. Our Western blots demonstrate efficient

inhibition of CaBP<sub>28k</sub> expression in RIN1046-38 cells using 200 nM PS-AS-ON as shown in Figure 24. The control untreated RIN1046-38 cells showed the same levels of expression of CaBP<sub>28k</sub> as the 50 nM PS-AS-ON transfected cells (Figure 24). In addition, we found greater reductions of the levels of CaBP<sub>28k</sub> expression with increasing concentrations of PS-AS-ON. The signal of CaBP<sub>28k</sub> expression was not detectable (below the sensitivity level of the chemiluminescence's detection) using 200 nM PS-AS-ON (Figure 24).

The effect of ON transfection on the expression of other calcium-binding proteins in RIN1046-38 cells is shown in Figure 25. We studied calmodulin (17 kDa), a nontargeted protein that is used as an internal control, to validate the specificity of the effects of PS-AS-ON transfection assays.  $\beta$ -actin (43 kDa) was used as a protein gel-loading control. The PS-NS-ON-transfected cells were included as a control to evaluate the sequence-specific and gene-specific effects of AS-ON transfection assays (Figure 25). Gene expression in the control RIN cells exposed to the 100 – 200 nM PS-NS-ON were similar to that of the untreated cells (Figure 25). It is noteworthy that calbindin-D<sub>9k</sub> expression was present in the 200 nM PS-AS-ON-transfected RIN1046-38 cells (Figure 25).

Previous studies reported that the PY-AS-ON were more potent and specific in their effects than the PS-AS-ON (Wagner et al., 1993; Raviprakash et al., 1995). Thus, it is of interest to compare the effects of the PY-AS-ON in RIN1046-38 cells to those of the PS-AS-ON. We applied a range of PY-AS-ON

concentrations from 2.5 to 20 nM in order to determine the most effective inhibitory concentration for CaBP<sub>28k</sub> expression in RIN cells (Figure 26). The immunoblottings showed gradual reductions in the CaBP<sub>28k</sub> expression levels in the RIN cells with increasing concentrations of PY-AS-ON until a complete inhibition is achieved using 20 nM (Figure 26). Thus, inhibition was achieved using PY-AS-ON at considerably lower concentration levels than PS-AS-ON. Furthermore, at 20 nM PY-AS-ON a positive expression of calbindin-D<sub>9k</sub> was observed as shown in Figure 26.

Taken together, Western blot analysis of the AS-ON transfection assays (of both PS- and PY-) revealed that when the CaBP<sub>28k</sub> expression is completely inhibited (below the sensitivity level of the chemiluminescence's detection) a strong increase in calbindin-D<sub>9k</sub> expression is detected (Figures 24, 25, and 26).

We further extended our study to include VDR (52 – 57 kDa) as shown in Figure 27. We found that VDR expression in the RIN cells transfected with AS-ON was much higher than that of the NS-ON transfections (Figure 27). However, when comparing the effects of using 200 nM PS-AS-ON to those of using 20 nM PY-AS-ON we found a much stronger immunodense reaction of VDR expression in the RIN cells transfected with the 200 nM PS-AS-ON (Figure 27).



### ***Microarray data analysis***

RIN 1046-38 cells transfected with 200 nM PS-AS-ON and control 200 nM PS-NS-ON-transfected cells were included in our microarray analysis (Table 4). Interestingly, the cDNA microarray analysis revealed up-regulation of the vitamin D receptor mRNA [ $\uparrow$  3-fold] and the calbindin-D<sub>9k</sub> mRNA [ $\uparrow$  4-fold] while no change occurred in the calmodulin or insulin mRNAs (Table 4).  $\beta$ -actin mRNA was used as a positive control and did not show any change in its level. Our microarray data further extend and confirm our Western blot findings and demonstrate that the increases in the VDR and calbindin-D<sub>9k</sub> expressions reported in the 200 nM PS-AS-ON-transfected RIN cells occurred at both the mRNAs and proteins levels (Table 4, and Figures 25 and 27).

### ***Insulin content of the RIN cells***

The total insulin contents (in ng/10<sup>6</sup> cells) of transfected RIN1046-38 cells were estimated to be as follows (mean  $\pm$  SEM): Lipofectamine™ 2000 (vehicle) treated, 46.32  $\pm$  3.90; 200 nM PS-NS-ON transfected, 47.20  $\pm$  5.97; 200 nM PS-AS-ON transfected, 45.43  $\pm$  6.69 (n = 5) (Figure 28). There was no statistically significant difference in the insulin contents of the three groups included in our study (Figure 28).

### ***Insulin secretion studies***

*In vitro* studies for the insulin secretory responses of RIN1046-38 cells were done on 200 nM PS-AS-ON transfected cells and controls [vehicle-treated

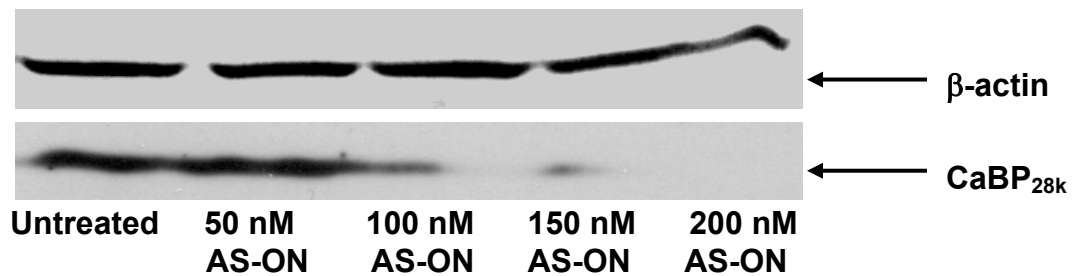
cells, and 200 nM PS-NS-ON-transfected cells]. Our insulin ELISA results showed a statistically significant increase in insulin release from the 200 nM PS-AS-ON-transfected cells ( $8.94 \pm 0.39$  ng/ $10^6$  cells) as compared to that of the vehicle-treated cells ( $5.78 \pm 0.12$  ng/ $10^6$  cells), and the 200 nM PS-NS-ON-transfected ( $5.68 \pm 0.16$  ng/ $10^6$  cells) in response to 0.5 mM carbachol stimulation (n = 5) (Table 6 and Figure 29).

We estimated the fractional insulin secretion of RIN cells as the percentage of the insulin secretion/the total cellular insulin content. As presented in Table 7 and Figure 30, the 200 nM PS-AS-ON-transfected RIN cells had a significantly higher fractional insulin secretory responses in the 60 min. stimulation period with 0.5 carbachol ( $20.94 \pm 2.42\%$ ) as compared to controls [vehicle-treated,  $12.70 \pm 1.33\%$ ; and 200 nM PS-NS-ON-transfected,  $12.62 \pm 1.57\%$ ; n = 5, p < 0.05].

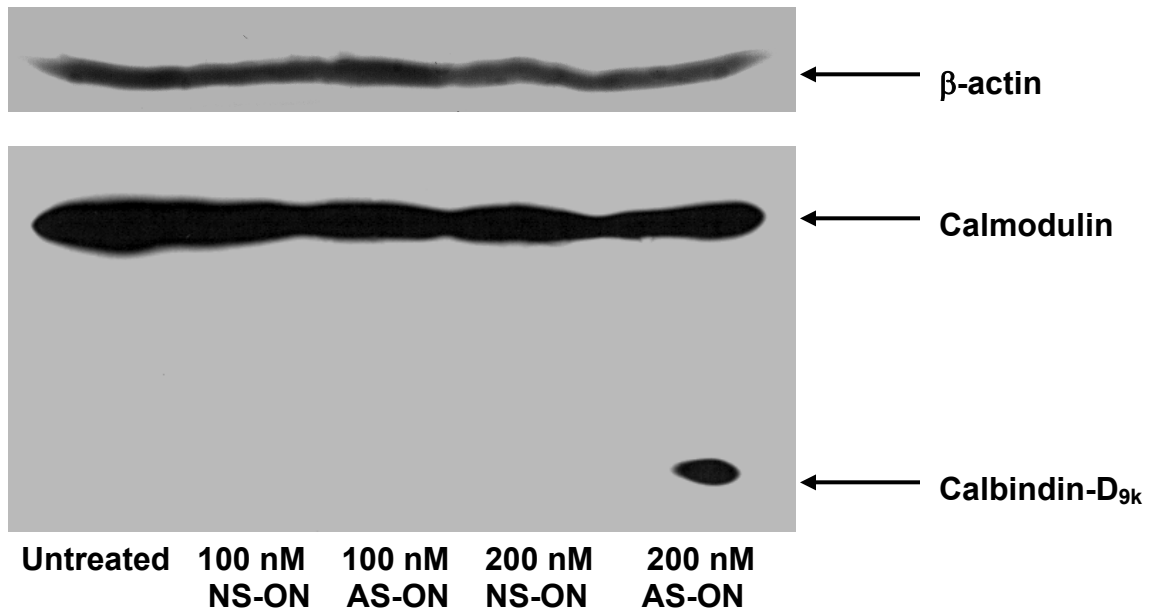
Upon increasing the glucose concentration from 2.8 mM to 17 mM plus 0.5 mM carbachol in the incubation medium, the 200 nM PS-AS-ON transfected RIN cells exhibited a significantly greater increase in insulin release as compared to that of the 200 nM PS-NS-ON-transfected cells over the 75 min. stimulation period (Table 8 and Figure 31).

We further compared the insulin secretory influence of 0.5 mM carbachol to that of the combined 0.5 mM carbachol plus 17 mM glucose in the RIN1046-38

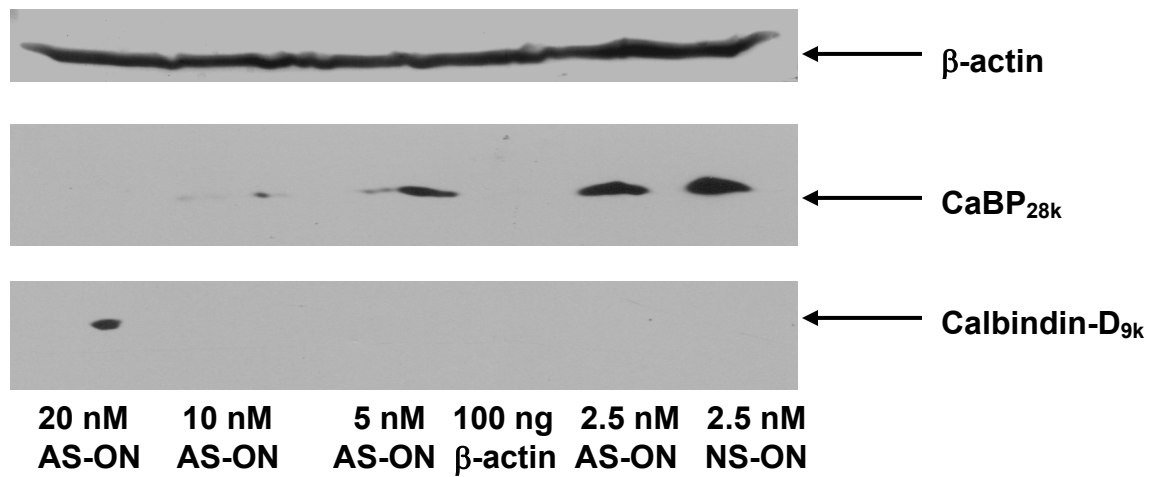
cells. Our data indicated that the CaBP<sub>28k</sub>-ablated cells (200 nM PS-AS-ON-transfected) have significantly higher insulin secretory responses under basal conditions, and in response to 0.5 mM carbachol and the combination of 0.5 mM carbachol plus 17 mM glucose than the control 200 nM PS-NS-ON-transfected cells ( $p < 0.001$ ) (Table 9 and Figure 32).



