

# Molecular and physiological regulation of adiponectin exocytosis in white adipocytes

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2015



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Cover illustration: Patch pipette attached to a 3T3-L1 adipocyte by Ali Komai.  
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ISBN: 978-91-628-9630-0 (printed version), 978-91-628-9631-7 (electronic version)  
E-published at <http://hdl.handle.net/2077/39573>  
Printed by Ineko AB, Gothenburg, Sweden 2015

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## ABSTRACT

In this thesis we have investigated the mechanisms of white adipocyte regulated exocytosis in health and disease, with a special focus on adiponectin. We have applied a combination of electrophysiological membrane capacitance measurements, biochemical measurements of released adipokines and gene expression analysis. Our work hypothesis was that since white adipocytes are endocrine cells, secretion of adipocyte hormones should be regulated in a way resembling how hormone secretion is controlled in other endocrine cell types. In **paper I** we show that adipocyte exocytosis is triggered by cAMP via activation of exchange proteins directly activated by cAMP (Epac). cAMP triggers secretion of a readily releasable pool of vesicles in a  $\text{Ca}^{2+}$ -independent manner. However, a combination of  $\text{Ca}^{2+}$  and ATP augments exocytosis via a direct effect on the release process and by recruitment of new releasable vesicles. We further demonstrate that recorded membrane capacitance increases can be largely correlated to release of adiponectin containing vesicles and that the regulation of adiponectin exocytosis is similarly controlled in primary human subcutaneous adipocytes. In **paper II** we show that the  $\text{Ca}^{2+}$ /ATP-dependent maturation of adiponectin vesicles is a temperature-dependent step and thus reduced by cooling. We suggest that the temperature-dependent effects reflect the need of ATP hydrolysis in order to provide energy for recruitment of new releasable vesicles as well as for phosphorylation of exocytotic proteins. Our study provides important mechanistic information about the regulation of white adipocyte stimulus-secretion coupling. In **paper III** we show that adiponectin exocytosis is physiologically stimulated via adrenergic signalling chiefly involving catecholamine activation of  $\beta_3$ -adrenergic receptors. We also demonstrate that Epac1 is the isoform expressed in white adipocytes. We moreover show that adrenergic stimulation of adiponectin exocytosis is disturbed in adipocytes isolated from obese/type-2 diabetic mice and that the disruption is due to a low abundance of  $\beta_3$ -adrenergic receptors in combination with a reduced expression of Epac1.

**Keywords:** White adipocytes, adiponectin secretion, exocytosis, stimulus-secretion coupling

**ISBN:** 978-91-628-9630-0



# Reports constituting this thesis

This thesis is based on the following papers referred to in the text by their roman numerals:

- I. Komai AM, Brännmark C, Musovic S, Olofsson CS. (2014). PKA-independent cAMP stimulation of white adipocyte exocytosis and adipokine secretion: modulations by  $\text{Ca}^{2+}$  and ATP. *J Physiol* 592, 5169-5186
  
- II. El Hachmane MF \*, Komai AM \*, Olofsson CS. (2015). Cooling reduces cAMP-stimulated exocytosis and adiponectin secretion at a  $\text{Ca}^{2+}$ -dependent step in 3T3-L1 adipocytes. *PLoS ONE*, 10(3): e0119530  
  
\* The authors contributed equally to this work
  
- III. Komai AM, Musovic S, El Hachmane MF, Johansson M, Peris E, Asterholm IW, Olofsson CS. White adipocyte adiponectin exocytosis is stimulated via  $\beta_3$  adrenergic signalling and activation of Epac1 – catecholamine resistance in obesity and type-2 diabetes. *Submitted*

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# Abbreviations

[Ca <sup>2+</sup> ] <sub>i</sub>	Intracellular Ca <sup>2+</sup> concentration
AC	Adenylate cyclase
AdipoR1	Adiponectin receptor 1
AdipoR2	Adiponectin receptor 2
AR	Adrenergic receptor
ATP	Adenosine triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid)
BAT	Brown adipose tissue
cAMP	Cyclic adenosine monophosphate
CL	CL 316,243
C <sub>m</sub>	Membrane capacitance
CREB	cAMP response element-binding protein
C/EBPs	CCAAT-enhancer-binding protein
DEXA	Dexamethasone
EC	Extracellular solution
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Epac	Exchange proteins directly activated by cAMP
ER	Endoplasmic reticulum
Forsk	Forskolin
GABA	Gamma-aminobutyric acid
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
G protein	Guanine nucleotide-binding protein
GTP	Guanosine triphosphate
HFD	High fat diet
HMW	High molecular weight
IBMX	3-isobutyl-1-methylxanthine
IL-6	Interleukin 6
IWAT	Inguinal white adipose tissue
LMW	Low molecular weight

