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Molecular Mechanisms Regulating Exocytosis

– Studies of Insulin Secretion and Neurotransmitter Release

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To my Family

ABSTRACT

In order to achieve normal physiology the secretion of hormones and neurotransmitters needs to be firmly controlled. The basic molecular machinery mediating regulated exocytosis of hormones and neurotransmitters is in principal revealed, but knowledge about how the individual components are spatially and temporarily coordinated is limited. This thesis was primarily aimed to clarify molecular mechanisms that modulate insulin secretion, with the emphasis on protein phosphorylation, but it also aimed to compare the regulation of related secretory machineries responsible for secretory granule and synaptic vesicle exocytosis.

Cyclin-dependent kinase 5 (Cdk5) associated with either p35 or p39 forms a multifunctional kinase that is primarily expressed in neurons. Cdk5 was identified in pancreatic β-cells where it functions as a positive regulator of insulin secretion. Although β-cells expressed both of the identified Cdk5 regulatory subunits, only Cdk5, activated by p39, enhanced insulin secretion. Co-expression of Cdk5, p35 or p39 with munc18-1, mutated in potential phosphorylation sites, revealed that Cdk5/p39 activity facilitated secretion by phosphorylating the syntaxin 1–interacting protein munc18-1.

The role of Cdk5 activity in spontaneous neurotransmitter release and in functional synapse formation was examined using the neuroblastoma/glioma cell line NG108-15, which when co-cultured with myotubes form cholinergic synapses. NG108-15 cells endogenously expressed Cdk5 and both of its activators. In contrast to the β-cell, which predominantly expressed the p39 activator, the NG108-15 cell mainly expressed the p35 protein. NG108-15 cells overexpressing a dominant negative mutant of Cdk5 showed a reduced mEPP frequency and had less ability to form functional synaptic-like structures with muscle cells as compared to non-transfected cells. Overexpression of either Cdk5/p35 or Cdk5/p39 enhanced both the mEPP frequency and functional synapse formation to a similar extent, indicating that Cdk5 activity facilitated spontaneous neurotransmitter release as well as functional synapse formation in NG108-15 cells.

Protein phosphatase 1 (PP1) is regarded as an important regulator of insulin exocytosis, but regulation of its activity in β-cells is unknown. RT-PCR, Western blotting and immunohistochemistry revealed expression of the endogenous PP1 inhibitors DARPP-32 and inhibitor-1 in β-cells, suggesting a potential role for DARPP-32 and inhibitor-1 in the regulation of PP1 activity in signal transduction and insulin exocytosis.

Keywords: Cdk5, cell line, DARPP-32, dephosphorylation, exocytosis, inhibitor-1, insulin, islet, kinase, munc18, myotube, p35, p39, phosphatase, phosphorylation, PP1, presynaptic mechanisms, secretion, subcellular localization.

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LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to by their Roman numbers in the text:

I. **Lilja L**, Yang SN, Webb DL, Juntti-Berggren L, Berggren PO, Bark C (2001) Cyclin-dependent Kinase 5 Promotes Insulin Exocytosis. *J. Biol. Chem.* 276: 34199-34205.

II. **Lilja L**, Johansson JU, Gromada J, Mandic SA, Fried G, Berggren PO, Bark C (2004) Cyclin-dependent Kinase 5 Associated with p39 Promotes Munc18-1 Phosphorylation and Ca^{2+}-dependent Exocytosis. *J. Biol. Chem.* 279:29534-29541.

III. Johansson JU, **Lilja L**, Chen XL, Higashida H, Meister B, Noda M, Zhong ZG, Yokoyama S, Berggren PO, Bark C (2005) Cyclin-dependent Kinase 5 Activators p35 and p39 Facilitate Formation of Functional Synapses. *Submitted Manuscript*.

IV. **Lilja L**, Meister B, Berggren PO, Bark C (2005) DARPP-32 and Inhibitor-1 are Expressed in Pancreatic β-cells. *Submitted Manuscript*.

Related publications and manuscripts:

Andersson J, Fried G, **Lilja L**, Meister B, Bark C (2000) Differential Sorting of SNAP-25a and SNAP-25b Proteins in Neuroblastoma Cells. *Eur. J. Cell Biol.* 79:781-789.

Zhang W, **Lilja L**, Bark C, Berggren PO, Meister B (2004) Mint1, a Munc-18-interacting Protein, is Expressed in Insulin-secreting β-cells. *Biochem. Biophys. Res. Commun.* 320:717-721.

Zhang W, **Lilja L**, Mandic SA, Smidt K, Gromada J, Takai Y, Bark C, Berggren PO, Meister B (2005) Tomosyn is Expressed in β-cells and Negatively Regulates Insulin Exocytosis. *Submitted Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
$[Ca^{2+}]_i$	Cytoplasmic free calcium concentration
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	Adenosine 3',5'-cyclic monophosphate
CAPS	Ca^{2+}-dependent activator protein for secretion
Cdk	Cyclin-dependent kinase
CK	Casein kinase
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kD
dBcAMP	Dibutyryladenosine cyclic monophosphate
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dn	Dominant negative
ECL	Enhanced chemiluminescence
GDP	Guanosine diphosphate
GLUT	Glucose transporter
GTP	Guanosine triphosphate
hGH	Human growth hormone
KRBH	Krebs-Ringer bicarbonate HEPES buffer
LDCV	Large dense core vesicle
MAPK	Mitogen-activated protein kinase
mEPP	Miniature endplate potential
mRNA	Messenger ribonucleic acid
Munc18	Mammalian homologue of *C. elegans unc-18*
NMJ	Neuromuscular junction
PA	Phosphatic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PP	Protein phosphatase
PTP	Protein tyrosine phosphatase
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RP	Reserve pool
RRP	Readily releasable pool
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SG	Secretory granule
SLMV	Synaptic-like microvesicles
SNAP-25	Synaptosomal-associated protein of 25 kD

SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SRP	Slowly releasable pool
SV	Synaptic vesicle
VAMP	Vesicle-associated membrane protein
VDCC	Voltage-dependent calcium channel
wt	Wild-type

INTRODUCTION

Background

Fusion of intracellular vesicles with the plasma membrane, a process referred to as exocytosis, is essential for numerous cellular events and forms the basis for intercellular communication in multicellular organisms. In all eukaryotic cells the constitutive secretory pathway continuously secretes molecules to the extracellular matrix and supplies the plasma membrane with newly synthesized lipids and proteins. In specialized secretory cells, such as neurons and pancreatic β-cells there exists an additional secretory pathway that is subjected to short-term regulation in order to allow release of neurotransmitters and hormones only in response to a physiological signal (Alberts et al., 2002). Although differently controlled, regulated exocytosis of synaptic vesicles and constitutive secretion in yeast are mediated by similar proteins, suggesting that vesicular fusion is performed and regulated by an evolutionary conserved core secretory machinery. Related protein machineries also function in all intracellular fusion events within the cell (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994).

In order to achieve normal physiology the secretion of hormones and neurotransmitters needs to be tightly controlled. Minor impairments in gene expression, protein localization and/or phosphorylation status of the proteins regulating exocytosis might alter cell-to-cell communication and result in a wide range of metabolic and neuropsychiatric diseases, such as type 2 diabetes mellitus and schizophrenia (Mirnics et al., 2000; Nagamatsu et al., 1999; Zhang et al., 2002). Clarification of cellular mechanisms that improve our understanding of the molecular interactions that regulate hormone secretion and neurotransmitter release might reveal therapeutically interesting targets for development of new drugs, with the possibility to adjust secretory deficiencies.

Regulated exocytosis

Neurotransmitter release at neuronal synapses and insulin secretion from pancreatic β-cells exemplifies two major types of regulated secretion that are distinguished by the morphological appearance of secretory vesicles, the release kinetics and the mode of vesicle biogenesis. Secretion of insulin occurs from secretory granules (SGs), which have an average diameter of about 350 nM (Olofsson et al., 2002). Regulated exocytosis of insulin is usually initiated on a millisecond time scale, but if exposed to elevated levels of glucose, these cells can continuously secrete insulin until the blood glucose level is normalized (Proks and Ashcroft, 1995; Smith et al., 1999; Ämmälä et al., 1993). SG formation that relies on *de novo* synthesis of substances to be secreted, is generated by budding of vesicles from the trans-Golgi network (Molinete et al., 2000; Tooze et al., 2001). The other type of regulated secretion is synaptic vesicle (SV) exocytosis that occurs at neuronal synapses. In general, SVs are <50 nm in diameter (Jahn and Südhof, 1994) and are generated by recycling between the plasma membrane and early endosomes (Kelly, 1993). Fusion of SVs in nerve terminals generates a very fast (within microseconds) but short-lived signal across the synapse (Bruns and Jahn, 1995; Sabatini and Regehr, 1996).

Compared to SG exocytosis neurotransmitter release is triggered by relatively high Ca^{2+} concentrations (Barg, 2003; Morgan and Burgoyne, 1997).

Although pancreatic β-cells and neurons primarily secrete substances by SGs and SVs, respectively, both cell types contain two types of secretory vesicles. Pancreatic β-cells secrete the neurotransmitter gamma–aminobutyric acid (GABA) stored in vesicles resembling SVs, and hence they are called synaptic-like microvesicles (SLMV) (Reetz et al., 1991; Thomas-Reetz et al., 1993). In neuroendocrine cells, the small class of secretory vesicles is also referred to as SLMV (De Camilli and Jahn, 1990). Neurons release neuropeptides and catecholamines, which are stored in large dense core vesicles (LDCVs) (Kandel et al., 2000).

Despite several differences, secretion of hormones and neurotransmitters proceeds by the same series of events (Fig. 1). First, vesicles are recruited to the plasma membrane, where the vesicle membrane and the plasma membrane initially come in physical contact in a process referred to as docking or tethering. The docked vesicles then undergo a series of ATP- and Ca^{2+}-dependent maturation steps, called priming, to gain competence for Ca^{2+}-triggered fusion. Finally, the vesicle membrane merges with the plasma membrane and subsequently the vesicle content is released to the extracellular space (Burgoyne and Morgan, 2003; Gerber and Südhof, 2002; Li and Chin, 2003; Südhof, 2004; Söllner, 2003).