**Figure 24.** Western blots showing the effects of using different concentrations of PS-AS-ON transfection in RIN1046-38 cells. 12% SDS-polyacrylamide gels were loaded with 200  $\mu$ g total protein per well of samples of RIN1046-38 cells [control untreated cells, and 50, 100, 150, 200 nM PS-AS-ON treated cells]. The nitrocellulose sheets were probed with the rabbit anti-CaBP<sub>28K</sub> antibody (bottom blot) and the mouse anti- $\beta$ -actin antibody (upper blot). CaBP<sub>28K</sub> protein was visualized as a single immunoreactive band at 28 kDa seen by the chemiluminescent peroxidase method. Greater reductions of the levels of CaBP<sub>28k</sub> expression are seen with increasing concentrations of PS-AS-ON until complete inhibition of CaBP<sub>28k</sub> expression is achieved using 200 nM PS-AS-ON (bottom blot).  $\beta$ -actin expression was used as a protein gel-loading control (upper blot). (n = 3) [a representative experiment is shown].

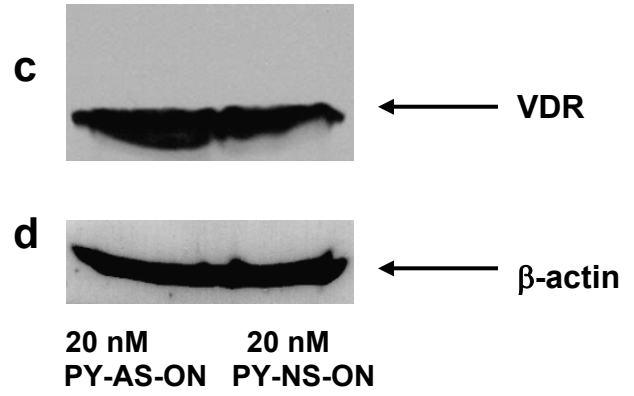
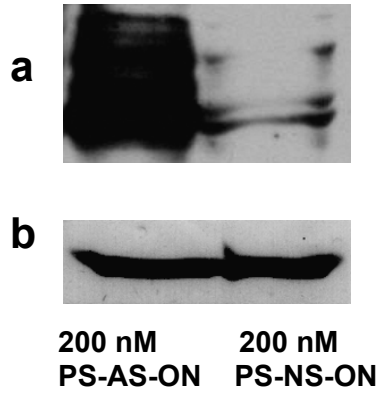


**Figure 25.** Effect of PS-ON transfection on calmodulin and calbindin-D<sub>9k</sub> expression in RIN1046-38 cells. 12% SDS-polyacrylamide gels were loaded with 200 µg total protein per well of samples of RIN cells. The nitrocellulose sheets were sequentially probed with both rabbit anti-calbindin-D<sub>9k</sub> antibody and mouse anti-calmodulin (bottom blot), and mouse anti-β-actin antibody (upper blot). The immunoreactions were visualized by the chemiluminescent peroxidase method. β-actin was used as a protein gel-loading control (upper blot). Calmodulin was used as a positive control, and did not show a marked change in its expression (bottom blot). Interestingly, only 200 nM PS-AS-ON transfected cells showed an expression of calbindin-D<sub>9k</sub> (bottom blot) [a representative experiment is shown].



**Figure 26.** Western blots showing the effects of using different concentrations of PY-AS-ON in RIN1046-38 cells. 12% SDS-polyacrylamide gels were loaded with 200 µg total protein per well of samples of RIN cells; one well was loaded with 100 ng β-actin as a standard. The nitrocellulose sheets were probed with rabbit anti-calbindin-D<sub>9k</sub> antibody (bottom blot), rabbit anti-CaBP<sub>28k</sub> antibody (middle blot), and mouse anti-β-actin antibody (upper blot). The immunoreactions were visualized by the chemiluminescent peroxidase method. β-actin was used as a protein gel-loading control (upper blot). CaBP<sub>28k</sub> expression showed marked reductions with increasing concentrations of PY-AS-ON until complete inhibition is seen at 20 nM PY-AS-ON (middle blot). Only 20 nM PY-AS-ON transfected cells showed expression of calbindin-D<sub>9k</sub> (bottom blot) [a representative experiment is shown].

**Figure 27.** Western blots showing the effect of ON transfection on the expression of VDR in RIN1046-38 cells. 12% SDS-polyacrylamide gels were loaded with 200  $\mu$ g total protein per well of samples of RIN cells. The cells were transfected with either PS-ON (200 nM AS vs. 200 nM NS), left panel, or PY-ON (20 nM AS vs. 20 nM NS), right panel. These concentrations of AS-ON were selected because they caused complete inhibition of CaBP<sub>28k</sub> expression (Figures 24 and 26). Blots were probed with rat anti-VDR (upper blots, a and c) and mouse anti- $\beta$ -actin antibodies (bottom blots, b and d). The immunoreaction product was visualized by the chemiluminescent peroxidase method.  $\beta$ -actin was used as a protein gel-loading control (b, d). VDR expression showed a much higher expression in RIN cells transfected with 200 nM PS-AS-ON as compared to that of control 200 nM PS-NS-ON transfections (a). 20 nM PY-AS-ON caused a small increase in the expression of VDR as compared to that of control 20 nM PY-NS-ON transfections (c). In comparison to the effects of 200 nM PS-AS-ON, the application of 20 nM PY-AS-ON caused a much smaller increase in the expression of VDR. [The experiments were repeated twice, representative experiments are shown].





**Table 4.** A comparison of the gene expression profiles of the 200 nM PS-AS-ON- and the 200 nM PS-NS-ON-transfected RIN1046-38 cells by microarray analysis.

mRNA Description	Microarray Results
	[200 nM PS-AS-/200 nM PS-NS-ON]
Calbindin-D <sub>9k</sub> mRNA	Up-regulated [↑ 4-fold]
Vitamin D receptor (VDR) mRNA	Up-regulated [↑ 3-fold]
Calmodulin mRNA	No change
Calcium/calmodulin-dependent serine-protein kinase II (CaM kinase II) mRNA	No change
Insulin mRNA	No change
S100 calcium-binding protein mRNA	No change
Secretogranin II mRNA	No change
Chromogranin A & B mRNAs	No change
β-actin mRNA	No change

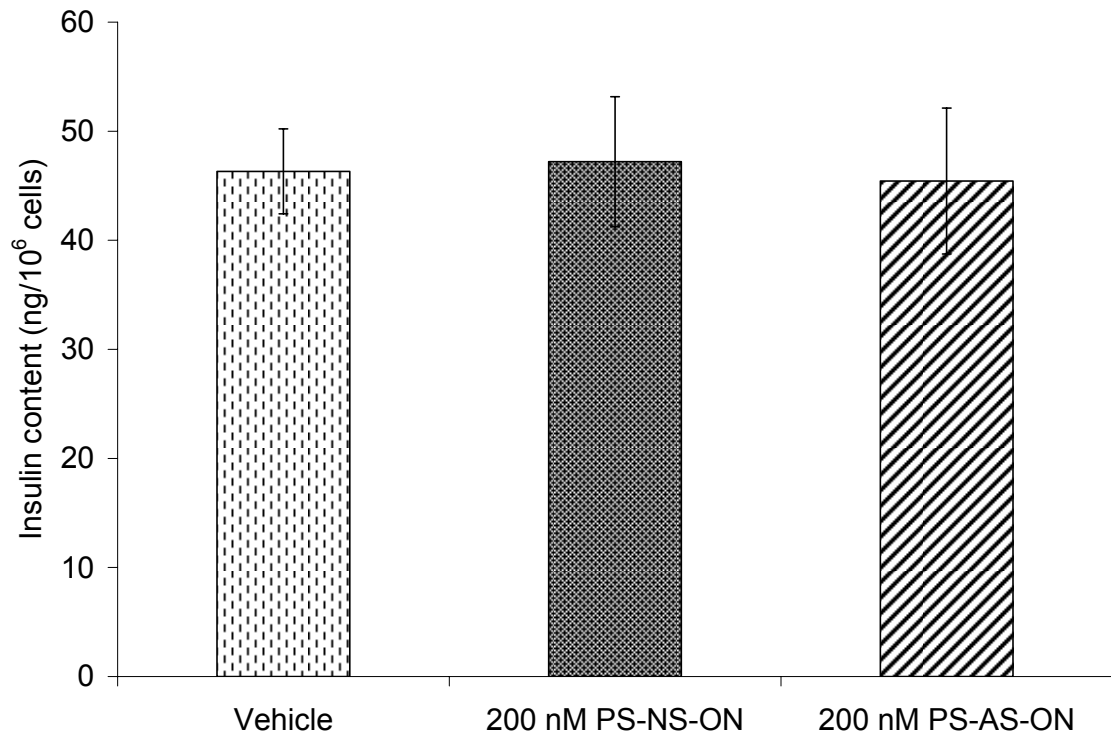
- The microarray analysis was done in the MU DNA Core Facility using six PancChip5.0 slides (assays were repeated three times)
- The PancChip is an endocrine pancreas specific microarray slide that has 14,688 genes (3,139 of them are non-redundant genes)
- β-actin mRNA was used as a positive control and showed no change in its expression level