NCS	Newborn calf serum
PDE	Phosphodiesterase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PPAR $\gamma$	Peroxisome proliferator activated receptor gamma
RT-PCR	Reverse transcriptase polymerase chain reaction
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins
TIRF	Total internal reflection fluorescence
TNF- $\alpha$	Tumor necrosis factor alpha
WAT	White adipose tissue
$\Delta C/\Delta t$	Rate of exocytosis (F/s)

# Introduction

In 2014 the World Health Organization announced that more than 1.9 billion adults were overweight and that 600 million of these were obese. The obese state can lead to many different pathological conditions such as cardiovascular diseases, type 2 diabetes and certain cancers (Lavie *et al.*, 2009). In fact, overweight and obesity is more closely linked to death than underweight. Obesity is characterized by an increase in white adipose tissue mass, the body's fat storing organ. Historically, adipose tissue has been viewed as an inert storage depot of fat to be used when energy requirements are not met by energy input (Kershaw & Flier, 2004). However, growing evidence during the last two decades shows that white adipose tissue secretes a wide variety of hormones and bioactive molecules with important roles in control of whole-body physiology (Trujillo & Scherer, 2006). One of the protein hormones secreted by the white adipocyte, adiponectin, has many improving effects in metabolic disease (Whitehead *et al.*, 2006). In obesity, there is a dysregulation of adiponectin secretion resulting in decreased levels of this protective hormone. 20 years after its discovery the mechanisms regulating adiponectin release remain unclear. In this thesis, we aim to investigate the intracellular mediators involved in the acute release of adiponectin and how this release might be disturbed in obesity and type 2 diabetes.

## Adipose tissue

The main types of adipose tissues are white adipose tissue (WAT) and brown adipose tissue (BAT). Another long known but less studied distinct type of adipose tissue is bone marrow adipose tissue (Fazeli *et al.*, 2013).

Adipose tissue is situated in different depots throughout the body with functional as well as morphological differences depending on both the adipose tissue type and location (Cousin *et al.*, 1993; Frayn *et al.*, 2003; Cinti, 2012). In humans, WAT is situated subcutaneously (under the skin) or viscerally (around the internal organs; Fig. 1). In rodents, there are several subcutaneous and visceral depots (depicted in

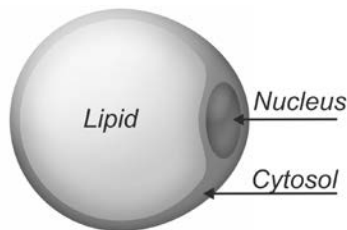
Fig. 3 of Cinti, 2005). WAT to BAT ratio is determined based on several factors such as age, nutrition and environmental conditions (Cinti, 2005; Berry *et al.*, 2013). WAT has several important roles in metabolism including energy storage and secretion of many bioactive molecules with central and peripheral effects. BAT has a protective role in cold environments by non-shivering heat production. The brown color of BAT is due to a high content of mitochondria which specifically express uncoupling protein 1 (UCP-1). Cold stimulates the release of norepinephrine from sympathetic nerves which in turn stimulates lipolysis. Subsequently, UCP-1 uncouples oxidative metabolism from ATP production with succeeding generation of heat (Cannon & Nedergaard, 2004).