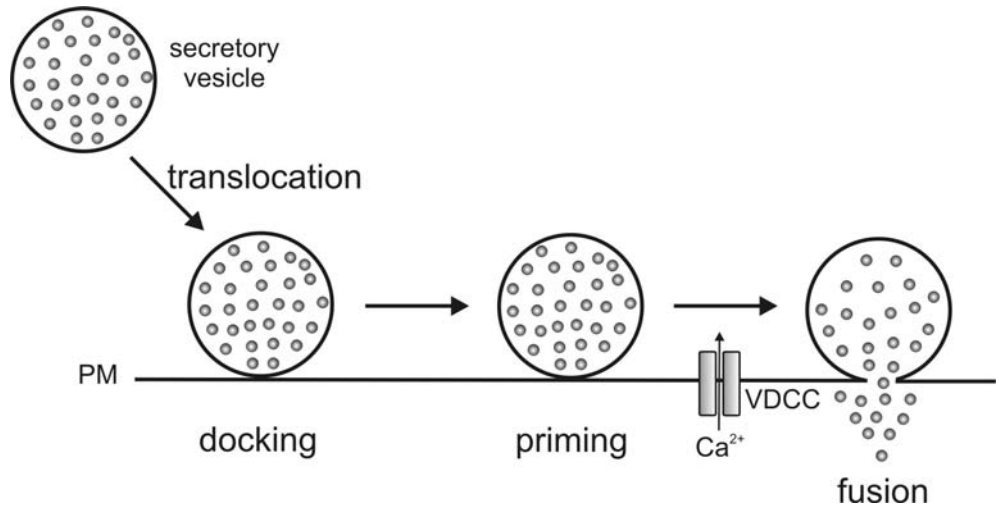

Figure 1. Exocytosis of secretory vesicles. Vesicles are transported to sites of secretion in the plasma membrane where they are docked and primed to achieve release competence. As a response to a physiological signal, the Ca^{2+} channels open. Hence vesicles fuse with the plasma membrane and release their cargo. Although not indicated in the figure, the transitions between the individual steps in the exocytotic pathway are believed to be reversible (Martin, 2003). PM, plasma membrane; VDCC, voltage-dependent Ca^{2+} channel.

Docking of vesicles

Vesicle docking can be defined in two ways. Morphological docking refers to when vesicles visualized by electron microscopy appear to interact with the plasma membrane (Gray, 1959; Plattner et al., 1997; Steyer et al., 1997). Alternatively, a

vesicle is defined as biochemically docked when a protein connection is established between the two merging membranes (Martin and Kowalchyk, 1997). The initial contact between the vesicle and the target membrane is mediated via an evolutionary conserved large multiprotein complex called the exocyst. The exocyst, which originally was identified in yeast comprises eight proteins; Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, Sec15p, Exo70p and Exo84p (Finger et al., 1998; Finger and Novick, 1998; Guo et al., 1999; TerBush et al., 1996). Mutations in any of the exocyst genes results in an accumulation of undocked secretory vesicles and defect exocytosis (EauClaire and Guo, 2003). The mammalian exocyst is ubiquitously expressed and regulates vesicle targeting and docking of vesicles to specialized sites of exocytosis in the plasma membrane (Grindstaff et al., 1998; Hsu et al., 1999; Hsu et al., 1996). The recruitment of vesicles to the target membrane is mediated by interactions between the exocyst proteins and vesicle-associated small GTPases. The small GTPases cycle between an inactive GDP-bound form and an active GTP-bound form (Takai et al., 2001). In different systems, separate families of small GTPases interact with different exocyst proteins (Lipschutz and Mostov, 2002; Novick and Guo, 2002). In yeast, Sec15p binds the GTP-bound form of the Rab GTPase Sec4p (Guo et al., 1999), whereas the homologous function in mammalian systems is mediated through an interaction between Sec5p and the GTP-bound form of Ral, a protein that is absent in yeast (Brymora et al., 2001; Moskalenko et al., 2002; Sugihara et al., 2002).

Besides from the exocyst components other proteins have been suggested to regulate targeting and tethering of vesicles to active exocytotic sites. For example, both Rab3, a small GTPase that is specifically expressed in secretory cells, including pancreatic β-cells and neurons (Geppert et al., 1994; Iezzi et al., 1999; Leenders et al., 2001; Nonet et al., 1997; Regazzi et al., 1996; Zerial and McBride, 2001), and the syntaxin 1-interacting protein munc18-1 are implicated in vesicle docking as well as other stages in the exocytotic process (Weimer et al., 2003; Voets et al., 2001). Munc18-1 belongs to the Sec1/munc18 (SM) protein family that were first discovered during genetic screens for membrane-trafficking mutants in *Caenorhabditis elegans* (*unc18*) and yeast (sec1) (Brenner, 1974; Novick et al., 1980). Different SM proteins specifically participate in all intracellular vesicle trafficking events (Jahn and Südhof, 1999; Toonen and Verhage, 2003). There are three mammalian isoforms of *unc18* (munc18-1, munc18-2 and munc18-3) (Garcia et al., 1994; Hata et al., 1993; Hata and Südhof, 1995; Pevsner et al., 1994; Tellam et al., 1995). The different munc18 isoforms interacts with specific isoforms of syntaxin and may thus confer additional specificity of vesicle targeting (Katagiri et al., 1995; Pevsner et al., 1994; Riento et al., 1998; Tamori et al., 1998; Tellam et al., 1997)

Priming of vesicles

The secretory vesicles exist in several distinct pools. Typically, only a few percentages of the vesicles are release-competent and can undergo exocytosis without further modification. These vesicles belong to the ready releasable pool (RRP). Thus, the majority of secretory vesicles belongs to the slowly releasable pool (SRP) or the reserve pool (RP) that need to undergo a series of ATP- and Ca^{2+}-dependent maturation steps to gain release competence (Barg et al., 2002; Rettig and Neher, 2002; Rorsman and Renström, 2003; Südhof, 2000; Südhof, 2004). This process,

referred to as priming, may involve changes in the lipid composition as well as protein rearrangements (Klenchin and Martin, 2000).

Priming of SVs and LDCVs appear to differ. SV priming is more efficient both when it comes to the number of primed vesicles out of the total number of docked vesicles and the rate of RRP replenishment (Martin, 2003 and references therein). In addition, as depletion of intracellular Mg-ATP, a component required for priming, results in a rapid loss of the RRP for LDCV in chromaffin cells but not for SVs in neurons, the RRP of SVs is regarded as more stable (Heidelberger et al., 2002; Xu et al., 1998).

It is generally believed that the priming reaction involves protein rearrangements resulting in assembly of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex (Fig. 2; the SNARE complex is further discussed below). Initially, syntaxin 1 is hold in a closed conformation in a complex with munc18-1. Munc13, an evolutionary conserved syntaxin 1-binding protein that is essential for SV priming (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999; Varoqueaux et al., 2002) and affects the size of the RRP in chromaffin and insulinoma cells (Ashery et al., 2000; Sheu et al., 2003), is believed to prime vesicles by promoting a conformational change of syntaxin 1 from the closed to the open state, thereby enable SNARE complex formation necessary for fusion. The mechanism of action is thought to be via its interaction with the Rab3 effector RIM1 (Koushika et al., 2001; Li and Chin, 2003; Wang et al., 1997). In addition to munc13/RIM1-mediated disassembly of the dimeric complex, protein phosphorylations of both syntaxin 1 and munc18-1 have been shown to modulate the affinity of this interaction (Fletcher et al., 1999; Fujita et al., 1996; Liu et al., 2004; Tian et al., 2003).

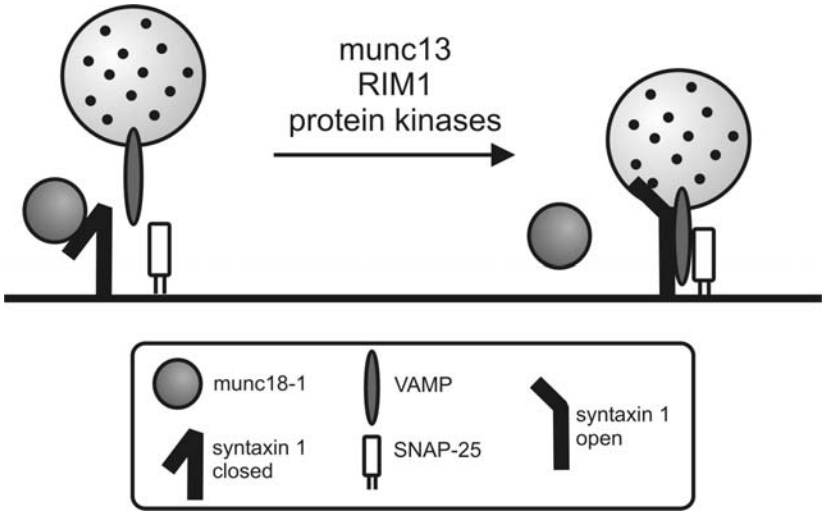

Figure 2. Activation of the t-SNARE syntaxin 1. Initially, munc18-1 is associated with the closed conformation of syntaxin 1. Actions of munc13, RIM1 and protein phosphorylation events have been proposed to displace munc18-1 from syntaxin, and thereby facilitate a conformational change of syntaxin 1 into the open state, which allows SNARE complex assembly.

Priming of LDCVs in chromaffin cells has been suggested to occur via a two-step priming reaction (Grishanin et al., 2004; Martin, 2003). The first step is Mg-ATP-dependent and involves synthesis of phosphatidylinositol 4, 5-bisphosphate (PIP_2), which is required for Ca^{2+}-triggered exocytosis LDCV (Hay et al., 1995; Holz and Axelrod, 2002). The precise function for PIP_2 in exocytosis has not been defined, but it has been proposed to guide PIP_2-binding proteins to specific locations in the plasma membrane. Several PIP_2-binding proteins implicated in the regulation of exocytosis have been identified, including Ca^{2+}-dependent activator protein for secretion (CAPS) (Grishanin et al., 2002; Grishanin et al., 2004; Loyet et al., 1998), synaptotagmin (Bai et al., 2004a; Schiavo et al., 1995; Tucker et al., 2003), rabphilin (Chung et al., 1998) and mints (Okamoto and Südhof, 1997). The second priming step of LDCVs is relatively slow and Ca^{2+}-dependent, and involves recruitment of CAPS to specific sites in the plasma membrane (Loyet et al., 1998; Martin, 2001). CAPS-1, which is essential for LDCV/SG exocytosis but not for SV exocytosis (Berwin et al., 1998), binds to PIP_2 in the plasma membrane via its pleckstrin homology (PH) domain and to the vesicle membrane via its C-terminal region, suggesting that CAPS bridges the merging membranes during LDCV fusion (Grishanin et al., 2002).