**Table 5.** Additional microarray data analysis of 200 nM PS-AS-ON-/200 nM PS-NS-ON-treated RIN cells.

Gene I.D.	Name	Regular ratio treated/control
16476	Mus musculus Jun oncogene mRNA	71.42
4161889	Mus musculus insulin-like growth factor binding protein 1 mRNA	46.10
20537	Mus musculus solute carrier family 5 (sodium/glucose cotransporter), member 1 mRNA	34.49
4242076	Unnamed protein product [Mus musculus] mRNA	26.51
3967219	Mus musculus prolyl endopeptidase mRNA	23.24
4217694	Phosphoenolpyruvate carboxykinase 1, cytosolic [Mus musculus] mRNA	19.29
4240796	Apolipoprotein A-IV mRNA	17.98
4192268	Mus musculus transthyretin mRNA	15.37
2644984	Mus musculus mRNA for integrin beta 1 subunit	14.24
2582271	Glucose-6-phosphate isomerase (GPI) (Phosphoglucose isomerase) (PGI) (Phosphohexose isomerase) (PHI) (Neuroleukin) (NLK) mRNA	14.15
3663150	Mus musculus transforming growth factor, beta 3 mRNA	12.76
23894	General transcription factor IIH, polypeptide 2 (44 kDa subunit); basal transcription factor 2, p44 subunit [Mus musculus] (NM_022011) mRNA	11.65
4210558	Heparin-binding growth factor 1 precursor (HBGF-1) (acidic fibroblast growth factor) (AFGF) mRNA	9.44
4195958	Apolipoprotein A-II precursor, C57BL/6-mouse mRNA	9.09
4008490	Paired box transcription factor Pax-6 – mouse mRNA	7.89
3500885	Mus musculus hypoxanthine guanine phosphoribosyl transferase mRNA	7.26
2631167	Mus musculus glucose-6-phosphatase, transport protein 1 mRNA	7.22
25058	Hexokinase 1 (EC 2.7.1.1) [Rattus norvegicus] mRNA	6.71
3497611	Mus musculus solute carrier family 5 (sodium/glucose cotransporter), member 1 mRNA	6.37
596564	Mus musculus promyelocytic leukemia mRNA, (BC020990)	6.00
576391	Mus musculus ubiquitin-conjugating enzyme E2I, mRNA	5.57
4218355	Mus musculus unknown protein for MGC:19143 mRNA	5.57
4238940	Mus musculus frizzled homolog 4 (Drosophila) mRNA	5.48
4191539	Peroxisome proliferator activated receptor gamma (PPAR-gamma) mRNA	4.54
4237050	Vitamin D-dependent calcium-binding protein, intestinal (Calbindin-D <sub>9k</sub> ) mRNA	4.42
4225392	Mucin glycoprotein MUC3 [Mus musculus] mRNA	4.34
30024406	Mus musculus cadherin 1 (Cdh1), mRNA	4.14

4237115	Unnamed protein product [Mus musculus] mRNA	3.88
3710866	Vitamin D receptor [Mus musculus] mRNA	3.32
3707718	Paired box protein Pax-9 mRNA	3.30
596464	Mus musculus RIKEN cDNA 1700009P03 gene mRNA	3.28
3587973	Mus musculus annexin A1 mRNA	3.24
4194027	Mus musculus unknown protein for MGC:29137 mRNA	3.20
4194295	Unnamed protein product [Mus musculus] mRNA	2.91
30013295	Insulin-like growth factor II precursor (multiplication stimulating polypeptide) (IGF-II) mRNA	2.83
56370	Neuronal protein NP25, transgelin 3 mRNA	2.57
4159971	Fatty acid-binding protein, liver (L-FABP) (14 kDa selenium-binding protein) mRNA	2.50
5066237	Mus musculus MAD homolog 2 (Drosophila) mRNA	2.46
3593200	Similar to myeloid ecotropic viral integration site-related gene 2 [Mus musculus] (BC003762) mRNA	2.33
4238555	Mus musculus alcohol dehydrogenase 1 (class I) mRNA	2.32
4192239	Hepatocyte growth factor receptor precursor (Met proto-oncogene tyrosine kinase) (c-met) (HGF receptor) (HGF-SF receptor) mRNA	2.20
6430786	Mus musculus unknown protein for MGC:46985 mRNA	0.50
6434212	(XM_128428) hypothetical protein XP_128428 [Mus musculus] mRNA	0.50
5669278	Unnamed protein product [Mus musculus] mRNA	0.49
6435574	Unnamed protein product [Mus musculus] mRNA	0.49
6433215	60S ribosomal protein L15 mRNA	0.49
5656666	(XM_157865) hypothetical protein XP_157865 [Mus musculus] mRNA	0.48
5660251	Baculoviral IAP repeat-containing protein 5 (apoptosis inhibitor survivin) (apoptosis inhibitor 4) (TIAP) mRNA	0.46
6436242	RAS-related protein RAB-26 [Mus musculus] mRNA	0.46
862806	Delta-like protein 4 precursor (Drosophila delta homolog 4) mRNA	0.43
6431732	Mus musculus Max dimerization protein mRNA	0.42
6436628	Betaine-homocysteine methyltransferase [Rattus norvegicus] [Mus musculus] mRNA	0.40
5682232	Hypothetical protein FLJ11230 [Mus musculus] mRNA	0.23

- Ratio value of 2.0 or above is considered as significant up-regulation, while 0.5 or below is considered as significant down-regulation
- A number of genes involved in glucose metabolism were up-regulated. This could be due to the increased turning-on of the transcription machinery of several genes that may need the additional energy originated from glucose metabolism in these AS-ON-transfected RIN cells



**Figure 28.** Insulin content of PS-ON transfected RIN1046-38 cells. This graphical representation reflects the results of the ELISA assays for insulin content of RIN1046-38 cells shown as mean  $\pm$  SEM. No statistically significant difference was found in the insulin content of RIN cells treated with vehicle, transfected with PS-AS-ON or with PS-NS-ON (vehicle v PS-NS-ON,  $p = 0.45$ ; vehicle v PS-AS-ON,  $p = 0.45$ ; PS-NS-ON v PS-AS-ON,  $p = 0.42$ ;  $n = 5$ ).

**Table 6.** Insulin secretion from RIN1046-38 cells (in ng/10<sup>6</sup> cells) in response to 0.5 mM carbachol.

	Basal insulin secretion (60 min.) [mean ± SEM]	0.5 mM carbachol stimulation (60 min.) [mean ± SEM]	Number of samples
Vehicle-treated RIN cells	3.03 ± 0.23 <sup>a,b</sup>	5.78 ± 0.12 <sup>d,e</sup>	5
200 nM PS-NS-ON transfected RIN cells	2.81 ± 0.09 <sup>a,c</sup>	5.68 ± 0.16 <sup>d,f</sup>	5
200 nM PS-AS-ON transfected RIN cells	3.61 ± 0.09 <sup>b,c</sup>	8.94 ± 0.39 <sup>e,f</sup>	5

<sup>a</sup> Basal: vehicle v PS-NS-ON, p = 0.21

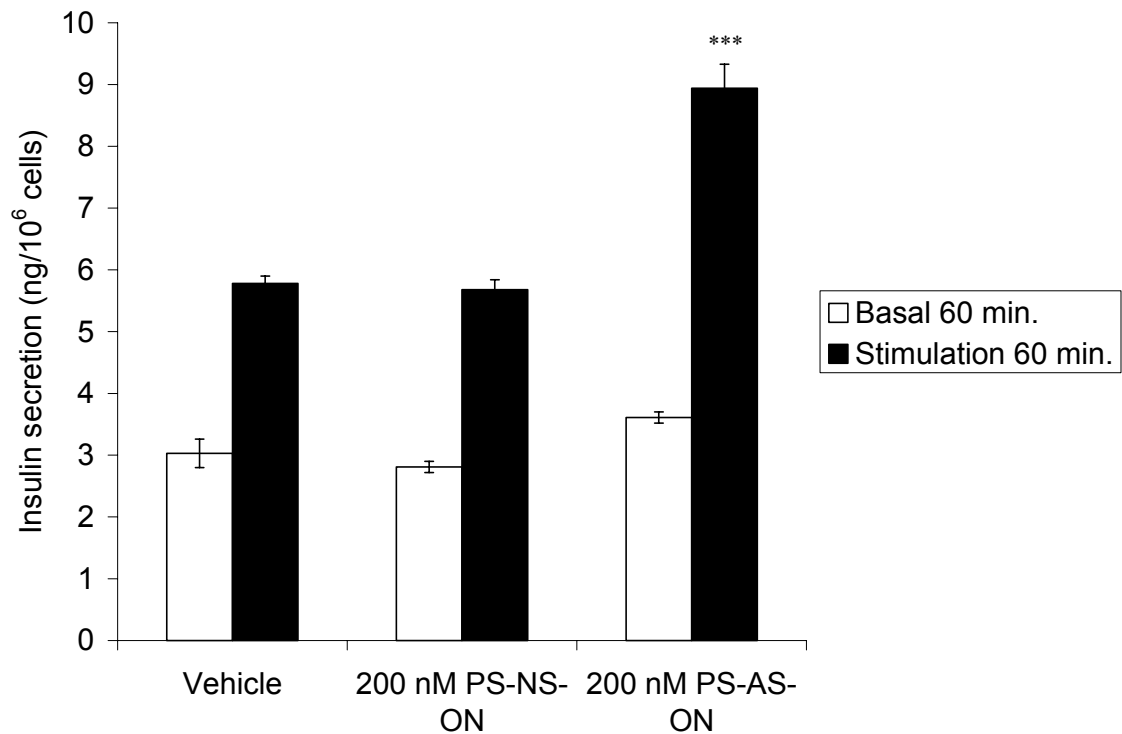
<sup>b</sup> Basal: vehicle v PS-AS-ON, p < 0.05

<sup>c</sup> Basal: PS-NS-ON v PS-AS-ON, p < 0.001

<sup>d</sup> 0.5 mM carbachol stimulation: vehicle v PS-NS-ON, p = 0.31

<sup>e</sup> 0.5 mM carbachol stimulation: vehicle v PS-AS-ON, p < 0.001

<sup>f</sup> 0.5 mM carbachol stimulation: PS-NS-ON v PS-AS-ON, p < 0.001



**Figure 29.** The effect of CaBP<sub>28k</sub> depletion on insulin secretion from the RIN1046-38 cells. The graphical representation reflects the results of the ELISA assays for insulin secretion into the basal incubation and stimulation media of RIN1046-38 cells shown as mean  $\pm$  SEM. RIN cells were incubated in the basal medium (modified Krebs-Ringer medium containing 2.8 mM glucose) for 60 min., then incubated in the stimulation medium (basal medium + 0.5 mM carbachol) for another 60 min. The RIN cells transfected with 200 nM PS-AS-ON showed a statistically significant higher insulin secretion level in response to 0.5 mM carbachol as compared to that of both the vehicle-treated and the 200 nM PS-NS-ON-transfected cells ( $p < 0.001$ ) [ $n = 5$ ].

**Table 7.** Fractional insulin secretion of RIN1046-38 cells (%insulin secretion/total cellular insulin content) in response to 0.5 mM carbachol.