**Fig. 1**  
1) Subcutaneous fat  
2) Abdominal muscle  
3) Visceral fat

### ***White adipose tissue cell types***

The white adipocyte (Fig. 2), the classical fat cell, is the main cell type of adipose tissue. The white adipocyte is a large and expandable cell and human adipocytes can vary from 25-250  $\mu\text{M}$  in size (Meyer *et al.*, 2013). The adipocyte is unique in its lipid storing properties and contains one large lipid droplet which comprises about 95% of the cell volume. The remaining 5% consist of the cell cytosol and nucleus, which are localized in close proximity to the plasma membrane. The adipose tissue can grow in size by hypertrophy or hyperplasia (Hausman *et al.*, 2001). Hypertrophy in this context is the enlargement of adipocytes due to increased lipid accumulation. Hyperplasia refers to an increase in the number of cells due to maturation of precursor cells.



**Fig. 2 The white adipocyte**



Besides adipocytes, adipose tissue contains other cell types collectively termed the stromal vascular fraction. The stromal vascular fraction includes preadipocytes, macrophages, monocytes, endothelial cells, pericytes as well as multipotent stem cells (Zuk *et al.*, 2002). Preadipocytes can be differentiated into mature adipocytes but the mechanisms involved in the recruitment and proliferation of preadipocytes are unclear (Hausman *et al.*, 2001; Arner & Spalding, 2010). Interestingly, it has been shown that despite a continuous turnover of adipocytes the number of adipocytes in humans can vary during childhood and adolescence but remains constant and tightly regulated in adulthood (Spalding *et al.*, 2008). Macrophages are involved in clearing dead adipocytes within the tissue and their proliferation increases specifically in adipose tissue with obesity (Cinti *et al.*, 2005; Amano *et al.*, 2014). Macrophages release inflammatory factors such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and chronic inflammation in obesity is closely related to insulin resistance (H. Xu *et al.*, 2003).

### ***White adipose tissue vascularisation and innervation***

Adipose tissue is highly vascularised and sufficient blood supply is essential for its proper function (Rupnick *et al.*, 2002; Nishimura *et al.*, 2007). Besides providing oxygen, lipids and nutrients to the WAT, blood enables the transportation of released free fatty acids and adipokines from the WAT to peripheral tissues. Likewise, adipose tissue can be exposed to various signaling molecules secreted into the blood stream. Microvascularisation of the adipose tissue itself enables paracrine signaling with neighbouring cells. The growth of WAT is highly dependent on angiogenesis which can be provided by endothelial cells and pericytes in the WAT (Rupnick *et al.*, 2002). Vascular endothelial growth factor A is proangiogenic and is secreted by neighbouring adipocytes and macrophages (Sung *et al.*, 2013).

The sympathetic innervation of WAT vasculature has been known for many years (Slavin & Ballard, 1978) and in 1995 WAT was shown to be directly innervated with postganglionic neurons from the sympathetic nervous system (Youngstrom & Bartness, 1995; Bamshad *et al.*, 1998). In this way WAT can be directly exposed to noradrenaline to activate its adrenergic receptors. Most studies conducted have not

shown any significant parasympathetic innervation of WAT, however, the existence of parasympathetic innervation is debated and remains to be clarified (Berthoud *et al.*, 2006; Giordano *et al.*, 2006; Kreier & Buijs, 2007).

## **Adipose tissue in lipid and glucose homeostasis**

One of the important and long-known roles of white adipose tissue is its function in maintenance of total glucose and lipid homeostasis. Adipocytes store excess calorie intake in the form of triacylglycerides (TAGs) to fuel the body by the process of lipolysis during fasting and energy deprivation. White adipose tissue is the primary site of energy storage and is the only organ evolved to do so, up to a certain limit, without losing its physiological properties. The metabolic direction of adipose tissue research has been extensively studied over the years and is reviewed in (Lafontan, 2008).

### ***The fasted state***

Catecholamines are the main physiological activators of lipolysis by binding to the adrenergic receptors on the plasma membrane of the white adipocytes. Adrenergic receptors (ARs) are members of the family of G protein-coupled receptors (GPCRs). There are two  $\alpha$ -AR ( $\alpha_1$  and  $\alpha_2$ ) and three  $\beta$ -AR ( $\beta_1$ ,  $\beta_2$ ,  $\beta_3$ ) subtypes of the receptors. All five adrenergic receptors are expressed in WAT. However, there are inter-depot (Mauriege *et al.*, 1987; Arner *et al.*, 1990) as well as inter-species (Lafontan *et al.*, 1995) differences in the presence of the receptor subtypes.