In β-cells ATP-dependent priming is coupled to glucose metabolism. Processing of proinsulin depends on intragranular acidification (Hutton, 1989), a reaction driven by a simultaneous action of the V-type H^+-ATPase and the ClC-3 Cl^- channel localized in the granular membrane (Barg et al., 2001). A few years ago, Renström and co-workers showed that blockade of the ClC3-channel by pharmacological agents or by antibodies inhibits priming whereas an increase in the ATP/ADP ratio generated by glucose metabolism enhances the activity of the Cl^- channel and facilitate acidification and priming of insulin granules (Barg et al., 2001).

Triggering of exocytosis

Unlike constitutive exocytosis where vesicle fusion occurs without an external stimulus, neurotransmitter- and hormone-filled vesicles undergo fusion as a response to a local rise in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Both in neurons and in pancreatic β-cells, generation of an action potential promotes opening of voltage-dependent calcium channels (VDCCs) (Ashcroft and Rorsman, 1989; Dean and Matthews, 1968; Katz, 1969; Yang and Berggren, 2005). In most synapses Ca^{2+} enters the cell through the P/Q- or N-type VDCCs (Iwasaki and Takahashi, 1998; Qian and Noebels, 2001; Regehr and Mintz, 1994; Takahashi and Momiyama, 1993; Wheeler et al., 1996), whereas in β-cells the L-type VDCC is the predominant Ca^{2+} channel (Mears, 2004; Satin, 2000; Yang and Berggren, 2005). The subsequent elevation in $[Ca^{2+}]_i$ triggers exocytosis of fusion competent vesicles and release of vesicle content (Wollheim and Sharp, 1981; Zucker, 1993; Ämmälä et al., 1993).

In pancreatic β-cells, the action potential is generated as a consequence of increased concentrations of circulating blood glucose (Fig. 3). Glucose enters the cell via glucose transporters (GLUTs) and is metabolized to generate ATP. In rodents glucose is mainly transported by GLUT2, but in human β-cells this function is carried out by GLUT1 (De Vos et al., 1995; Schuit, 1997). The resulting increase in ATP/ADP ratio induces the closure of ATP-dependent K^+ channels (K_{ATP}). The plasma membrane depolarizes, which promotes opening of L-type VDCCs. Ca^{2+}

entry generates a rapid and sustained rise in $[Ca^{2+}]_i$ that triggers biphasic insulin release (Barg, 2003; Lang, 1999; Mears, 2004; Rorsman and Renström, 2003). The first phase requires a fast and marked elevation of $[Ca^{2+}]_i$ and corresponds to exocytosis of the RRP of insulin-containing secretory granules. The sustained second phase of secretion requires signals additional to the $[Ca^{2+}]_i$ rise, and corresponds to the recruitment/maturation of secretory granules from the SRP or the RP (Henquin, 2000; Rorsman and Renström, 2003; Straub and Sharp, 2002).

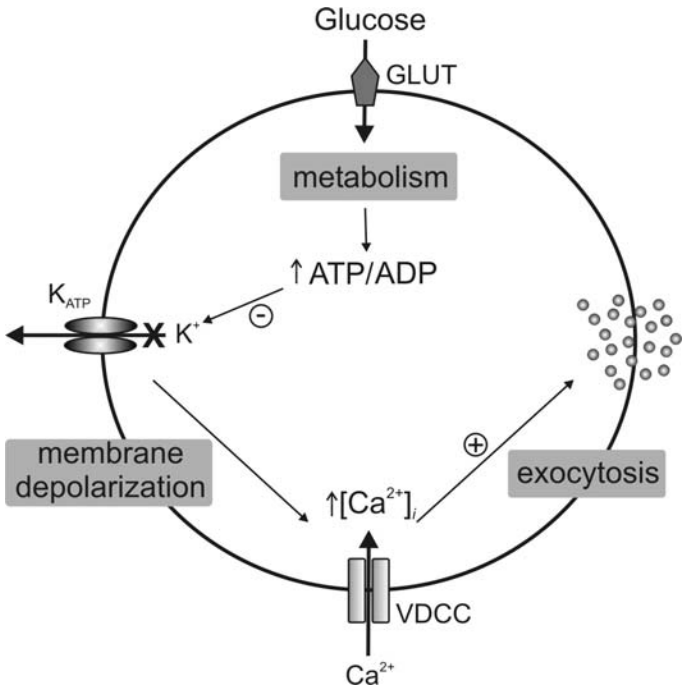

Figure 3. Stimulus-secretion coupling in pancreatic β-cells. Glucose is actively transported into the β-cell via glucose transporters, where it is metabolized to generate ATP. The ATP/ADP ratio increases, which promotes closure of K_{ATP} channels and subsequent depolarization of the plasma membrane. This leads to opening of VDCCs and an increase in $[Ca^{2+}]_i$ that triggers insulin release.

Synaptotagmins represent the most probable candidates to mediate Ca^{2+}-triggered exocytosis. Thirteen different synaptotagmin isoforms have been characterized in human (Südhof, 2002), and database searches have identified six additional potential family members (Craxton, 2001). Typically, synaptotagmins contain an N-terminal transmembrane domain, a variable linker region and two cytoplasmic Ca^{2+}-binding domains; called C2A and C2B (Perin et al., 1990; Südhof, 2002). The synaptotagmins are either localized to the secretory vesicle membrane or to the plasma membrane and they exhibit distinct Ca^{2+} affinities (Fukuda et al., 2004; Sugita et al., 2002; Südhof, 2002). Synaptotagmin I and II are integral membrane proteins of SVs that interact directly with the t-SNAREs, syntaxin 1 and SNAP-25 at all stages during SNARE complex assembly (discussed below) (Bai and Chapman, 2004). Synaptotagmin I and II are believed to be the major Ca^{2+} sensors responsible for SV exocytosis (Augustine, 2001; Koh and Bellen, 2003). When binding Ca^{2+}, the

C2A and C2B domains of synaptotagmin I insert into the plasma membrane (Bai et al., 2002), with the guidance of PIP_2 localized on the inner leaflet of the target membrane (Bai et al., 2004a). These interactions bring the opposing lipid bilayers into close proximity. Interactions with the SNAREs directly target the Ca^{2+} sensor to sites of membrane fusion. In addition to playing a key role in Ca^{2+}-sensing, synaptotagmins might influence assembly of trans-SNARE complexes (Littleton et al., 2001; Mahal et al., 2002) as well as modulating fusion pore dynamics (Bai et al., 2004b; Wang et al., 2001; Wang et al., 2003).

Regulated exocytosis of insulin granules does not depend on synaptotagmin I or II. Initially synaptotagmin III was suggested to be the Ca^{2+} sensor in β-cells (Brown et al., 2000; Gao et al., 2000; Mizuta et al., 1997), but recent studies suggest that synaptotagmin V,VII or IX might also act as Ca^{2+} sensors (Gao et al., 2000; Iezzi et al., 2004). As β-cells contain two populations of SGs with distinct Ca^{2+} sensitivities (Wan et al., 2004; Yang and Gillis, 2004), several Ca^{2+} sensors might operate to generate a gradual stimulation of exocytosis over a wide range of $[Ca^{2+}]_i$ (Barg and Rorsman, 2004). A similar heterogeneity in Ca^{2+} sensitivity of exocytosis is also observed in adrenal chromaffin cells (Yang et al., 2002).

Membrane fusion

The central players in all fusion events are the SNARE proteins, a protein family in which all members contain at least one homologous approximately 60 amino acid α-helical coiled-coil domain, called the SNARE motif (Jahn and Südhof, 1999; Rothman, 1994; Weimbs et al., 1997). Based on their localization on vesicle or target membranes the SNARE proteins were initially divided into v-SNAREs and t-SNAREs (Söllner et al., 1993b), but later on they were reclassified as R-SNAREs and Q-SNAREs according to the conserved arginine or glutamine residue in the center of their SNARE motifs (Fasshauer et al., 1998).

SV and SG exocytosis are both mediated by a core complex comprised of three SNARE proteins: vesicle-associated membrane protein (VAMP, also called synaptobrevin) localized to the secretory vesicle, and syntaxin 1 and synaptosomal-associated protein of 25 kD (SNAP-25) in the plasma membrane. VAMP and syntaxin 1 are transmembrane proteins that contain a single SNARE motif (Weimbs et al., 1997). SNAP-25, which contains two SNARE motifs is anchored to the plasma membrane via palmitoylation (Hess et al., 1992; Weimbs et al., 1997; Veit et al., 1996). The four SNARE motifs derived from these three proteins assembles into a parallel four-stranded helical bundle to form the SNARE complex (Sutton et al., 1998). According to the SNARE hypothesis, different members of the SNARE families are localized to distinct membrane compartments and form unique SNARE complexes that enhance the fidelity of vesicle trafficking and fusion (Rothman, 1994; Söllner et al., 1993b). A genomic screening revealed that the human genome contains 35 different SNAREs, divided into four subfamilies (Bock et al., 2001).

Although there is a general agreement that SNARE proteins are of critical importance for secretory vesicle exocytosis, their precise role in membrane fusion remains uncertain. Originally, interactions between v-SNAREs and t-SNAREs were thought to mediate vesicle docking and account for the specificity of membrane fusion (Rothman, 1994; Söllner et al., 1993a; Söllner et al., 1993b). However, in later studies where the SNARE proteins are either absent or cleaved by botulinum toxins

fusion is abolished, but vesicles are still targeted to the presynaptic membrane and dock normally at specialized release sites (Banerjee et al., 1996; Broadie et al., 1995). Analysis of structural data proposed that trans-SNARE complexes may function in a zipper-like fashion, bringing the vesicle and plasma membrane in close proximity (Hanson et al., 1997; Lin and Scheller, 1997; Sutton et al., 1998). In fact, purified membranes containing only cognate SNARE proteins undergo membrane fusion, although the rates are low (McNew et al., 1999; McNew et al., 2000; Weber et al., 1998). These data imply that the SNAREs constitute the minimal machinery necessary for membrane fusion, but other components are probably required to support secretion *in vivo*. However, VAMP-2 and SNAP-25 knock-out mice are deficient in evoked synaptic transmission but exhibit stimulus-independent neurotransmitter release, suggesting that the SNARE proteins are not necessary for SV fusion (Schoch et al., 2001; Washbourne et al., 2002).