	Fractional basal insulin secretion (60 min.) [mean ± SEM]	Fractional stimulation insulin secretion (60 min.) [mean ± SEM]	Number of samples
Vehicle-treated RIN cells	6.69 ± 0.99 <sup>a,b</sup>	12.70 ± 1.33 <sup>d,e</sup>	5
200 nM PS-NS-ON transfected RIN cells	6.22 ± 0.74 <sup>a,c</sup>	12.62 ± 1.57 <sup>d,f</sup>	5
200 nM PS-AS-ON transfected RIN cells	8.54 ± 1.04 <sup>b,c</sup>	20.94 ± 2.42 <sup>e,f</sup>	5

<sup>a</sup> Fractional basal: vehicle v PS-NS-ON, p = 0.36

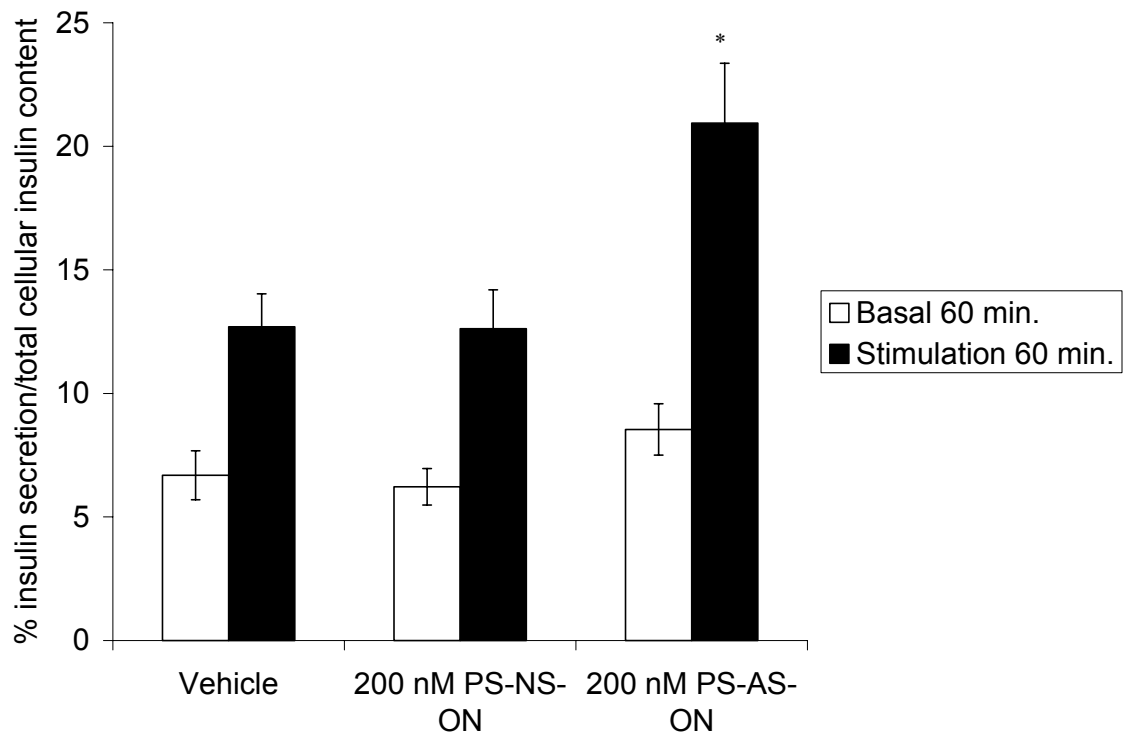
<sup>b</sup> Fractional basal: vehicle v PS-AS-ON, p = 0.12

<sup>c</sup> Fractional basal: PS-NS-ON v PS-AS-ON, p = 0.06

<sup>d</sup> Fractional stimulation (0.5 mM carbachol): vehicle v PS-NS-ON, p = 0.48

<sup>e</sup> Fractional stimulation (0.5 mM carbachol): vehicle v PS-AS-ON, p < 0.05

<sup>f</sup> Fractional stimulation (0.5 mM carbachol): PS-NS-ON v PS-AS-ON, p < 0.05



**Figure 30.** Graphical representation of the fractional insulin secretion from the transfected RIN1046-38 cells based on the insulin ELISA assay results. The values shown as mean  $\pm$  SEM in this chart reflect the percentage of insulin secretion/total cellular insulin content. RIN cells transfected with 200 nM PS-AS-ON show a statistically significant ( $p < 0.05$ ) higher percentage of fractional insulin secretion in response to 0.5 mM carbachol as compared to that of both the vehicle-treated and the 200 nM PS-NS-ON-transfected cells [ $n = 5$ ].



**Table 8.** Insulin secretion of RIN1046-38 cells (mean  $\pm$  SEM in ng/10<sup>6</sup> cells) in response to 0.5 mM carbachol + 17 mM glucose.

	200 nM PS-NS-ON transfected RIN cells	200 nM PS-AS-ON transfected RIN cells	Number of samples
1 <sup>st</sup> 25 min. basal	2.69 $\pm$ 0.22 <sup>a</sup>	3.62 $\pm$ 0.18 <sup>a</sup>	6
1 <sup>st</sup> 25 min. stimulation	11.85 $\pm$ 0.50 <sup>b</sup>	17.62 $\pm$ 1.30 <sup>b</sup>	6
2 <sup>nd</sup> 25 min. stimulation	5.48 $\pm$ 0.24 <sup>c</sup>	12.22 $\pm$ 0.50 <sup>c</sup>	6
3 <sup>rd</sup> 25 min. stimulation	4.07 $\pm$ 0.44 <sup>d</sup>	5.98 $\pm$ 0.92 <sup>d</sup>	6
Last 25 min. basal	3.00 $\pm$ 0.18 <sup>e</sup>	4.42 $\pm$ 0.66 <sup>e</sup>	6

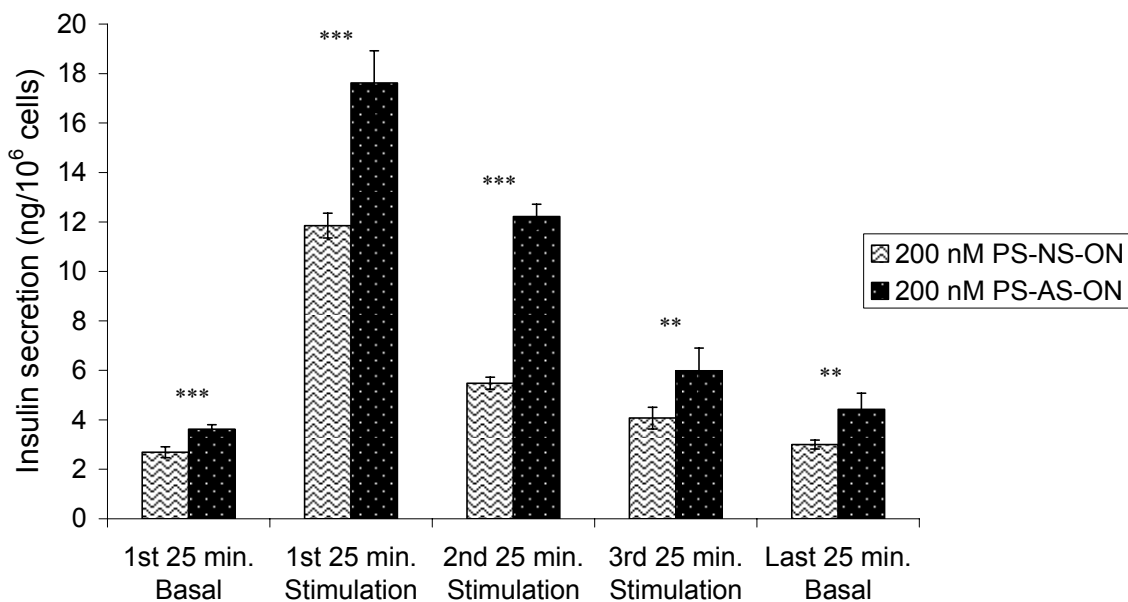
<sup>a</sup> 1<sup>st</sup> 25 min. basal: PS-NS-ON v PS-AS-ON, p < 0.001

<sup>b</sup> 1<sup>st</sup> 25 min. stimulation: PS-NS-ON v PS-AS-ON, p < 0.001

<sup>c</sup> 2<sup>nd</sup> 25 min. stimulation: PS-NS-ON v PS-AS-ON, p < 0.001

<sup>d</sup> 3<sup>rd</sup> 25 min. stimulation: PS-NS-ON v PS-AS-ON, p < 0.005

<sup>e</sup> Last 25 min. basal: PS-NS-ON v PS-AS-ON, p < 0.005



**Figure 31.** Insulin secretion from the PS-ON transfected RIN1046-38 cells in response to 17 mM glucose and 0.5 mM carbachol. This chart reflects the results of the ELISA assays for insulin secretory responses from RIN cells shown as mean  $\pm$  SEM. The 200 nM PS-AS-ON transfected cells showed significantly greater amounts of insulin release compared to that of control 200 nM PS-NS-ON transfected cells. (\*\* indicates  $p < 0.005$ ; \*\*\* indicates  $p < 0.001$ ) [n = 6].

**Table 9.** A summary of the insulin secretion results (mean  $\pm$  SEM) from the ON transfected RIN1046-38 cells (in ng/10<sup>6</sup> cells).