Binding of catecholamines to  $\beta$ -adrenergic receptors activates adenylate cyclases (ACs), enzymes which catalyse the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA) and subsequently a set of different lipases that catabolize triacylglycerides into free fatty acids and glycerol that is released into the blood stream. Free fatty acids in the circulation are then taken up by peripheral tissues and  $\beta$ -oxidized to produce energy in the form of ATP (Arner, 1999; Duncan *et al.*, 2007).

## ***The fed state***

Insulin is secreted by pancreatic  $\beta$ -cells in response to elevations in blood glucose. Insulin switches metabolism from the catabolic to the anabolic state, and thus inhibits lipolysis and stimulates both glucose and free fatty acid uptake (Rutkowski *et al.*, 2015). Insulin binds to insulin receptors present on the adipocyte plasma membrane and activates insulin receptor substrate proteins that recruit phosphoinositide 3-kinase (PI3K) to the plasma membrane. PI3K phosphorylates and activates numerous proteins which ultimately leads to the activation of protein kinase B (PKB; also known as Akt) signaling pathway. Consequently, PKB phosphorylates and activates phosphodiesterase 3B (PDE3B), an enzyme that catalyses the hydrolysis of cAMP. In this way, cAMP-dependent activation of PKA and lipolysis is inhibited. PKB also stimulates the translocation of glucose transporter 4 containing vesicles to the plasma membrane, enabling the entry of glucose into the adipocyte (Taniguchi *et al.*, 2006).

Dietary fats or synthesised fatty acids from non-lipid substrates are the sources of fatty acids delivered to the white adipose tissue. The production of fatty acids from non-lipid substrates, *de novo* lipogenesis, mainly occurs in the liver even if WAT is also capable of this process. Fatty acids circulate in the form of triacylglycerides transported by lipoproteins. Lipoproteins are too large to penetrate the adipose tissue capillaries and triacylglycerides must therefore undergo lipoprotein lipase-dependent lipolysis before being delivered to the adipocytes as free fatty acids. Lipoprotein lipase is synthesised by the adipocytes and upregulated in the fed state. Once the free fatty acids are inside the adipocyte, they are re-esterified and stored in the lipid droplet as triacylglycerides (Ameer *et al.*, 2014).

## **Adipose tissue as an endocrine organ**

Over the last two decades, adipose tissue has been firmly established as an endocrine and highly complex organ. Adipose tissue can affect whole-body physiology in an auto-/para- and endocrine fashion by its wide range of secretory products. More than a hundred different biologically active molecules are secreted by WAT including hormone-like proteins, inflammatory cytokines, anti-inflammatory factors and sex

steroids (Kershaw & Flier, 2004; Fantuzzi, 2005; Balistreri *et al.*, 2010). The peptides and proteins that are produced and released by adipose tissue are commonly referred to as *adipokines*.

Adipokines have central as well as peripheral effects, and have gained a lot of attention due to their pleiotropic and important roles in inflammation, immunity, cardiovascular health and metabolism. Some adipokines have gained increased attention due to their beneficial functions in obesity and obesity-related maladies (Ronti *et al.*, 2006; Waki & Tontonoz, 2007; Balistreri *et al.*, 2010). Adipocyte peptide and protein hormones include (but are not restricted to) leptin, adiponectin, adipisin, apelin and visfatin. Some of these adipocyte peptide hormones are further described below.

The traditional view of white adipose tissue as a passive energy reservoir was revised with the discovery of the adipokine adipisin (also known as complement factor D) in 1987 (Cook *et al.*, 1987). Adipsin has recently been shown to be important for  $\beta$ -cell function and glucose homeostasis, and it has been reported that adipsin levels are decreased in type 2 diabetic patients (Lo *et al.*, 2014). Moreover, adipsin has an important role in innate immunity and is crucial for complement activation by the alternative pathway (Xu *et al.*, 2001).

The endocrine role of adipose tissue was further confirmed with the discovery of leptin in 1994 (Zhang *et al.*, 1994). Leptin is secreted by white adipocytes (also secreted by the gastric mucosa; Cammisotto & Bendayan, 2007) and leptin levels correlate with adipose tissue mass and are thus increased in obesity (Maffei *et al.*, 1995). Leptin is the most studied adipokine and has a central role in regulating food intake and energy expenditure by binding to leptin receptors in the brain to signal long-term satiety (Tartaglia *et al.*, 1995). Moreover, leptin can indirectly affect glucose homeostasis by acting on the pancreatic  $\beta$ -cells to reduce insulin secretion (Marroqui *et al.*, 2012).