Regulated exocytosis of neurotransmitters and hormones proceeds through formation of a fusion pore that connects the lumen of the vesicle with the extracellular space (Lindau and Alvarez de Toledo, 2003). Extensive regulation of the fusion pore opening results in at least two types of exocytosis, full and incomplete fusion (An and Zenisek, 2004). In full fusion, the fusion pore dilates and consequently the vesicle membrane becomes fully incorporated and flattened into the plasma membrane and is then retrieved through a clathrin-dependent process. Alternatively, a transient opening of the fusion pore results in incomplete fusion, often referred to as kiss-and-run exocytosis (An and Zenisek, 2004; Burgoyne and Morgan, 2003; Fesce et al., 1994). It is well established that both SVs and LDCV can be released by full fusion (Brodin et al., 2000; Cremona and De Camilli, 1997; Gundelfinger et al., 2003; Heuser, 1989; Lindau and Almers, 1995; Zenisek et al., 2002). Recently generated evidences show that kiss-and-run exocytosis of both SVs and LDCV can occur, at least under certain conditions (Aravanis et al., 2003; Gandhi and Stevens, 2003; Lindau and Alvarez de Toledo, 2003; Rutter and Tsuboi, 2004; Staal et al., 2004). However, whether kiss-and-run exocytosis occurs in β-cells is a matter of debate (Ma et al., 2004; Tsuboi and Rutter, 2003). Fusion pore dynamics are probably more important for SG than for SV exocytosis. SV are small and even a transient opening of the fusion pore will empty the SV completely, but probably not the SG. The difference in size between chemical neurotransmitters and hormones also influences the amount of signal substances released during a transient opening of the fusion pore (Burgoyne and Barclay, 2002). However, kiss-and-run exocytosis may be crucial for the fast recycling of SVs (Südhof, 2004). The composition of the fusion pore is unknown, but most likely both lipids and proteins are involved (An and Zenisek, 2004; Cho et al., 2004; Jena et al., 2003).

Protein phosphorylation regulates exocytosis

A powerful way to regulate protein function is the covalent addition of a phosphate group to amino acid side chains, typically tyrosines, threonines or serines. Because of the negative charge of a phosphate group, phosphorylation of a protein can induce major conformational changes that affect the binding of ligands and dramatically change the activity of the protein through an allosteric effect. Alternatively, addition of a phosphate group can directly generate a binding site for other proteins. Either way, phosphorylation events control activity, structure and

cellular localization of many proteins. This regulation is so extensive that more than one-third of the 10,000 or so proteins in a typical mammalian cell are estimated to be phosphorylated at any given time (Alberts et al., 2002).

There is no doubt that protein phosphorylation plays a significant role in regulated exocytosis of SVs and LDCVs/SGs. cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) enhance exocytosis in essentially all examined cell types (Burgoyne and Morgan, 2003). In addition, Ca^{2+}/calmodulin-dependent protein kinases (CaMK), mitogen-activated protein kinases (MAPK), casein kinases (CK) and protein tyrosine kinases are implicated in the regulation of secretion (Jones and Persaud, 1998; Lin and Scheller, 2000; Turner et al., 1999). Both the SNARE proteins themselves and many SNARE-regulators are phosphorylated *in vitro* by at least one protein kinase, but often these proteins are utilized as substrates for several protein kinases. However, the physiological significance of these phosphorylations is only known for a few examples (Chheda et al., 2001; Evans et al., 2001; Foletti et al., 2001; Graham and Burgoyne, 2000; Lonart and Südhof, 1998). See figure 4 for a summary of protein kinases and substrates within the exocytotic machinery.

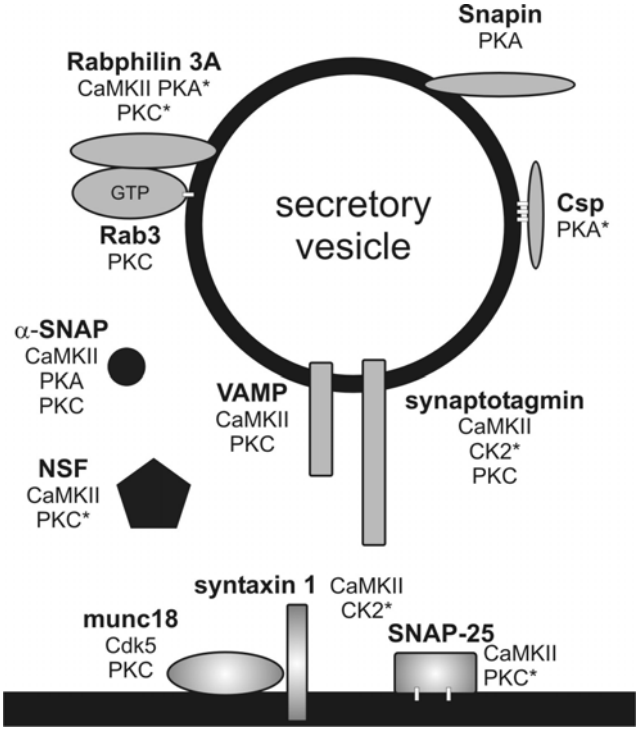

Figure 4. Key proteins within the exocytotic machinery that are phosphorylated by protein kinases. When a protein has been shown to be an *in vivo* substrate the kinase is indicated by an asterisk. Modified from Burgoyne and Morgan, 2003.

Cyclin-dependent kinase 5 (Cdk5) is a member of the large family of proline-directed serine/threonine protein kinases that recently has started to emerge as an important regulator of secretion (Chergui et al., 2004; Fletcher et al., 1999; Rosales et al., 2004; Tomizawa et al., 2002; Xin et al., 2004; Yan et al., 2002). Although identified as a sequence homologue to the conventional Cdks (Lew et al., 1992; Meyerson et al., 1992; Xiong et al., 1992), the key regulators of eukaryotic cell cycle

progression, Cdk5 is involved in mechanisms unrelated to cell division. Whereas the activity of conventional Cdks depends on association with a cyclin protein (Hunter and Pines, 1994; Lees, 1995; Morgan and Burgoyne, 1997; Nigg, 2001), Cdk5 is activated by either of its non-cyclin regulatory subunits, called p35 and p39 (Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994). Cdk5 is a ubiquitously expressed protein, but its kinase activity is mainly associated with post-mitotic neurons where p35 and p39 are expressed (Hellmich et al., 1992; Ino et al., 1994; Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994; Tsai et al., 1993). Until now, more than 30 different proteins with diverse functions have been identified as substrates for Cdk5, and novel Cdk5 substrates are continuously added to this list (Fu et al., 2004; Honma et al., 2003; Kansy et al., 2004; Li et al., 2004; Lim et al., 2003; Morabito et al., 2004; Moy and Tsai, 2004). It appears that Cdk5 functions in many essential neuronal processes, including neuronal migration, axon guidance, cytoskeletal dynamics, cell adhesion, dopamine signaling, synapse formation at the neuromuscular junction, apoptosis, neurosecretion and endocytosis (Cheng and Ip, 2003; Cheung and Ip, 2004; Cruz and Tsai, 2004; Dhavan and Tsai, 2001; Nguyen and Bibb, 2003; Smith and Tsai, 2002). In addition, Cdk5 activity has also been demonstrated in several non-neuronal tissues, including differentiating muscle cells, lens epithelial and fiber cells and in the adult prostate (Fu et al., 2001; Gao et al., 1997; Lazaro et al., 1997; Musa et al., 2000; Musa et al., 1998; Philpott et al., 1997; Session et al., 2001).

Protein dephosphorylation regulates exocytosis

In order to achieve an adequate secretion, there needs to be a balance between the rate of phosphorylation by protein kinases and the subsequent dephosphorylation by protein phosphatases (PPs) of components of the exocytotic machinery. Originally PPs where thought to be unregulated, but it is clear that enzyme activities of PPs are tightly controlled (Cohen, 2002; Oliver and Shenolikar, 1998; Sim et al., 2003; Sontag, 2001). PPs are classified according to their substrate specificity into serine/threonine PPs and protein-tyrosine phosphatases (PTPs). Based on the substrates, inhibitors of the enzymes, and their requirement for different divalent cations serine/threonine PPs are classified into four major groups, called PP1, PP2A, PP2B and PP2C (reviewed in Cohen, 1989). Although there is growing evidence for a role of PTPs in regulated secretion (Gogg et al., 2001; Hermel et al., 1999; Kapp et al., 2003; Roberts et al., 2001; Wimmer et al., 2004; Östenson et al., 2002), the importance of serine/threonine PPs as regulators of secretion is more established.

PP1 regulates numerous cellular functions, including membrane fusion (Peters et al., 1999). The catalytic subunit (PP1c) interacts with more than 50 different regulatory subunits. Most of these subunits direct the PP1c to specific subcellular compartments, but some subunits function as modulators of PP1 activity (Cohen, 2002). The ubiquitously expressed inhibitor-1 and its neuronal homologue dopamine- and cAMP-regulated phosphoprotein of 32 kD (DARPP-32) are the best-characterized endogenous inhibitors of PP1. PKA phosphorylation of inhibitor-1 and DARPP-32 on Thr^{35} and Thr^{34}, respectively, are critical for their inhibitory function. Once phosphorylated they inhibit PP1 activity, thus enhancing protein phosphorylation events catalyzed by PKA as well as other kinases (Hemmings et al., 1984; Huang and Glinsmann, 1976). DARPP-32 activity is regulated by phosphorylation by other kinases than PKA (Fig. 5). Phosphorylations by CK1 and

CK2 strengthen the inhibitory effect on PP1 by increasing the state of phosphorylation on Thr^{34} (Desdouits et al., 1995; Girault et al., 1989; Hemmings et al., 1990). Phosphorylation of Thr^{75} by Cdk5 turns the protein into a potent inhibitor of PKA (Bibb et al., 1999). Like DARPP-32, inhibitor-1 is also a substrate for Cdk5, but there are conflicting reports regarding the consequence of this modification. Cdk5-phosphorylated inhibitor-1 was initially claimed to function as a potent inhibitor of PP1 activity, but data generated by Greengard and co-workers suggest that this modification may convert the protein into a less efficient substrate for PKA (Bibb et al., 2001; Huang and Paudel, 2000).