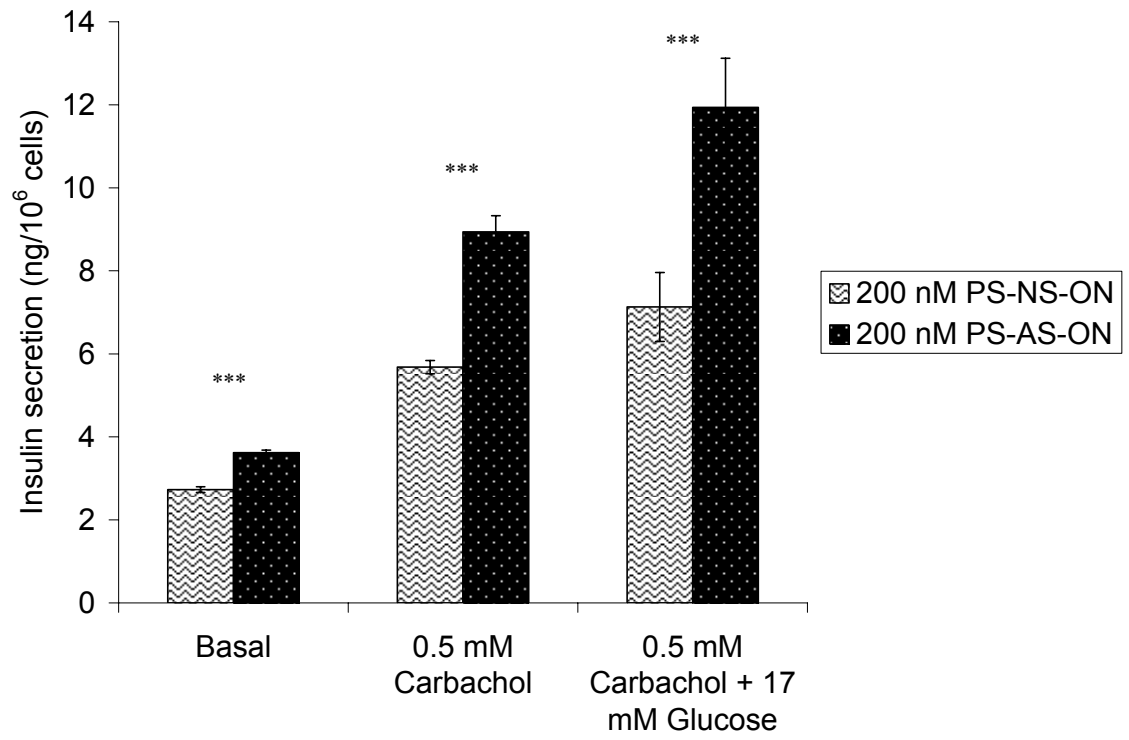
	200 nM PS-NS-ON transfected RIN cells	200 nM PS-AS-ON transfected RIN cells	Number of samples
Basal insulin secretion (pooled data of the 60 min. and 1 <sup>st</sup> 25 min.)	2.73 $\pm$ 0.07 <sup>a</sup>	3.62 $\pm$ 0.06 <sup>a</sup>	11
0.5 mM carbachol stimulation (60 min.)	5.68 $\pm$ 0.16 <sup>b</sup>	8.94 $\pm$ 0.39 <sup>b</sup>	5
0.5 mM carbachol + 17 mM glucose stimulation (pooled data of the 75 min.)	7.13 $\pm$ 0.83 <sup>c</sup>	11.94 $\pm$ 1.18 <sup>c</sup>	6

<sup>a</sup> Basal: PS-NS-ON v PS-AS-ON, p < 0.001

<sup>b</sup> 0.5 mM carbachol stimulation: PS-NS-ON v PS-AS-ON, p < 0.001

<sup>c</sup> 0.5 mM carbachol + 17 mM glucose stimulation: PS-NS-ON v PS-AS-ON, p < 0.001

**Figure 32.** The effects of the PS-ON transfection assays on the insulin secretion from RIN1046-38 cells are combined in this chart. This graphical representation reflects the results of the ELISA assays for insulin secretion after basal incubation and after stimulation from RIN1046-38 cells; the results are shown as mean  $\pm$  SEM. RIN cells were incubated in basal medium containing 2.8 mM glucose, and then in either the stimulation medium of basal medium + 0.5 mM carbachol, or the stimulation medium of 17 mM glucose + 0.5 mM carbachol. RIN cells transfected with 200 nM PS-AS-ON show statistically significant greater insulin secretion in the basal, 0.5 mM carbachol, and 17 mM glucose + 0.5 mM carbachol stimulation conditions, as compared to those of the 200 nM PS-NS-ON transfections ( $p < 0.001$ ) [n = 11 basal samples, n = 5 samples of 0.5 mM carbachol stimulation, n = 6 samples of 0.5 mM carbachol + 17 mM glucose stimulation].



## DISCUSSION

Our previous studies that were done on the CaBP<sub>28k</sub>-KO mice as an *in vivo* model of CaBP<sub>28k</sub>-ablation (Chapter 2) indicated an important role for CaBP<sub>28k</sub> in suppressing the glucose-induced  $\Delta [Ca^{2+}]_i$  in the pancreatic  $\beta$ -cells. In the islet  $\beta$ -cells lacking CaBP<sub>28k</sub>, we found greater rises in the  $[Ca^{2+}]_i$  in response to glucose stimulation as compared to controls (Chapter 2). However, the genomic effects of the lack of CaBP<sub>28k</sub> were not studied. In chapter 3, the model we have adopted, an *in vitro* model of CaBP<sub>28k</sub>-ablation, allows us a more precise evaluation of the effects of the absence of CaBP<sub>28k</sub> in  $\beta$ -cells. Our application of the antisense technology was done in order to assess the function of CaBP<sub>28k</sub> in the cultured RIN1046-38 cells.

We produced complete inhibition of CaBP<sub>28k</sub> using AS-ON in the RIN1046-38 cells and believe that this is the first report of this finding. The complete inhibition of CaBP<sub>28k</sub> expression was achieved using 200 nM PS-AS-ON and 20 nM PY-AS-ON (see Figures 24 and 26). PY-AS-ON transfections have previously been applied to inhibit the expression of different genes both *in vitro* and *in vivo* (Busuttil et al., 1996). Complete inhibition of the SV40 large T antigen (TAg) expression in African green monkey kidney cells, CV-1 cells, was achieved using 50 nM PY-AS-ON (Wagner et al., 1993), while 67% inhibition of p27<sup>Kip1</sup> expression in CV-1 cells was achieved using 30 nM PY-AS-ON (Coats et al., 1996; Flanagan et al., 1999). Another group of researchers described graded

inhibition in the expression of luciferase in HeLa X1/5 cells using PY-AS-ON: no inhibition occurred at 3 nM PY-AS-ON, while 50% inhibition was produced using 9 nM and complete inhibition using 27 nM (Flanagan et al., 1996; Lewis et al., 1996). The concentration of the PY-AS-ON we have applied in our RIN1046-38 cells transfection assays that completely inhibited the CaBP<sub>28k</sub> expression was 20 nM, which is close to the effective inhibitory concentration of 27 nM PY-AS-ON previously used in transfection assays (Flanagan et al., 1996; Lewis et al., 1996). However, Busuttill et al. (1996) applied higher concentrations of the PY-AS-ON (100 – 400 nM), with efficient inhibition of protein kinase C- $\alpha$  and - $\delta$  in cultured rat aortic vascular smooth muscle cells produced at 400 nM. The application of higher concentrations of the PY-AS-ON ( $\geq 20 \mu\text{M}$ ) lead to the nonspecific inhibition of a nontargeted gene ( $\beta$ -gal), in addition to the target gene (TA $\alpha$ ) in the CV-1 cells (Wagner et al., 1993).

Based upon our experience with RIN cells (Rhoten and Sergeev, 1994), a 50% change in the level of CaBP<sub>28k</sub> was associated with a change in the  $[\text{Ca}^{2+}]_i$  responses to secretagogues. Our findings in Chapter 2 showed that the CaBP<sub>28k</sub>-KO (CaBP<sub>28k</sub>-ablated) mice islets had much greater rises in the  $[\text{Ca}^{2+}]_i$  responses to glucose than those of the WT mice islets. In Chapter 3, we have knocked down (ablated) CaBP<sub>28k</sub> expression of the RIN1046-38 cells. Taken together, it would be likely to find associated changes in the  $[\text{Ca}^{2+}]_i$  responses in our CaBP<sub>28k</sub>-ablated RIN1046-38 cells. We report that expression of calbindin-D<sub>9k</sub> (protein and mRNA) in transfected RIN1046-38 cells was increased when the

expression of CaBP<sub>28k</sub> was completely abolished (Figures 24, 25, and 26; and Table 4). Calbindin-D<sub>9k</sub> is a major cytosolic protein that acts as a shuttle for Ca<sup>2+</sup> transfer from the luminal to the basolateral poles of the intestinal epithelial cell (Kumar, 1995). Calbindin-D<sub>9k</sub> also increases ATP-dependent Ca<sup>2+</sup> transport in the duodenal basolateral membrane (Walters, 1989; Walters et al., 1990) and can bind to the regulatory calmodulin-binding domain of the plasma membrane Ca<sup>2+</sup> pump (Christakos et al., 1989; Gross and Kumar, 1990; Bouhtiauy et al., 1994). Calbindin-D<sub>9k</sub> was also found to play an important role in the transport of Ca<sup>2+</sup> in the distal tubule of the kidney where it enhances the ATP-dependent Ca<sup>2+</sup> transport through the basolateral membrane (Bouhtiauy et al., 1994). Calbindin-D<sub>9k</sub> levels were determined in the rat pancreas by radioimmunoassay to be  $9.8 \pm 2.7$  ng/mg protein (Thomasset et al., 1982). Calbindin-D<sub>9k</sub> can act as an intracellular Ca<sup>2+</sup> buffer to protect the cells from the toxic effects of increased cytoplasmic calcium concentrations and stabilize [Ca<sup>2+</sup>]<sub>i</sub> (Thomasset, 1997). Armbrrecht et al. (2003) illustrated that the increase in the expression of calbindin-D<sub>9k</sub> protein in the rat duodenum was strongly correlated with both increased Ca<sup>2+</sup> transport and Ca<sup>2+</sup> uptake.

Additionally, dietary Ca<sup>2+</sup> levels were found to modulate the gene expression of intestinal calbindin-D<sub>9k</sub> in the rat (Freund and Bronner, 1975; Bronner et al., 1986). The effect of Ca<sup>2+</sup> on calbindin-D<sub>9k</sub> gene expression has been confirmed by *in vitro* studies that showed an increase in the production of calbindin-D<sub>9k</sub> mRNA upon increasing the Ca<sup>2+</sup> concentration in the medium of



fetal rat intestinal organ cultures, in the presence or absence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Bréhier and Thomasset, 1990). Ca<sup>2+</sup> as a second messenger encodes information about the magnitude (amplitude), frequency, and spatial organization of concentration changes (Lechleiter et al., 1991). In addition, Li et al. (1997) reported that a reduction of calbindin-D<sub>9k</sub> expression in the intestine and kidney was observed in normocalcemic VDR-ablated mice, suggesting that it is a consequence of VDR deficiency rather than hypocalcemia. However, a diet high in Ca<sup>2+</sup> leads to normalization of intestinal calbindin-D<sub>9k</sub> expression in the VDR-ablated mice, suggesting the involvement of Ca<sup>2+</sup> in the regulation of calbindin-D<sub>9k</sub> gene expression (Li et al., 1998).

cDNA microarray analysis done on 200 nM PS-AS-ON-transfected RIN cells revealed that the VDR and calbindin-D<sub>9k</sub> mRNAs were up-regulated when compared to that of control 200 nM PS-NS-ON transfections (Table 4). We also detected the enhanced expression of VDR protein in the CaBP<sub>28k</sub>-ablated RIN1046-38 cells using Western blotting analysis (Figure 27). Our data also illustrates a strong correlation between the calbindin-D<sub>9k</sub> mRNA levels with its protein expression in the RIN1046-38 cells. This correlation is similar to that previously found by Armbrecht et al. (2003), who reported that in the rat duodenum the changes in calbindin-D<sub>9k</sub> protein expression levels were highly correlated with its mRNA expression levels. We also found a strong correlation between the VDR mRNA levels with its protein expression in the RIN1046-38 cells. The expression of both the VDR protein and mRNA in normal untreated

RIN1046-38 cells has been previously reported (Christakos et al., 1991; Lee et al., 1994). Both  $1,25\text{-(OH)}_2\text{D}_3$  and  $\text{Ca}^{2+}$  were previously reported to regulate the expression of VDR (Brown et al., 1995; Zineb et al., 1998; Healy et al., 2003). VDR mediates the signal of  $1,25\text{-(OH)}_2\text{D}_3$  by binding to the VDREs in the DNA as a homodimer or as a heterodimer composed of one VDR subunit and one RXR receptor subunit (Nishikawa et al., 1995; Christakos et al., 2003b). VDR interacts with many nuclear transcription factors and coactivators including the general transcription factor TFIIB, a component of the basal transcription complex, which plays a critical role in ligand-dependent transcription (Blanco et al., 1995; MacDonald et al., 1995; Masuyama et al., 1997).