In 1995, Scherer and colleagues were the first to describe a novel protein highly specific to adipocytes which was later named adiponectin (Scherer *et al.*, 1995). Shortly thereafter other groups reported the existence of adiponectin in adipocytes

from mouse and rat (Hu *et al.*, 1996), in human plasma (Nakano *et al.*, 1996) and in adipose tissue (K. Maeda *et al.*, 1996). Since its discovery, adiponectin has shown great potential as an adipokine with anti-diabetic, anti-atherogenic and anti-inflammatory properties.

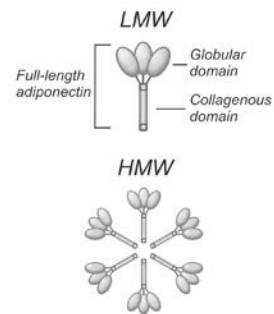
## **Adiponectin**

Adiponectin is a 30-kDA, 247 amino acid long peptide encoded by the AdipoQ gene (also called apM1, Acrp30). The production of adiponectin is regulated by adipogenetic transcriptional factors such as C/EBPs (Saito *et al.*, 1999), sterol regulatory element binding protein 1c (Seo *et al.*, 2004) and peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ; N. Maeda *et al.*, 2001).

Adiponectin (Fig. 3) consists of an N-terminal signalling peptide region followed by a collagenous domain and a C-terminal globular domain (Scherer *et al.*, 1995). Full-length adiponectin is present in the circulation as a low molecular weight (LMW) trimer, hexamer or a high molecular weight (HMW) multimer complex (Tsao *et al.*, 2003). After synthesis in the endoplasmic reticulum (ER), the assembly of different adiponectin isoforms is tightly regulated and dependent on the ER-chaperones ERp44 and Ero1-La

(Z. V. Wang *et al.*, 2007; Hampe *et al.*, 2015). Multimerisation of full-length adiponectin to higher order complexes requires extensive post-translational modifications (Y. Wang *et al.*, 2006) and once secreted the complexes stay stable and do not inter-convert between different forms (Schraw *et al.*, 2008). In humans and rodents, the HMW to LMW ratio as well as total adiponectin levels are higher in females than in males (Pajvani *et al.*, 2003; Gui *et al.*, 2004; Santaniemi *et al.*, 2006).

Adiponectin is produced and secreted by mature adipocytes. Adiponectin is highly abundant in blood (mg/l range) and accounts for ~0.01% of total serum protein in humans (Arita *et al.*, 1999). The half-life of adiponectin in humans has not been determined but the half-life of human adiponectin in rabbits is reported to be 14.3 h



**Fig. 3 Adiponectin structure and isoforms**

and 17.5 h for the LMW and HMW, respectively (Peake *et al.*, 2005). In another study, wild-type and recombinant adiponectin had a half-life of ~75 min in mice and differences in clearance rates for the different isoforms were also reported (Halberg *et al.*, 2009).

Although adiponectin expression has also been detected in skeletal muscle, endothelial cells and cardiac myocytes (Delaigle *et al.*, 2004; Pineiro *et al.*, 2005; Wolf *et al.*, 2006) the contribution by these alternative sources to circulating levels is uncertain. In context, mice lacking adipose tissue do not exhibit significant plasma levels of adiponectin pinpointing the adipose tissue as the main source of adiponectin (F. Wang *et al.*, 2013).

### ***Metabolic effects of adiponectin***

Most studies investigating the mechanisms mediating the metabolic effects of adiponectin are conducted using different animal or *in vitro* models. Those investigations therefore chiefly use *in vitro* produced full-length adiponectin or its cleaved globular form. The physiological significance of globular adiponectin has been debated as mainly full-length adiponectin multimers are released into circulation. Nevertheless, studies of this kind have provided some insight into the important role of adiponectin in the regulation of whole body physiology.