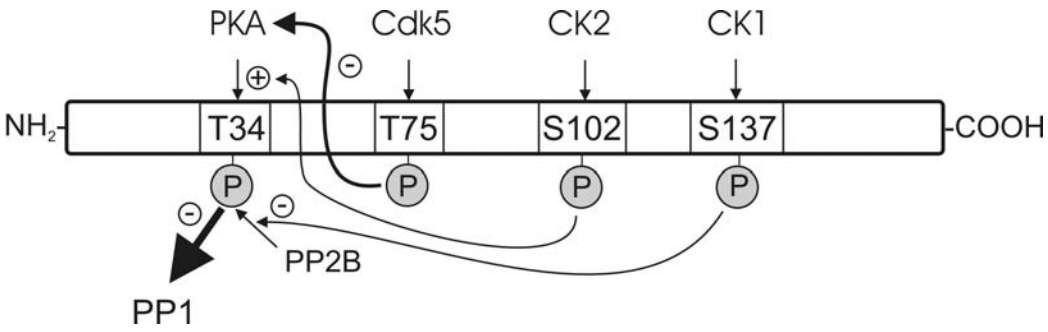

Figure 5. DARPP-32 is phosphorylated at multiple phosphorylation sites. Phosphorylation at Thr^{34} by PKA converts DARPP-32 into a potent inhibitor of PP1. Phosphorylation at Ser^{137} by CK1 turns DARPP-32 into a poorer substrate for PP2B-catalyzed dephosphorylation of Thr^{34}, whereas phosphorylation at Ser^{102} by CK2 converts DARPP-32 into a better substrate for PKA. The effect by CK1 and CK2 is substrate-specific. Cdk5 phosphorylation of DARPP-32 at Thr^{75} converts DARPP-32 into an inhibitor of PKA, reducing its ability to phosphorylate any substrate. Modified from Greengard, 2001 and Greengard et al., 1999.

Islets and β-cell lines have been reported to express PP1, PP2A and PP2B, but so far PP2C has not been detected (Jones and Persaud, 1998; Sim et al., 2003). Most evidences for a role of PPs in the regulation of insulin secretion have been obtained using PP inhibitors and depending on the cell type and the mode of stimulation different studies has generated contradictory results. Treatment of β-cells with okadaic acid, an inhibitor of PP1 and/or PP2A has been shown to both enhance and suppress insulin secretion. In most studies where glucose was used to stimulate insulin release okadaic acid inhibits secretion, but if exocytosis was induced by other secretagogues the secretory response is enhanced (Sim et al., 2003). Likewise, both inhibitory and stimulatory effects on insulin exocytosis have been observed in response to PP2B inhibition (Sim et al., 2003).

AIMS

The main goal of this study was to identify molecular mechanisms that modulate regulated exocytosis of secretory granules from pancreatic β-cells by combining cell and molecular biology techniques with electrophysiology. The study also aimed to compare the regulatory mechanisms operating in exocytosis of secretory granules and synaptic vesicles.

The specific aims of the study were to:

1. Examine the expression, subcellular distribution of Cdk5 and its neuronal activators p35 and p39 in pancreatic β-cells.
2. Investigate a potential role of Cdk5 in insulin secretion.
3. Determine if p35 and p39 stimulates insulin secretion to a similar extent.
4. Identify substrates or mechanisms by which Cdk5 promotes insulin exocytosis.
5. Study the expression, subcellular distribution of Cdk5, p35 and p39 in the neuroblastoma/glioma cell line NG108-15 and investigate their role in synaptic vesicle exocytosis.
6. Characterize expression of endogenous PP1 inhibitors in pancreatic β-cells.

METHODOLOGIES

Animal models

Tissues from adult *ob/ob* mice (C57BL/6J) or lean mice were used for RT-PCR, immunocytochemistry and Western blotting experiments in paper I, II and IV as well as for intracellular calcium measurements, insulin secretion measurements and electrophysiological recordings in paper I. In paper II islet cells isolated from female NMRI mice were used for capacitance measurements. In paper III rat myotubes were isolated from Wistar rats. In paper IV immunohistochemistry was performed on pancreatic sections from male Sprague-Dawley rats. Local ethical committees have approved all studies.

Cell culture

All primary cells and cell lines used in this study were cultured at 37°C in a humidified atmosphere containing 5% CO_2 in cell culture media specified below.

Isolation and culture of primary pancreatic β-cells

Primary islets were isolated from NMRI, ob/ob or lean mice by collagenase digestion (Lacy and Kostianovsky, 1967). For immunocytochemistry, perifusion and capacitance measurements, a cell suspension was prepared essentially as previously described (Lernmark, 1974). The cells were seeded onto coverslips or into non-adherent petri dishes (perifusion experiments) and cultured for 1-4 days in RPMI 1640 culture medium containing 11 mM glucose supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

Isolation and culture of rat muscle cells

Rat hindlimb muscle cells derived from newborn Wistar rats were isolated by trypsinization and cultivated in Dulbecco's modified Eagle medium (DMEM) for 7 days to allow formation of myotubes (20-30 μm in diameter and >100 μm long) as described previously (Higashida et al., 1981; Puro and Nirenberg, 1976). Already fused and contractile muscle cells were overlayed with NG108-15 cells. The co-cultures were maintained in DMEM supplemented with 10% horse serum and 0.25 mM dibutyryladenosine cyclic monophosphate (dBcAMP, Sigma).

Culturing of cell lines

The rat β-cell line INS-1E was cultured in complete medium composed of RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 50 μm 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Mouse insulinoma MIN6 cells were cultured in DMEM (Invitrogen), containing 11 mM glucose supplemented with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 64 μM 2-mercaptoethanol.

NG108-15 cells, which is a hybrid between the mouse neuroblastoma cell line N18TG2 and the rat glioma cell line C6-BU-1 (Nelson et al., 1976), were cultured in DMEM (Invitrogen), supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine. Cellular differentiation was induced by culturing the cells in 0.25-0.5 mM dBcAMP (Sigma). NG108IIA1 cells, a clone of NG108-15 cells that stably overexpress synapsin IIa was cultured under the same condition as the original cell line.

Preparation of expression vectors

Detailed descriptions of all plasmids used in the different experiments are found in the respective methodology section of paper I-III.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from mouse brain (C57BL6 or *ob/ob*), pancreatic islets (*ob/ob*) or NG108-15 cells using the RNeasy Mini Kit (Qiagen) or GenElute™ Mammalian Total RNA kit (Sigma), according to the manufacturer's instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using the SuperScript™ RT-PCR System (Invitrogen). Detailed description of the primers and RT-PCR protocols used are described in the respective methodology section of the four papers. PCR products were visualized on ethidium bromide-stained 1.5-2% agarose gels. 1 Kb DNA ladder (Invitrogen) was used as size marker. The amplified DNA fragments were purified from the gel and sequenced by an ABI Prism 377 sequencer (Applied Biosystems) using appropriate primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Immunocytochemistry and immunohistochemistry

Male Sprague-Dawley rats (b. wt. 80-100 g; B & K Universal) were perfused via the ascending aorta with Ca^{2+}-free Tyrode's solution (37^{O}C), followed by an ice-cold mixture of formalin-picric acid (4% paraformaldehyde and 0.4% picric acid in 0.16 M phosphate buffer, pH 6.9). The pancreas was fixed in the same fixative for 90 min and rinsed for at least 24 hours in 0.1 M phosphate buffer (pH 7.4) containing 10% sucrose, 0.02% Bacitracin and 0.01% sodium azide. Sections (14 μm) were cut in a cryostat (Dittes). Isolated primary pancreatic β-cells or NG108-15 cells were fixed in 4% ice-cold paraformaldehyde, permeabilized in 0.4% saponin and blocked in 10% goat serum. The fixed cells and the pancreatic sections were stained with appropriate primary and secondary antibodies as described in the respective papers. The stainings were analyzed with a Leica laser scanning confocal microscope or Bio-Rad RadiancePlus confocal laser scanning system. The images were processed with Adobe Photoshop softwares.

Subcellular fractionation

Cell homogenization

To separate soluble and membrane fractions, islets, brain tissue or NG108-15 cells were homogenized in buffer containing (in mM): 20 HEPES, 2 EDTA, 1 $MgCl_2$ and protease inhibitors (pH 7.4). Samples were left on ice for 40 min before

ultracentrifuged at 100,000-130,000 *x g* for 40 min and the supernatant was saved as the soluble fraction. Pellets were dissolved in 1% Triton X-100 and then centrifuged at 17,000 *x g* for 10 min to remove cell debris. For whole-cell lysates NG108-15 cells were lysed on ice for 40 min in 1% Triton X-100 PBS buffer with protease inhibitor cocktail and centrifuged to remove cell debris. To analyze protein expression during differentiation, NG108-15 cells were harvested in PBS. Soluble and detergent-insoluble cytoskeletal fractions were separated as described (Walker and Menko, 1999), with modifications (Gao et al., 2001). NG108-15 cells were harvested in PBS and lysed on ice for 40 min in a 1% Triton X-100 buffer (10 mM imidazole, 100 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, 0.5 mM NaF, 0.1 μM okadaic acid and protease inhibitor cocktail, pH 7.4). To separate soluble and Triton X-100 insoluble (cytoskeletal-associated) fractions, samples were ultracentrifuged at 130,000 *x g* for 20 min. The insoluble fraction was washed with the Triton X-100 buffer and then solubilized in RIPA buffer (5 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl and protease inhibitor cocktail, pH 7.4). All protein preparation procedures were repeated at least three times. Bio-Rad protein assay (Bio-Rad) was used for all protein concentration determinations.

Sucrose density gradients

Unstimulated *ob/ob* islets, glucose-stimulated *ob/ob* islets, undifferentiated NG108-15 cells or dBcAMP-differentiated NG108-15 cells were washed and homogenized in homogenization buffer containing (in mM): 20 HEPES, 1 $MgCl_2$, 250 D-sucrose, 2 EDTA, 1 PMSF, as well as 5 μg/ml each of antipain, aprotinin, leupeptin and pepstatin, pH 7.4 or protease inhibitor cocktail as described by the manufacturer (Roche Diagnostics). The homogenates were centrifuged shortly to pellet nuclei and the resulting supernatants were loaded onto a 4.4 ml linear sucrose density gradient (prepared from 0.6 M and 2 M sucrose stock solutions). The gradient was centrifuged at 35,000 rpm for 18 hours in a Beckman L8-55 ultracentrifuge in a SW50 rotor and 15-16 fractions (300 μl each) were collected from the top of the gradient. The linearity of the gradients was examined by measuring the refractive index of each fraction. Protein estimation of homogenates was performed using a protein microassay according to the manufacturer's instructions (Bio-Rad).