The association between the increased expression of VDR mRNA and protein with the expression of calbindin- $\text{D}_{9k}$  mRNA and protein in our  $\text{CaBP}_{28k}$ -ablated RIN cells marks the interrelationships between these factors. It has been postulated that VDR modulates the expression of calcium binding proteins which control the  $[\text{Ca}^{2+}]_i$  (Christakos et al., 1979; Christakos and Norman, 1980; Mayer et al., 1981). Moreover, earlier developmental studies have shown that the induction of the VDR mRNA in the rat kidney is correlated with the induction of  $\text{CaBP}_{28k}$  mRNA, while the induction of the VDR mRNA in the rat intestine is associated with the induction of the calbindin- $\text{D}_{9k}$  mRNA (Christakos et al., 1991). The increase in the expression levels of the VDR, and both  $\text{CaBP}_{28k}$  and calbindin- $\text{D}_{9k}$  at the same developmental stage suggests that the induction of the VDR has an important role in regulating the gene expression of both  $\text{CaBP}_{28k}$

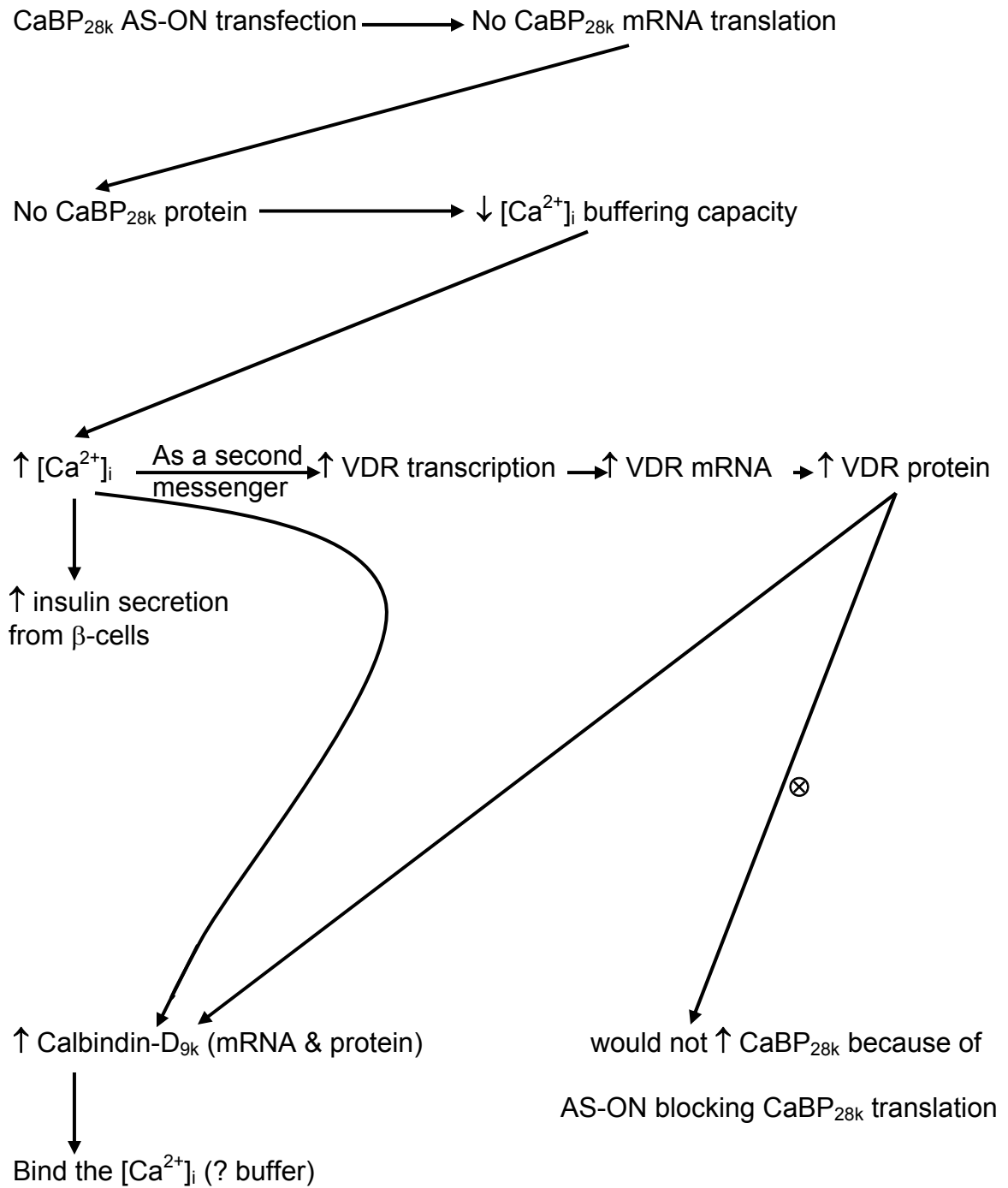
and calbindin-D<sub>9k</sub> (Huang et al., 1989; Christakos et al., 1991). In addition, in the vitamin D-deficient adult rat, the repletion of vitamin D led to the up-regulation of VDR and CaBP<sub>28k</sub> in the kidney and calbindin-D<sub>9k</sub> in the intestine (Christakos et al., 1991). Li et al. (1998) reported that mice lacking the VDR have markedly reduced levels of calbindin-D<sub>9k</sub> mRNA in the duodenum, kidney, brain, and lungs, when compared to control wild-type mice. However, CaBP<sub>28k</sub> mRNA levels in the VDR-ablated mice were only moderately reduced in the kidney and not affected in the lungs and brain, compared to control wild-type mice. These data demonstrate that VDR regulates calbindin-D<sub>9k</sub> gene expression and that additional tissue-specific factors may modulate the effects of VDR on CaBP<sub>28k</sub> gene expression (Li et al., 1998). Furthermore, Hemmingsen et al. (2002) concluded that rat intestinal calbindin-D<sub>9k</sub> was increased significantly in response to high plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub> level and was significantly decreased in response to low plasma 1,25-(OH)<sub>2</sub>D<sub>3</sub>. Their study also showed that the effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on the intestinal calbindin-D<sub>9k</sub> was modulated by the plasma Ca<sup>2+</sup> concentrations (Hemmingsen et al., 2002).

Notably, our study presents, for the first time, that cultured beta cells express calbindin-D<sub>9k</sub>. The synthesis of calbindin-D<sub>9k</sub> was previously reported to be under the control of the 1,25-(OH)<sub>2</sub>D<sub>3</sub> in the rat intestine (Thomasset et al., 1982; Perret et al., 1985; Bréhier and Thomasset, 1990). Previous investigations also found that the 1,25-(OH)<sub>2</sub>D<sub>3</sub> controls the expression of the calbindin-D<sub>9k</sub> gene at the transcriptional and the posttranscriptional levels (Dupret et al., 1987).

A consensus binding site for the VDR-RXR heterodimer has been characterized in the calbindin-D<sub>9k</sub> gene (Nishikawa et al., 1994; Colnot et al., 1995). Lee et al. (1994) reported that 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment of the RIN1046-38 cells led to inhibition of cell growth and up-regulation of VDR expression as compared to controls.

Our findings suggest that the regulatory mechanisms of CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> levels in RIN cells may interact so that the absence of CaBP<sub>28k</sub> expression leads to increased calbindin-D<sub>9k</sub> expression. RIN cells may respond to the depletion of CaBP<sub>28k</sub>, which is required for binding free [Ca<sup>2+</sup>]<sub>i</sub>, by synthesizing an alternative Ca<sup>2+</sup>-binding protein, calbindin-D<sub>9k</sub>. This is compatible with the idea that CaBP<sub>28k</sub> or an alternative Ca<sup>2+</sup>-binding protein such as calbindin-D<sub>9k</sub> plays a critical role in β-cells. In the nervous system CaBP<sub>28k</sub> is known to bind and buffer [Ca<sup>2+</sup>]<sub>i</sub>, and protect neurons against Ca<sup>2+</sup>-induced cytotoxicity (Mattson et al., 1991). Although the endoplasmic reticulum (ER) in pancreatic beta-cells is of major importance in buffering Ca<sup>2+</sup> for the continuous regulation of Ca<sup>2+</sup> cycling (Nilsson et al., 1987), additional buffering of Ca<sup>2+</sup> may be required. The high affinity Ca<sup>2+</sup>-binding functions of calbindin-D<sub>9k</sub> are well established (Delorme et al., 1983; Bruns et al., 1986; Juffer and Vogel, 2000; Armbrrecht et al., 2003). Calbindin-D<sub>9k</sub> can act as an intracellular Ca<sup>2+</sup> buffer to protect cells from the toxic effects of increased cytoplasmic calcium concentrations and stabilize [Ca<sup>2+</sup>]<sub>i</sub> (Thomasset, 1997). Calbindin-D<sub>9k</sub> can also bind to the regulatory calmodulin-binding domain of the plasma membrane Ca<sup>2+</sup>

pump (Christakos et al., 1989; Gross and Kumar, 1990; Bouhtiauy et al., 1994). Thus, calbindin-D<sub>9k</sub> could compensate for the effects of CaBP<sub>28k</sub> ablation and modulate the rises in [Ca<sup>2+</sup>]<sub>i</sub>. The known functions of both CaBP<sub>28k</sub> and Calbindin-D<sub>9k</sub> are to regulate [Ca<sup>2+</sup>]<sub>i</sub> levels in the cell. Evidence of functional compensation between CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> could be seen in the mammalian kidney where the calbindin-D<sub>9k</sub> gene is expressed in both mouse and rat kidneys (Thomasset et al., 1982; Delorme et al., 1983; Schreiner et al., 1983; Rhoten et al., 1985; Bindels et al., 1991). However, calbindin-D<sub>9k</sub> is less active in the rat kidney, where CaBP<sub>28k</sub> gene is highly expressed and predominates in Ca<sup>2+</sup> regulation (Schreiner et al., 1983; Bindels et al., 1991; Thomasset, 1997). In addition, in the rat intestine no CaBP<sub>28k</sub> mRNA is expressed and calbindin-D<sub>9k</sub> may fulfill the functions of CaBP<sub>28k</sub> (Hunziker, 1986). Calbindin-D<sub>9k</sub> is known to play an important role in buffering [Ca<sup>2+</sup>]<sub>i</sub> in the duodenal enterocytes (Schroder et al., 1996). Additional research will help in understanding the exact role of calbindin-D<sub>9k</sub> in the AS-ON transfected RIN1046-38 cells and lead to a better understanding of calbindin-D<sub>9k</sub> functions in other cells. A hypothesis of the mechanisms underlying the interplay between CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> in AS-ON transfected RIN1046-38 cells is illustrated in Figure 33. These proposed mechanisms include potential roles of VDR and calbindin-D<sub>9k</sub> expression in the AS-ON transfected RIN1046-38 cells. This model is in accordance with recent studies indicating that several factors are involved in the control of the expression of calbindin-D<sub>9k</sub>, including the VDR and Ca<sup>2+</sup> levels (Walters et al., 1999).