The two main receptors by which adiponectin mediate its effects are AdipoR1 and AdipoR2 (Yamauchi *et al.*, 2003; Yamauchi *et al.*, 2007). AdipoR1 is predominantly present in skeletal muscle and has a higher affinity for the globular domain of adiponectin whereas AdipoR2 is highly expressed in the liver and has a higher affinity for the full-length protein (Yamauchi *et al.*, 2003). Adiponectin receptors are widely distributed and have, in addition to skeletal muscle and liver, been shown to be present in adipose tissue (Rasmussen *et al.*, 2006), heart (Ding *et al.*, 2007; Palanivel *et al.*, 2007), pancreas (Staiger *et al.*, 2005) and in the brain (Kubota *et al.*, 2007).

Increased plasma triglyceride levels, hyperglycemia and insulin resistance are all symptoms of the metabolic syndrome, prediabetes or type 2 diabetes (Grundy, 2012). Combined results of several studies indicate that adiponectin has great potential to

improve many of these pathological conditions. Skeletal muscle, which is the body's major source of glucose uptake, is highly affected by obesity and insulin resistance in this tissue is itself a contributor to type 2 diabetes (DeFronzo & Tripathy, 2009). Skeletal muscle benefits from adiponectin in several ways. Adiponectin acts on skeletal muscle to increase free fatty acid oxidation (Fruebis *et al.*, 2001; Yamauchi *et al.*, 2002), glucose uptake (Ceddia *et al.*, 2005) and mitochondrial number and function (Ceddia *et al.*, 2005). Insulin acts on the liver to inhibit glucose output; an insulin resistant liver will itself contribute to increased blood glucose levels (Titchenell *et al.*, 2015). Adiponectin decreases liver glucose output by increasing liver insulin sensitivity and by suppressing genes involved in gluconeogenesis (Berg *et al.*, 2001; Combs *et al.*, 2001; Yamauchi *et al.*, 2002; Miller *et al.*, 2011).

There are several proposed roles of adiponectin in inflammation. Adiponectin has been shown to down-regulate macrophage recruitment to the inflammatory tissue and decrease the production of pro-inflammatory cytokines such as TNF- $\alpha$  and interleukin 6 (Yokota *et al.*, 2000; Park *et al.*, 2008). Adiponectin can also inhibit the expression of TNF- $\alpha$  in the liver and by autocrine effects in the adipocytes (A. Xu *et al.*, 2003; Ajuwon & Spurlock, 2005). Furthermore, adiponectin can increase the production of anti-inflammatory cytokines in leukocytes, monocytes and macrophages (Kumada *et al.*, 2004; Wolf *et al.*, 2004; Wulster-Radcliffe *et al.*, 2004).

The role of adiponectin in the central nervous system is not fully understood. AdipoR1 and AdipoR2 are expressed in the hypothalamus of rodents (Kubota *et al.*, 2007; Guillod-Maximin *et al.*, 2009) and humans (Kos *et al.*, 2007). Adiponectin has also been detected in rodent (Kubota *et al.*, 2007) and human (Kos *et al.*, 2007; Kusminski *et al.*, 2007) cerebrospinal fluid. On the contrary, in a previous study adiponectin was not detectable in human cerebrospinal fluid and it did not pass the blood brain barrier (Spranger *et al.*, 2006).

### ***Disturbed adiponectin levels in obesity and type 2 diabetes***

Low adiponectin levels are now established as a marker of the metabolic syndrome (Ryo *et al.*, 2004). Unlike most adipokines adiponectin levels decrease in the obese

state, a correlation demonstrated in several studies (Hu *et al.*, 1996; Arita *et al.*, 1999; Weyer *et al.*, 2001). Associations have also been observed between adiponectin and different adipose tissue depots where adiponectin levels exhibit an independent negative correlation to visceral fat mass (Gavrila *et al.*, 2003; Lihn *et al.*, 2004; Cote *et al.*, 2005). Moreover, visceral adipocyte size negatively correlates with serum HMW adiponectin (Meyer *et al.*, 2013).