Gel electrophoresis and Western blotting

Equal amounts of each protein fractions, lysates or homogenates were separated on SDS-PAGE or NU-PAGE (Invitrogen) gels. The separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in PBS containing 0.025% Tween-20 (VWR) and 3% blotto (Amersham Biosciences) or 5% milk powder for one hour and then probed with primary antibodies overnight at 4ºC as described in each paper. After washing, membranes were incubated with horseradish peroxidase-conjugated immunoglobulins for 45-90 min at room temperature. Immunoreactive bands were detected by enhanced chemiluminescence (ECL plus, Amersham Biosciences) after exposure to Hyperfilm (Amersham Biosciences) or by using a CCD camera (LAS 1000, Fuji Photo Film CO., Ltd.), which provides optimal linearity of signal intensity. When appropriate, obtained signals were quantified using the Image Gauge 3.12 software (Fuji Photo Film CO., Ltd.).

Transfections

Transfections were carried out 2-4 days before the experiment was performed or as otherwise stated, using different techniques according to the manufactures instructions. For plasmid transfections of primary β-cells, clonal β-cell lines and NG108-15 cells in mono-culture, Lipofectamine 2000 was used (Invitrogen). Co-transfections of green fluorescent protein (1 μg/ml; Clontech) and antisense or sense oligonucleotides were performed with the Oligofectamine technique (Invitrogen). NG108-15 cells that subsequently were seeded onto differentiating myotubes in order to establish a co-culture were transfected with Lipofectamine or Lipofectamine Plus (Invitrogen). The NG108IIA1 clone was generated using the calcium phosphate precipitation method (Sambrook et al., 1989).

Measurements of secretion

Perifusion of β-cell aggregates and insulin RIA

β-cells isolated from *ob/ob* mice or lean mice were preincubated for 4 hours in RPMI 1640 culture medium supplemented with 10 μM roscovitine, or with an equal volume of DMSO as control. β-cell aggregates were mixed with Bio-Gel P4 polyacrylamide beads (Bio-Rad), in a 0.5 ml column at 37°C. The cells were perifused at a rate of 0.2 ml/min with a HEPES buffer containing (in mM): 125 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.28 $CaCl_2$ and the indicated concentrations of glucose and KCl. Roscovitine and/or DMSO were included in all solutions during the experiment. Samples were collected at 2 min intervals for insulin measurements. Insulin release was assayed by solid phase radioimmunoassay (RIA), using rat insulin as a standard (Novo Nordisk). The basal level of insulin secretion was determined as the mean value from 10 data points of the first 20 min at 3 mM glucose.

Human growth hormone release assay

Transfected INS-1E cells were incubated for 2 hours in glucose-free culture medium. The cells were then washed twice and preincubated for 30 min at 37ºC in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) consisting of (in mM): 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES and 0.1% BSA (pH 7.4 using NaOH). Following preincubation, cells were washed in glucose-free KRBH and then incubated for 30 min in KRBH in the presence of 3 or 10 mM glucose. Subsequently, the supernatant was removed and centrifuged at 100 *x g* for 5 min. Human growth hormone (hGH) in the supernatant from this centrifugation is referred to as secreted hGH. The cells in the dishes were resuspended in 0.5 ml ice-cold phosphate-buffered saline containing 1 mM EDTA and transferred to the pellet remaining from the initial centrifugation. Cells were then lysed by six freeze-thaw cycles, and insoluble material was pelleted as described above. hGH in the supernatant from this centrifugation is referred to as cellular hGH that was not secreted. hGH levels in the various samples were measured using ELISA according to the manufacturer's instructions (Roche Diagnostics). All experiments were performed in triplicates, and the average percent of total hGH released was calculated.

Capacitance Measurements in single mouse β-cells

Single mouse pancreatic β-cells from adult *ob/ob* (paper I) or NMRI (paper II) mice were transfected with plasmids as indicated in the individual papers using the Lipofectamine 2000 technique (Invitrogen). Two to four days after transfection, cells expressing enhanced green fluorescent protein (eGFP) were selected for whole cell patch clamp capacitance measurements. Electrodes were made from borosilicate glass capillaries coated with Sylgaard at their tips. The pipette resistance when filled with the pipette solutions was 2-4 MΩ. The zero-current potential was adjusted before establishment of the seal with the pipette in the recording bath. The holding potential was –70 mV. Exocytosis was measured as increases in cell capacitance using an EPC-9 patch clamp amplifier and the Pulse software (HEKA Elektronik). Cells were perfused continuously with the extracellular solution with a flow-rate of 1.5-2 ml/min during the course of an experiment. The temperature of the extracellular solution was 32-33°C when measured in the position of recording electrodes. The extracellular solution was composed of (in mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES and 3 (paper I) or 5 (paper II) D-glucose (pH 7.4 with NaOH). The pipette solution consisted of (in mM): 110 (paper I) or 125 (paper II) potassium glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES, 0.5 (paper I) or 3 (paper II) Mg-ATP, 10 EGTA, 7 $CaCl_2$, 0.1 cAMP (paper I) (pH 7.15 with KOH). The free Ca^{2+} concentration of the resulting buffer was 340 nM (paper I) or 500 nM (paper II) using the binding constants of Martell & Smith (Martell and Smith, 1971).

Measurements of miniature endplate potentials (mEPPs)

Non-, Cdk5-, dnCdk5-, Cdk5+p35- or Cdk5+p39-transfected NG108-15 cells were overlaid at a density of $2x10^4$ cells per 35 mm dish on the already fused and contracting muscle cells (Chen et al., 2001; Yano et al., 1984; Zhong et al., 1997). The co-cultures were maintained in DMEM supplemented with 10% horse serum and 0.25 mM dBcAMP for 1-7 days. The co-culture medium was replaced with a recording medium (10 mM HEPES-buffered DMEM supplemented with 2 mM $CaCl_2$ and 0.1 mM choline chloride), as described previously (Nelson et al., 1976). Postsynaptic activities were studied electrophysiologically by a conventional intracellular recording method with sharp microelectrodes filled with 1 M potassium citrate (5-20 MΩ). Membrane potentials of myotubes were amplified via an Axoclamp 2A amplifier (Axon Instruments). Membrane potentials of DC-coupled or high gain RC-coupled recordings were continuously monitored on a Nihon Koden thermal array recorder (model RTA-1100), with frequency characteristics of DC to 1 kHz. The noise level was usually less than 0.2 mV. The presence of miniature endplate potentials (mEPPs) in a given myotube that showed a synaptic-like connection to NG108-15 cells under a phase microscopy was judged by monitoring waveform on a storage oscilloscope as described (Chen et al., 2001). The same criteria for recognizing functionally connected pairs was used as described previously (Chen et al., 2001; Nirenberg et al., 1983). The numbers of mEPPs were usually counted during the initial 2 min from the beginning of establishing a stable recording. The existence of mEPP frequency above 2/min in a given myotube was considered to be a criteria for a synapse-positive pair (Chen et al., 2001; Higashida et al., 1981; Nelson et al., 1976; Nirenberg et al., 1983). Average mEPP frequency was calculated

from synapse-positive muscle cells during early (day 1-3) and late (day 4-7) phases of cell co-culturing.

$[Ca^{2+}]_i$ measurements

β-cells were pretreated with 10 μM roscovitine or DMSO for 3.5-5 hours and loaded with 2 μM of the fluorescent Ca^{2+} indicator fura-2/AM for 45 min. The cells, attached to coverslips, were then transferred to a perfusion chamber and stimulated with 25 mM KCl for 2 min. Thereafter cells were stimulated with glucose to verify that the recorded cells were glucose responsive β-cells. The microscope (Zeiss, Axiovert 35M) was equipped with a photon counting photometer and connected to a SPEX fluorolog-2 CM1T11I system, allowing dual wavelength excitation fluorimetry. Emissions at the two excitation wavelengths of 340 nm (F340) and 380 nm (F380) were used to calculate the fluorescence ratio (F340/F380), yielding relative changes in $[Ca^{2+}]_i$. The emitted light, selected by a 500–530 nm bandpass filter, was directed to a CCD imaging system. Cells from each group were averaged for each time point and a composite recording was plotted. Rate of initial response, peak delta ratio and area under the curve were analyzed.

Statistics

Results are presented as mean values ± S.E.M. for indicated number of experiments. Statistical significance was evaluated using Student's *t*-test for paired data. For multiple comparisons Dunnett's test or one-way analysis of variance (ANOVA) followed by least significant difference (LSD) were performed. In paper III, homogeneity of variances was tested with Fisher's F test followed by Student's *t*-test using two-way analysis of homogeneous variance. A p value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION

Cdk5 and its activators are expressed in β-cells (paper I and II)

Cdk5 is a multifunctional serine/threonine protein kinase that mainly has been associated with functions related to neuronal development, even though recent data indicate a role in synaptogenesis and neurotransmission (Smith and Tsai, 2002). We have found that the mRNAs and proteins for Cdk5 and its activators p35 and p39 are expressed in mouse β-cells. Whereas Cdk5 and p39 immunoreactivities in β-cell homogenates were almost as intense as in brain homogenates, p35 immunoreactivity was barely detectable unless the p35 protein was enriched by subcellular fractionation. Habener and co-workers recently demonstrated expression of the p35 subunit in INS-1 cells and in rat and human islets (Ubeda et al., 2004). In agreement with our data, they state that the expression level of p35 is low under physiological conditions. However, when INS-1 cells were exposed to 20-30 mM glucose for 24 hours, the mRNA and protein levels of p35 were increased (Ubeda et al., 2004). It should be noticed that prolonged incubation of primary β-cells in elevated glucose concentrations induces apoptosis (Efanova et al., 1998), and that Cdk5 activity is implicated in neuronal cell death (Weishaupt et al., 2003). Recently, p39 was found to be the predominantly expressed Cdk5 activator in anterior pituitary cells (Xin et al., 2004), suggesting a general importance of the p39 protein in endocrine cells. In the present study we have examined the expression levels of Cdk5, p35 and p39 in islets derived from adult mice. Since numerous studies have demonstrated that Cdk5 activity is essential for the development of the nervous system (Chae et al., 1997; Ko et al., 2001; Ohshima et al., 1996), it would be interesting to examine if the expression levels of the Cdk5 activators are differently regulated during islet development.