**Figure 33.** A diagrammatic illustration of the hypothesis of the possible cascade of events occurring in AS-ON transfected RIN1046-38 cells.

Our data suggest that CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> may be able to functionally compensate for one another in AS-ON transfected RIN cells. Previous studies showed that CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> are products of two independent genes (Hunziker, 1986). The CaBP<sub>28k</sub> mRNA is 2.0 – 3.1 kilobases long and does not cross-hybridize with calbindin-D<sub>9k</sub> ( $\approx$  0.7 kilobase) (Hunziker, 1986). Both CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> bind Ca<sup>2+</sup> with high affinity ( $1 - 10 \times 10^{-7}$  M) (Bredderman and Wasserman, 1974; Fullmer and Wasserman, 1980). The effects of AS-ON transfections in RIN cells suggests that the system may be able to restore some of the regulatory functions of these molecules by independently switching off/on the expression of CaBP<sub>28k</sub>/calbindin-D<sub>9k</sub>.

We further extended our study to investigate the effects of ON transfections on the insulin content and the insulin secretory functions of the RIN1046-38 cells. We are the first to elucidate the effects of CaBP<sub>28k</sub>-AS-ON transfection on insulin content and release by RIN1046-38 cells. Using ELISA, we estimated the insulin content of our control vehicle-treated RIN cells to be  $46.32 \pm 3.90$  ng/10<sup>6</sup> cells, while that of the PS-NS-ON transfected cells to be  $47.20 \pm 5.97$  ng/10<sup>6</sup> cells, and that of the PS-AS-ON transfected cells was  $45.43 \pm 6.69$  ng/10<sup>6</sup> cells. We did not find any significant difference between the three groups included in our study. However, our results are close to those of previous research that used a radioimmunoassay to measure the insulin content of control RIN1046-38 cells, which was reported to be  $52 \pm 3$  ng/10<sup>6</sup> cells (Lee et al., 1994).

Our data showed significantly increased basal and stimulated insulin secretion in response to 0.5 mM carbachol from the PS-AS-ON transfected RIN cells compared to controls (vehicle-treated and PS-NS-ON transfections) [Table 6 and Figure 29]. Using ELISA, we estimated the insulin secretion of the RIN1046-38 cells that were incubated for 60 min. in the basal buffer with 2.8 mM glucose to be (in ng/10<sup>6</sup> cells): 3.03 ± 0.23 for the vehicle-treated cells, 2.81 ± 0.09 for the PS-NS-ON transfected cells, and 3.61 ± 0.09 for the PS-AS-ON transfected cells. These findings are close to those of previous studies using radioimmunoassay that measured the insulin secretion of control RIN1046-38 cells incubated for 90 min. in the basal buffer with 1 mM glucose to be 2.89 ± 0.36 ng/10<sup>6</sup> cells (Rodriguez-Pena et al., 1997). Both the glucose concentrations of 1 mM and 2.8 mM were considered as nonstimulatory for insulin secretion from β-cells (Hermans et al., 1987; Yada et al., 1992, 1995). Moreover, we measured by ELISA the insulin secretory responses of RIN cells that were incubated for 60 min. in the stimulation buffer with 2.8 mM glucose + 0.5 mM carbachol to be (in ng/10<sup>6</sup> cells): 5.78 ± 0.12 for the vehicle-treated cells, 5.68 ± 0.16 for the PS-NS-ON transfected cells, and 8.94 ± 0.39 for the PS-AS-ON transfected cells. Our findings are in accord with studies using radioimmunoassay to measure insulin secretory responses of control RIN1046-38 cells that were incubated for 90 min. in a buffer containing 0.1 mM carbachol to be 3.81 ± 0.52 ng/10<sup>6</sup> cells (Rodriguez-Pena et al., 1997). Since we have incubated our RIN cells in a buffer with a higher concentration of carbachol (0.5 mM) as the stimulating reagent, it is expected to find our insulin secretion values



higher than those of Rodriguez-Pena et al., 1997. Our insulin ELISA results showed a much greater increase in insulin release from the PS-AS-ON- compared to the PS-NS-ON-transfected RIN cells in response to secretagogues. The microarray analysis (Table 4) indicated that no change occurred in the insulin mRNA. These results imply that the PS-AS-ON transfection did not affect insulin synthesis, but rather increased the insulin secretory responses of the cultured RIN1046-38 cells.

We found significantly higher insulin secretory responses to stimulation with 0.5 mM carbachol plus 17 mM glucose in all the time periods of measurement of 200 nM PS-AS-ON transfected RIN cells compared to that of control 200 nM PS-NS-ON transfected cells (Table 8 and Figure 31). AS-ON transfected (CaBP<sub>28k</sub>-ablated) RIN cells showed a much higher insulin secretory response to the secretagogues as compared to that of the controls. This is consistent with previous observations that showed significant increases in insulin secretion from the CaBP<sub>28k</sub>-KO mice islets, as compared to controls, when depolarized with a high concentration of KCl [45 mM] (Sooy et al., 1999). Moreover, our previous research has shown a significant increase in the  $\Delta [Ca^{2+}]_i$  from the CaBP<sub>28k</sub>-KO mice islets, as compared to controls, when the  $\beta$ -cells were exposed to a high concentration of glucose (Chapter 2; Parkash et al., 2002). It has been established that Ca<sup>2+</sup> plays an important role in the glucose-induced insulin secretion (Figure 12; Gilon et al., 1993; Jones and Persaud, 1998; Easom, 1999; Lang, 1999; Lingappa and Farey, 2000; Barg et al., 2002). In

addition, in  $\beta$ -cells the  $[Ca^{2+}]_i$  and insulin secretions were found to oscillate in synchrony during stimulation by glucose (Gilon et al., 1993; Henquin et al., 2002). It was also reported that omission of  $Ca^{2+}$  from the incubation medium (Grotsky and Bennett, 1966; Milner and Hales, 1967) or addition of blockers of L-type voltage-dependent  $Ca^{2+}$ -channels abolished glucose-stimulated insulin secretion (Devis et al., 1975). In our earlier study on  $\beta$ HC-13 CaBP40 cells, we showed that the overexpression of calbindin- $D_{28k}$  caused almost a complete absence of the typical glucose-induced rise in  $[Ca^{2+}]_i$  in these cells, while the control  $\beta$ HC-13 cells responded to the addition of high glucose with a very substantial increase in the  $\Delta [Ca^{2+}]_i$  (Parkash et al., 2002). The present study, two earlier studies (Rhoten and Sergeev, 1994; Reddy et al., 1997) and our more recent studies (Sooy et al., 1999; Parkash et al., 2002) indicate the importance of CaBP $_{28k}$  in stabilizing the  $[Ca^{2+}]_i$ , specifically in the  $\beta$ -cell. Taken together, all these studies indicate that CaBP $_{28k}$  plays a significant role in controlling glucose-induced insulin secretion from pancreatic  $\beta$ -cells.

However, RIN cells do not recognize glucose by itself as a potent secretagogue and have only a modest response to glucose stimulation (Halban et al., 1983; Praz et al., 1983; Sooy et al., 1999). In addition, the insulin content of RIN cells was only 0.1% of that of normal mice islets compared to the  $\beta$ TC-3 cells (a  $\beta$ -tumor cell line-3, from an adenoma created in transgenic mice expressing the simian virus 40, SV40, large T-antigen oncogene under control of the rat insulin II promoter) that have insulin content 20% of that of normal mice

islets (Hanahan, 1985; Efrat et al., 1988; D'Ambra et al., 1990; Sooy et al., 1999). D'Ambra et al. (1990) estimated the insulin content of  $\beta$ TC-3 cells to be  $3100 \pm 294$  ng/100  $\mu$ g protein which is, as expected, a much higher level than that of our control RIN1048-38 cells (vehicle-treated RIN cells,  $240.67 \pm 11.26$  ng/100  $\mu$ g protein; and 200 nM PS-NS-ON transfected RIN cells,  $213.75 \pm 22.99$  ng/100  $\mu$ g protein). RIN1046-38 cells express high levels of CaBP<sub>28k</sub> ( $0.8 \pm 0.2$   $\mu$ g/mg protein, Lee et al., 1994, using radioimmunoassay). This is similar to the chicken pancreas ( $1.06 \pm 0.2$   $\mu$ g/mg protein, Sergeev and Rhoten, unpublished data) that made them both appropriate models for investigating the functional role of CaBP<sub>28k</sub> in the  $\beta$ -cells.

Our present results demonstrate that in the presence of a high glucose concentration (which stimulates insulin release, 17 mM) and 0.5 mM carbachol produced a greater insulin secretion from both the 200 nM PS-AS-ON- and the 200 nM PS-NS-ON-treated RIN cells, than that evoked by 0.5 mM carbachol in the basal glucose concentration (non-stimulatory level, 2.8 mM) (Table 9 and Figure 32). This is in accordance with previous observations that carbachol increases insulin secretion mildly in the presence of basal glucose concentrations but is much more effective in the presence of glucose concentrations which are stimulatory for insulin secretion (Bergman and Miller, 1973; Hermans et al., 1987; Regazzi et al., 1990; Yada et al., 1995; Babb et al., 1996; Nesher et al., 2002). Elevated glucose and carbachol have been shown to stimulate rises in  $[Ca^{2+}]_i$  in rat pancreatic  $\beta$ -cells (Peter-Riesch et al., 1988; Theler et al., 1992; Wang et al.,

1992; Yada et al., 1992; Babb et al., 1996; Nesher and Cerasi, 2002; Nesher et al., 2002), and in RIN cells in association with insulin release (Biden and Wollheim, 1986; Wollheim and Biden, 1986; Biden et al., 1987, 1988). Carbachol requires that the  $\beta$ -cell membrane be sufficiently depolarized, e.g., by elevated glucose, to reach the threshold potential at which  $\text{Ca}^{2+}$  channels are activated. Hence carbachol can be considered as a mild initiator as well as a strong potentiator of insulin secretion (Hermans et al., 1987; Gylfe, 1991; Yada et al., 1995; Babb et al., 1996; Martin et al., 1997). It should be kept in mind, however, that the insulintropic action of carbachol can also involve activation of PKC, which is likely to contribute to the potentiating effect of carbachol in insulin secretion (Prentki and Matschinsky, 1987; Peter-Riesch et al., 1988; Persaud et al., 1989).

We need to expand our future study to include the  $\text{CaBP}_{28\text{k}}\text{-KO}$  and WT mice we have previously deployed in Chapter 2. This future research will include the study of VDR and calbindin- $\text{D}_{9\text{k}}$  protein expression in the pancreas of these mice using Western blot analysis. We will also conduct immunocytochemical analysis of their expressions in the pancreatic  $\beta$ -cells. In addition, cDNA microarray analysis will be needed to compare the gene expression profile of the  $\text{CaBP}_{28\text{k}}\text{-KO}$  mice pancreas to that of the WT mice pancreas. These future studies which will help to determine whether the changes we found in the expression of both VDR and calbindin- $\text{D}_{9\text{k}}$  in our *in vitro* model of  $\text{CaBP}_{28\text{k}}\text{-}$  ablation also occurs in the whole organism with *in vivo*  $\text{CaBP}_{28\text{k}}\text{-}$ ablation.

Nonetheless, the quantitative measurements (using ELISA) of the levels of the VDR and calbindin-D<sub>9k</sub> proteins in both AS-ON- and NS-ON-transfected RIN1046-38 cells will be required to quantify the influence of ON transfection on the expression of these factors. Also, measurements of [Ca<sup>2+</sup>]<sub>i</sub> will help to evaluate the possible role of Ca<sup>2+</sup> as a second messenger affecting the genetic profile of ON-transfected RIN cells. Quantitative measurement of both CaBP<sub>28k</sub> and calbindin-D<sub>9k</sub> proteins (using ELISA) in the RIN1046-38 cells before and after AS-ON transfection will be needed to estimate the degree of switching off/on of CaBP<sub>28k</sub>/calbindin-D<sub>9k</sub> expression in the RIN cells. It is well known that one molecule of calbindin-D<sub>9k</sub> binds 2 molecules of Ca<sup>2+</sup>, while one molecule of CaBP<sub>28k</sub> is known to bind 4 molecules of Ca<sup>2+</sup>. The proposed experiments would help to verify whether calbindin-D<sub>9k</sub> is synthesized as a complete or partial compensation for the variations in CaBP<sub>28k</sub> levels in transfected RIN1046-38 cells.

## **CONCLUSIONS**

It has been established that the interactions between  $[Ca^{2+}]_i$  and the cytoskeleton of pancreatic  $\beta$ -cells result in release of insulin granules through the process of exocytosis. Yet, the need to improve insulin secretory responsiveness to a glucose challenge is thought to be contributing to the treatment of type 2 diabetes. This thesis describes studies done to evaluate the interplay among  $[Ca^{2+}]_i$ , insulin concentration and secretion, and CaBP<sub>28k</sub> expression levels to help clarify the potential role of CaBP<sub>28k</sub> in the pancreatic  $\beta$ -cells in the control of insulin secretion. The three chapters presented here describe various experimental models for exploring the possible roles of CaBP<sub>28k</sub> in the  $\beta$ -cells using: (1) an avian model {chicken}, (2) *in vitro* studies with a mammalian model {CaBP<sub>28k</sub>-KO and WT mice}, and (3) tissue culture of mammalian  $\beta$ -cells {RIN1046-38 cell line}. The results of this study indicate the important influence of CaBP<sub>28k</sub> in the  $\beta$ -cells. This summary will briefly review the results of the three studies, indicate the importance of these results in understanding the possible roles of CaBP<sub>28k</sub> in  $\beta$ -cells, and present ideas for future studies that could be performed to look at additional aspects of the functional roles of CaBP<sub>28k</sub> in  $\beta$ -cells.

(1) It is has been known that the chicken pancreas expresses comparatively higher levels of CaBP<sub>28k</sub> than mammals, and the insulin release threshold of the chicken  $\beta$ -cells is elevated with reduced insulin output in response to glucose stimulation. We investigated the distribution of CaBP<sub>28k</sub> and insulin in the different lobes of chicken pancreas, and the cytosolic

interrelationships between insulin and CaBP<sub>28k</sub> in pancreatic  $\beta$ -cells. Western blot analysis for CaBP<sub>28k</sub> expression revealed the highest levels of expression in ventral and dorsal lobes, while the lowest expression was in the splenic lobe, which is similar to the results of ELISA for insulin concentrations in chicken pancreatic lobes. Immunohistochemistry demonstrated a greater intensity of reaction product for localization of CaBP<sub>28k</sub> than insulin in pancreatic islets. Confocal microscopic studies detected colocalization and a strongly positive correlation between insulin and CaBP<sub>28k</sub> in the  $\beta$ -cells. Our findings of similarities in the way of distribution of both insulin and CaBP<sub>28k</sub> and their colocalization suggest a possible role for CaBP<sub>28k</sub> in  $\beta$ -cells that could be contributing to the type 2 diabetes-like characteristics of chickens. Future studies could be designed to describe the changes in  $[Ca^{2+}]_i$  and insulin secretory responses to glucose stimulation in the CaBP<sub>28k</sub>-ablated (CaBP<sub>28k</sub>-AS-ON transfected) chicken pancreatic islets as compared to controls.

(2) In addition, *in vitro* studies were conducted to look at the glucose-induced changes in  $[Ca^{2+}]_i$  of pancreatic islets from CaBP<sub>28k</sub>-KO compared to WT mice. The pancreatic islets of CaBP<sub>28k</sub>-KO mice exposed to increasing glucose concentrations in the incubation medium from 2.8 mM to 30 mM, levels that mimic the transition from fasting to hyperglycemic states, showed significantly greater rises in  $[Ca^{2+}]_i$  as compared to WT. Qualitative differences were also seen in the kinetics of  $[Ca^{2+}]_i$  between CaBP<sub>28k</sub>-KO and WT mice islets. It has been established that the  $[Ca^{2+}]_i$  of the pancreatic  $\beta$ -cells controls the exocytosis



of insulin granules in response to glucose stimulation. With previous studies showing coincidence of insulin secretion with the rises in  $[Ca^{2+}]_i$  following secretagogue stimulation of the  $\beta$ -cells (Pralong et al., 1990; Gilon et al., 1993; Jones and Persaud, 1998; Easom, 1999; Lang, 1999), it is reasonable to believe that the levels of CaBP<sub>28k</sub> in the pancreatic islet  $\beta$ -cells could be contributing to the control of glucose-stimulated insulin secretion from the  $\beta$ -cells. Future studies will be needed to look at the expression of VDR and calbindin-D<sub>9k</sub> in the pancreatic islets as well as other organs, e.g. kidneys, of CaBP<sub>28k</sub>-KO as compared to WT mice. Furthermore, cDNA microarray analysis will be needed to compare the gene expression profile of the CaBP<sub>28k</sub>-KO mouse pancreas to that of the WT mouse pancreas.

(3) The third study characterized the effects of CaBP<sub>28k</sub>-AS-ON transfection on the genomic and nongenomic profiles of a cultured pancreatic  $\beta$ -cell line (RIN1046-38 cells). Western blots demonstrated complete inhibition of CaBP<sub>28k</sub> expression using 200 nM PS-AS-ON as well as using 20 nM of the more potent PY-AS-ON transfections. cDNA microarray analysis was done on the 200 nM PS-AS-ON- as compared to control 200 nM PS-NS-ON-transfected RIN1046-38 cells. We found up-regulation of both vitamin D receptor (VDR) and calbindin-D<sub>9k</sub> mRNAs in 200 nM PS-AS-ON-transfected RIN1046-38 cells. Western blotting showed overexpression of VDR and expression of calbindin-D<sub>9k</sub> in both 200 nM PS-AS-ON and 20 nM PY-AS-ON-transfected RIN1046-38 cells. It is noteworthy that this study presents for the first time that cultured  $\beta$ -cells express calbindin-

D<sub>9k</sub> in response to complete inhibition of CaBP<sub>28k</sub>. ELISA detected much greater increases in the insulin secretory responsiveness to 0.5 mM carbachol alone or in combination with 17 mM glucose in the 200 nM PS-AS-ON- compared to 200 nM PS-NS-ON-transfected RIN cells. Future studies should determine, using quantitative measurements, the levels of VDR and calbindin-D<sub>9k</sub> proteins in both AS-ON- and NS-ON-transfected RIN1046-38 cells. This will be required to quantify the influence of ON transfection on the expression of these factors. It is well known that one molecule of calbindin-D<sub>9k</sub> binds 2 molecules of Ca<sup>2+</sup>, while one molecule of CaBP<sub>28k</sub> is known to bind 4 molecules of Ca<sup>2+</sup>. The proposed experiments would help to verify whether calbindin-D<sub>9k</sub> is synthesized as a complete or partial compensation for the variations in CaBP<sub>28k</sub> levels in transfected RIN1046-38 cells. Moreover, measurements of [Ca<sup>2+</sup>]<sub>i</sub> will help in characterizing the possible role of Ca<sup>2+</sup> as a second messenger affecting the genetic profile of ON-transfected RIN cells. The ideas presented here support previous work, which suggested that several factors including the VDR and Ca<sup>2+</sup> levels are involved in the control of the expression of calbindin-D<sub>9k</sub> (Walters et al., 1999).

*In toto*, these studies suggest that CaBP<sub>28k</sub> plays an essential role in the β-cells such that the cells synthesize an alternative calcium-binding protein, calbindin-D<sub>9k</sub>, when the expression of CaBP<sub>28k</sub> is abolished. This induction of calbindin-D<sub>9k</sub> may be mediated by VDR overexpression. Additional studies are required to help in understanding the possible interactions and roles of calbindin-

D<sub>9k</sub>, [Ca<sup>2+</sup>]<sub>i</sub>, and VDR in the AS-ON-transfected β-cells, and to examine the mechanisms leading to the expression of calbindin-D<sub>9k</sub> and overexpression of VDR in the CaBP<sub>28k</sub>-ablated RIN1046-38 cells. Exploring the molecular basis of these changes in the CaBP<sub>28k</sub>-ablated cells may clarify several *a priori* assumptions on the role of CaBP<sub>28k</sub> in cultured β-cells which could foster the development of a new working model for studying β-cell functions.

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