Next we investigated the subcellular distribution of Cdk5 and its activators in pancreatic β-cells. Immunocytochemistry revealed that Cdk5, p35 and p39 exhibit a granular staining pattern homogenously spread in the cytoplasm. Double staining with insulin proved that all three proteins were specifically expressed in β-cells and partially co-distributed with this hormone. To further analyze the subcellular distribution of Cdk5 and the activators and to examine if glucose stimulation changed their localization, we performed linear sucrose gradients on unstimulated and glucose-stimulated mouse islets. Experimental data showed that in unstimulated islets Cdk5 immunoreactivity was detected in virtually all fractions, but enriched in cytosol- and plasma membrane-containing fractions. However, after glucose stimulation the Cdk5 immunoreactivity localized to the plasma membrane was notably diminished. These data might imply that during basal secretion Cdk5 is accumulated at the plasma membrane to support exocytosis of the RRP. However, during the second phase of insulin secretion, when SGs are recruited from the SRP and/or RP, plasma membrane-associated Cdk5 is continuously released to the cytosol. As described in neurons (Humbert et al., 2000a; Humbert et al., 2000b), p35 and p39 were localized to distinct but partially overlapping subcellular compartments. p35 partially co-distributed with the plasma membrane marker syntaxin 1, whereas p39 co-distributed in part with both syntaxin 1 and insulin. Unlike Cdk5, p35 and p39 did not redistribute in response to elevated glucose concentration. A possible explanation

for these results might be that there is a strong protein-protein interaction between the activators and the substrates. Alternatively, in order to achieve a fast and efficient regulation of Cdk5 activity, p35 and p39, which are short-lived proteins (Patrick et al., 1998; Patzke and Tsai, 2002), might be degraded directly after use and hence a possible translocation of the proteins were not detected in this set of experiments. The membranous localization of the regulatory subunits in β-cells agree with the finding that both p35 and p39 are myristoylated in their N-terminals (Patrick et al., 1998; Patzke and Tsai, 2002), a modification that is known to anchor proteins to membranes. As the majority of Cdk5 protein is found in non-activator containing fractions our data suggest that the interaction between Cdk5 and the regulatory subunits is transient. The activators have been proposed to directly target the activity of Cdk5 to the substrates (Cheng et al., 2002; Floyd et al., 2001; Nikolic et al., 1996). However, as p39 co-distributed with a pool of insulin granules, and Cdk5 was found in plasma membrane-containing fractions, it is intriguing to speculate that the activity of the kinase might also be regulated by the recruitment of the activator to target membranes.

Cdk5/p39 activity enhances insulin secretion (paper I and II)

To investigate if Cdk5 was involved in the regulation of insulin secretion Cdk5 activity was inhibited chemically by using a potent Cdk5 inhibitor (roscovitine), or by transient expression of a dominant negative (dn) Cdk5 mutant. In both cases insulin release measured by insulin RIA or whole cell capacitance recordings was impaired as compared with controls. In paper I overexpression of wild-type (wt) Cdk5 did not significantly increase membrane capacitance as compared with mock transfection. However, when cAMP was excluded from the pipette solution the rate of exocytosis was stimulated in wtCdk5-transfected cells (paper II). The fact that roscovitine treatment of primary β-cells did not affect the rise in $[Ca^{2+}]_i$ prior to fusion but still inhibited exocytosis (Figs. 3 and 5, paper I) suggested that Cdk5 might influence insulin secretion at a biochemical step after Ca^{2+} entry. An alternative interpretation of the data could be that inhibition of Cdk5 activity reduces the number of docked and/or primed vesicles. Recently, it was demonstrated that roscovitine treatment of anterior pituitary cells reorganizes the cortical actin cytoskeleton and thereby limits the ability of SGs to approach the plasma membrane (Xin et al., 2004).

Inhibition of Cdk5 activity has also been shown to affect secretion in other cell types. Treatment with Cdk5 inhibitors severely impairs secretion from neutrophils, pituitary cells and chromaffin cells (Fletcher et al., 1999; Rosales et al., 2004; Xin et al., 2004). On the contrary, Cdk5 inhibitors facilitate neurotransmitter release from several neuronal cell types (Chergui et al., 2004; Tomizawa et al., 2002; Yan et al., 2002), possibly by increasing the Ca^{2+} influx through P/Q-type VDCCs (Tomizawa et al., 2002). Cdk5 may also diminish the secretory response by phosphorylating P/Q-type VDCCs. The addition of a phosphate group to the P/Q-type VDCC disrupts its interaction with SNAP-25 and synaptotagmin, which is essential for efficient neurotransmission (Tomizawa et al., 2002). In addition, regulated secretion was recently shown to be unaffected by Cdk5 activity in digitonin-permeabilized PC12 cells. Neither overexpression of a dnCdk5 mutant, wtCdk5, the p35 activator or co-transfection of Cdk5 and p35 changed the secretion of exogenous hGH (Barclay et al., 2004). However, as digitonin treatment of chromaffin cells induces a leakage of

cytoplasmic proteins that are crucial for exocytosis, the lack of effect might be explained by the utilization of permeabilized cells (Sarafian et al., 1987).

To examine if both p35 and p39 mediated Cdk5-enhanced insulin secretion p35 and p39 were transiently overexpressed in primary mouse β-cells and secretion was evaluated by capacitance measurements. Interestingly, overexpression of p39, but not p35, stimulated secretion. Inhibition of Cdk5, p35 and p39 protein translation by anti-sense oligonucleotide treatment confirmed the importance of endogenous Cdk5/p39 activity in the regulation of insulin secretion. Cdk5 activity has also been demonstrated to regulate endocytosis of SVs, although its regulatory role is controversial. Tan et al. showed that Cdk5 activity was necessary for SV recycling whereas Tomizawa et al. suggested that Cdk5 suppresses SV endocytosis (Tan et al., 2003; Tomizawa et al., 2003). Therefore, to confirm that the positive effect of Cdk5 on insulin granule fusion that we had observed by membrane capacitance measurements was specifically caused by enhanced exocytosis and not reduced endocytosis, we measured exogenous secretion of hGH from INS-1E cells (Fig. 5, paper II). These data confirmed that Cdk5/p39 activity enhanced secretion in β-cells.

Overexpression of p35 did not stimulate insulin secretion in β-cells, although overexpression of p25, the proteolytic derivate of p35, stimulates secretion of exogenous hGH from chromaffin cells (Fletcher et al., 1999). However, p25 lacks the N-terminal myristoylation signal that anchors the activator to membranous compartments and is therefore not appropriately targeted within the cell. In addition, p25 has a longer half-life than p35 (Patrick et al., 1999). Thus, conversion of p35 to p25 leads to changes in cellular localization of catalytically active Cdk5, hyperphosphorylation of substrates and unspecific substrate recognition (Patrick et al., 1999). Recently, Ubeda et al. demonstrated that Cdk5/p35 activity stimulates the activity of the insulin gene promoter in INS-1 cells (Ubeda et al., 2004), indicating that expression of the p35 activator also is important in β-cell physiology.

Phosphorylation of munc18-1 mediates Cdk5-enhanced insulin secretion (paper II)

Cdk5 has been suggested to regulate neurotransmission through an interaction with the syntaxin-binding protein munc18-1 (Fletcher et al., 1999; Shuang et al., 1998). To examine if Cdk5/p39 enhanced insulin secretion by phosphorylating munc18-1, primary β-cells were transiently transfected with munc18-1 templates, alone or in combination with Cdk5 and the different Cdk5 activators. The stimulatory action of Cdk5 and p39 was specifically abolished when β-cells were co-transfected with munc18-1 templates mutated at the Cdk5 phosphorylation site, but not at the PKC phosphorylation sites (paper II, Fig. 6). Since the aim of this work was to examine effects of Cdk5 on exocytosis the experiments were designed to avoid activation of other kinases that could mask potential effects of Cdk5. Therefore, it is possible that PKC was not activated in this set of experiments and consequently no effect of the PKC phosphorylation mutant of munc18-1 was detected. However, our data do not rule out that PKC phosphorylation of munc18-1 could represent an important mechanism to modify the secretory response. Barclay et al. have recently demonstrated that both PKC and Cdk5 phosphorylation of munc18-1 regulate the late stages of exocytosis by modifying the opening time of the fusion pore. These

phosphorylation events narrow the kinetics of fusion, possibly representing an increase in the extent of kiss-and-run exocytosis (Barclay et al., 2004; Barclay et al., 2003). In addition, overexpression of a dnCdk5 mutant increases the conductance of the fusion pore, but mutational silencing of the Cdk5 phosphorylation site in munc18-1 reveals that this effect is achieved by a mechanism unrelated to Cdk5 phosphorylation of munc18-1 (Barclay et al., 2004). Therefore, additional Cdk5 phosphorylation targets modulate the late stages of single exocytotic events.

The function of munc18-1 in regulated exocytosis is heavily debated. Gene-targeted silencing studies have implicated a role for munc18-1 during docking at the neuromuscular junction in *C. elegans* and in chromaffin cells (Weimer et al., 2003; Voets et al., 2001). However, analysis of central synapses in the knock-out mouse and overexpression studies of munc18-1 mutants in chromaffin cells have pointed to a critical late stage role for munc18-1 subsequent to vesicle docking (Barclay et al., 2004; Barclay et al., 2003; Fisher et al., 2001; Verhage et al., 2000). The yeast Sec1 protein has also been suggested to operate after SNARE complex assembly (Carr et al., 1999; Grote et al., 2000). Although essential for exocytosis, several studies suggest that munc18 proteins may also serve as negative regulators of secretion (Dresbach et al., 1998; Li et al., 2000; Schulze et al., 1994; Zhang et al., 2000). The role of munc18-1 in secretion has mainly been associated with its interaction with syntaxin 1, an interaction that is necessary for appropriate trafficking of syntaxin 1 from Golgi to the plasma membrane (Rowe et al., 1999) and controls the availability of syntaxin 1 in SNARE complex formation (Dulubova et al., 1999; Misura et al., 2000; Yang et al., 2000). Recently, Ciufo et al. demonstrated that munc18-1 performs multiple functions during membrane trafficking, both via syntaxin-dependent and syntaxin-independent mechanisms (Ciufo et al., 2004). Several proteins implicated in regulated exocytosis, including DOC2 (Verhage et al., 1997), mint (Ciufo et al., 2004; Okamoto and Südhof, 1997), granuphilin A (Coppola et al., 2002), and phospholipase D (PLD) (Lee et al., 2004) have been reported to interact with munc18-1. The interaction between PLD and munc18-1 might be of particular importance for regulated exocytosis. PLD is a membrane-bound enzyme that hydrolyzes phosphatidylcholine to generate phosphatic acid (PA), a multifunctional lipid that is thought to play a role in many cellular functions including membrane trafficking. PA may affect secretion either as an intracellular second messenger or as a cone-formed lipid that promotes negative membrane curvature and thereby facilitate fusion (Liscovitch et al., 2000; Roth et al., 1999; Salaün et al., 2004). PLD1 is expressed in pancreatic β-cells and is intimately coupled to and required for insulin secretion, presumably at a very distal step in the exocytotic pathway (Hughes et al., 2004). In chromaffin cells, binding of munc18-1 to PLD potently inhibits basal PLD activity, but stimulation with epidermal growth factor (EGF) abolishes this interaction immediately and hence PLD is activated (Lee et al., 2004). Whether munc18-1 interacts with PLD1 and regulates its activity in β-cells remains to be studied.

Apart from munc18-1 several other proteins that modulate secretion have been described as substrates for Cdk5. Cdk5 phosphorylates synapsin 1 (Matsubara et al., 1996), a neuronal phosphoprotein that tethers SVs to the cytoskeleton and controls the number of vesicles available for release (Greengard et al., 1993). Synapsin 1 is also expressed in pancreatic β-cells and regulates insulin secretion (Longuet et al., 2004). Cdk5 facilitates regulated exocytosis from anterior pituitary cells by phosphorylating a GDP/GTP exchange factor (GEF) named Trio. This modification

increases the GEF activity of Trio, which results in localized activation of Rac. Thereby, Cdk5 may play a role in the reorganization of the actin cytoskeleton that must accompany regulated exocytosis (Bokoch, 2003; Xin et al., 2004). Moreover, Cdk5 phosphorylates a number of proteins such as Pak 1, cables, β-catenin, tau and nudel that directly or indirectly influence the dynamics of the cytoskeleton (Smith, 2003). Intrestingly, the triggering factor munc13-1 has a consensus site for Cdk5 phosphorylation (Barclay et al., 2004). As Cdk5 is described as a multifunctional kinase in neurons and neuroendocrine cells, it is not farfetched to believe that Cdk5 phosphorylates numerous substrates also in the β-cell, and thereby modulates additional cellular processes besides insulin secretion.

In conclusion, our data suggest that Cdk5/p39 activity enhance insulin secretion by phosphorylating munc18-1. However, as munc18-1 has been shown to regulate the exocytotic pathway at multiple stages, possibly through interactions with a number of proteins, elucidation of the mechanism of action in β-cells requires further investigation. Moreover, since deregulated Cdk5 activity has been associated with neurodegenerative diseases (Shelton and Johnson, 2004), it is intriguing to speculate that clarification of the regulatory role of Cdk5 in insulin secretion might reveal molecular defects that are associated with type 2 diabetes mellitus.

Cdk5/p35 and Cdk5/p39 activities enhance the frequency of spontaneous neurotransmitter release (paper III)

In this study we aimed to examine if Cdk5 activity also regulated SV exocytosis. As a model system we used the neuroblastoma/glioma cell line NG108-15 that upon *in vitro* differentiation acquires a neuron-like phenotype (Higashida, 1988; Kasai and Neher, 1992). When co-cultured with differentiating muscle cells, functional d-tubocurarine-sensitive cholinergic synapses are formed (Higashida et al., 1981; Kimura and Higashida, 1992; Nelson et al., 1976). By measuring miniature endplate potentials (mEPPs) in postsynaptic myotubes demonstrating cell-cell contacts with emerging processes from NG108-15 cells, it is possible to analyze the frequency of spontaneous transmitter release and thus the rate of formation of synaptic-like contacts. Hence, these cells constitute an *in vitro* model system for studying SV release at the neuromuscular junction (NMJ).

The basic characterization of the cell line showed that the mRNAs and proteins for Cdk5 and both of its activators were expressed in the NG108-15 cells. As described for other neuronal cells (Fu et al., 2002; Munoz et al., 2000; Paglini et al., 1998; Tomizawa et al., 1996), the protein levels of Cdk5 and p35 in NG108-15 cells were increased after cellular differentiation. However, the p39 protein level remained low. Thus, in contrast to our observation in β-cells (paper II, Fig. 1B), p35 seems to be the predominantly expressed Cdk5 activator in mono-cultures of NG108-15 cells. Nevertheless, when NG108-15 cells are co-cultured with myotubes the expression levels and/or localization of the Cdk5 activators might be altered to promote formation of active synaptic contacts and neurotransmitter release. Since Cdk5 and p35 have been reported to be expressed in developing muscle cells and localize to adult NMJs (Fu et al., 2001; Lazaro et al., 1997), it is problematical to evaluate the expression levels and subcellular localization of these proteins in NG108-15 cells that have been co-cultivated with myotubes. Moreover, in the present study we also

observed intense p39 immunoreactivity in the differentiating muscle cells, suggesting that the p39 protein is expressed in developing rat myotubes and thus is potentially involved in formation of NMJs.

Next, the role of Cdk5 and its activators in functional synapse formation and in spontaneous neurotransmitter release was examined. NG108-15 cells overexpressing dnCdk5 had a reduced ability to form functional synapses with muscle cells as compared to non-transfected cells and cells that were transfected with wtCdk5. Furthermore, the mEPP frequency was significantly lower in dnCdk5-transfected cells, indicating that Cdk5 activity facilitates spontaneous neurotransmitter release in NG108-15 cells. Using the same protocol we examined if p35 and p39 were equally important for Cdk5-mediated function in synaptic activities. Overexpression of either Cdk5/p35 or Cdk5/p39 in NG108-15 cells significantly increased the rate of functional synapse formation and the mEPP frequency as compared with non-transfected cells. Although the immunocytochemistry suggested that the cellular localization of p35 and p39 in NG108-15 cells were similar, subcellular fractionations of mono-cultured cells revealed that p35 and p39 distributed to organelles of different densities. As in the β-cell, Cdk5 was primarily localized to low- and middle-density fractions, and p39 was mainly found in high-density fractions. However, the cellular localization of p35 in NG108-15 cells was different. Unless overexpressed, p35 was exclusively found in low-density fractions. As the activators have been suggested to directly target the activity of Cdk5 to the substrates (Cheng et al., 2002; Floyd et al., 2001; Nikolic et al., 1996), the different subcellular localization of p35 and p39 in NG108-15 cells implies that Cdk5/p35 and Cdk5/p39 might mediate positive effects on spontaneous quantal release and synapse formation by targeting different substrates.

Taken together, in this *in vitro* system of the neuromuscular junction, Cdk5 activated by either of its two regulatory subunits facilitated both the number of active synaptic contacts and the frequency of spontaneous release. In future studies it would be interesting to investigate whether Cdk5 activity also enhances evoked secretion of neurotransmitters, and if that is the case if p35 and p39 are equally important in mediating this function.

Expression of endogenous PP1 inhibitors in pancreatic β-cells (paper IV)

PP1 activity has been suggested to have a key role in the regulation of exocytosis in several secretory systems, including the pancreatic β-cell (Peters et al., 1999; Sim et al., 2003). However, the regulation of PP1 activity in β-cells is unknown. In this study we demonstrated expression of two endogenous inhibitors of PP1 activity, inhibitor-1 and its neuronal homologue DARPP-32, in pancreatic β-cells. The experimental data suggested that inhibitor-1 is the major PP1 inhibitor in β-cells, but low a level of DARPP-32 was also detected. Consistently with the subcellular localization of DARPP-32 and inhibitor-1 in other tissues (Cohen, 2002; Hemmings et al., 1984; Huang and Glinsmann, 1976), sucrose density fractionation revealed that both proteins were mainly localized to the cytosol in islets of Langerhans. In addition, inhibitor-1 was found in the plasmamembrane-containing fractions, suggesting that the PP1 inhibitor is targeted to sites of exocytosis.

Apart from characterizing the expression of DARPP-32 and inhibitor-1 in pancreatic β-cells, we also aimed to elucidate the role and regulation of these proteins in stimulus-secretion coupling. Initially, we intended to investigate if there was a correlation between stimulation of secretion and the phosphorylation status of DARPP-32 and inhibitor-1. Due to techniqual difficulties, immunoblotting with phosphorylation-state specific antibodies did not reveal if DARPP-32 and inhibitor-1 were phosphorylated in β-cells or if the phosphorylation status changed as a result of stimulated secretion. Part of the problem was probably related to the low expression level of DARPP-32 in β-cells, and therefore it is possible that overexpression studies could solve this issue. The importance of endogenous PP1 inhibitors in insulin secretion might also be further investigated using anti-sense RNA or small interfering (si)RNA techniques in combination with capacitance measurements.

Although DARPP-32 and inhibitor-1 are the best-characterized inhibitors of PP1, additional endogenous PP1 inhibitors are described. (reviewed in Cohen, 2002). One example of a differently regulated PP1 inhibitor is inhibitor-2. Inhibitor-2 is ubiquitously expressed and is localized to the cytosol and to the nucleus (Cohen, 1989; Cohen, 2002; Huang and Glinsmann, 1976). In contrast to DARPP-32 and inhibitor-1, inhibitor-2 inhibits PP1 activity in its unphosphorylated form. When phosphorylated by protein kinases, such as glycogen synthase kinase 3 (GSK-3), MAPK and Cdk5, the catalytic subunit of PP1 is activated (Agarwal-Mawal and Paudel, 2001; Hemmings et al., 1982; Sakashita et al., 2003; Wang et al., 1995).

In summary, the presence of endogenous PP1 inhibitors in β-cells suggests that PP1 activity is controlled in these endocrine cells. Given the many substrates for protein kinases that are described in the secretory pathway for insulin release and the broad specificity of PP1, it is likely that DARPP-32 and inhibitor-1 directly or indirectly influence insulin secretion.

CONCLUSIONS

- Cdk5 is expressed in pancreatic β-cells and acts as a positive regulator of insulin secretion.

- p39 is the predominantly expressed Cdk5 activator in pancreatic islets but p35 is also present.

- Cdk5/p39 activity promotes Ca^{2+}-dependent insulin secretion from primary β-cells by phosphorylation of munc18-1.

- Cdk5, p35 and p39 proteins are expressed in the neuroblastoma/glioma cell line NG108-15 and here the p35 protein appears to be the major Cdk5 activator.

- Cdk5/p35 and Cdk5/p39 activities enhance formation of functional synaptic-like structures and increase the mEPP frequency.

- Endogenous PP1 inhibitors, DARPP-32 and inhibitor-1, are present in insulin-secreting pancreatic β-cells, indicating that they have a potential role in the regulation of PP1 activity and insulin exocytosis.

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