Reduced production and plasma levels of adiponectin are closely linked to insulin resistance (Weyer *et al.*, 2001) and low levels of adiponectin (hypoadiponectinemia) correlate better with insulin resistance than with adiposity (Hotta *et al.*, 2000; Weyer *et al.*, 2001; Klöting *et al.*, 2010). Furthermore, several studies implicate low levels of HMW rather than total levels of adiponectin as a predictor of the development of type 2 diabetes (Pajvani *et al.*, 2004; Murdolo *et al.*, 2009).

Chronic inflammation in the white adipose tissue has been suggested to play a causal role in the development of insulin resistance and type 2 diabetes (Hotamisligil, 2006). Previous studies have shown an inverse correlation between the inflammatory cytokine TNF-  $\alpha$  and adiponectin levels (Kern *et al.*, 2003; Hector *et al.*, 2007). In a recent study in mice TNF-  $\alpha$  was shown to decrease HMW adiponectin but not LMW adiponectin (He *et al.*, 2015).

In obesity, impaired vascularisation to cope with the demand of expanded adipocytes might lead to hypoxia (Kabon *et al.*, 2004; Spencer *et al.*, 2011). Studies in 3T3-L1 as well as human cultured adipocytes have shown diminished adiponectin expression and secretion under hypoxic conditions (Chen *et al.*, 2006; B. Wang *et al.*, 2007).

### ***Regulation of adiponectin expression and secretion***

A few studies have shown adiponectin gene expression to be downregulated during continuous exposure to  $\beta$ -AR- or cAMP-agonists (Fasshauer *et al.*, 2001; Delporte *et al.*, 2002; Cong *et al.*, 2007; Fu *et al.*, 2007). In one of the first studies that investigated the effect of adrenergic exposure on adiponectin secretion, 10 hour incubations of visceral adipose tissue explants with a  $\beta$ 3-AR- or cAMP-agonist decreased adiponectin secretion (Delporte *et al.*, 2002). The  $\beta$ -AR-induced suppression of adiponectin expression in 3T3-L1 adipocytes was further shown to be



mediated by PKA (Fasshauer *et al.*, 2001). Similar observations have been reported in primary rat adipocytes exposed to the  $\beta$ -AR agonist isoprenaline. In these experiments, 24 hour exposure to isoprenaline inhibited adiponectin secretion in a dose-dependent manner while stimulating lipolysis (Cong *et al.*, 2007). The inhibiting effect of isoprenaline on adiponectin secretion was shown after 4 hours of treatment and could be reversed when adipocytes were exposed to insulin in combination with the  $\beta$ -AR agonist. Furthermore, the reversing effect of insulin on isoprenaline-suppressed adiponectin secretion was shown to be mediated by a PI3K-mediated breakdown of PDEs (Cong *et al.*, 2007). PDE3B activation has previously been shown to be PI3K-dependent and is the basis for the opposing effects of cAMP-increasing  $\beta$ -AR-agonists and insulin (Degerman *et al.*, 1996).

On the gene level, insulin has been shown to decrease (Fasshauer *et al.*, 2002), increase (Halleux *et al.*, 2001; Seo *et al.*, 2004; Cong *et al.*, 2007) or to have no effect (Pereira & Draznin, 2005; Li *et al.*, 2013) on adiponectin expression. These disparities might be explained by different experimental conditions as well as variances in the adipocyte source (different animal species and/or adipose depot or cultured adipocytes). More consistently, several investigations have shown an insulin-mediated stimulation of adiponectin secretion at time points ranging from half an hour up to 24 hours (Bogan & Lodish, 1999; Motoshima *et al.*, 2002; Pereira & Draznin, 2005; Cong *et al.*, 2007; Blümer *et al.*, 2008; Li *et al.*, 2013; Lim *et al.*, 2015). The stimulatory effect of insulin on adiponectin secretion has further been shown to be mediated via PI3K (Pereira & Draznin, 2005; Blümer *et al.*, 2008; Lim *et al.*, 2015) and inhibition of this pathway also downregulated adiponectin gene expression (Pereira & Draznin, 2005).

Already in the first study identifying adiponectin in 1995, Scherer and colleagues suggested that the adipokine is secreted via regulated exocytosis (Scherer *et al.*, 1995). Further studies reported the presence of a secretory compartment containing adiponectin and that short-term secretion of adiponectin (measurable elevated release after 30-60 min) could be stimulated by insulin (Bogan & Lodish, 1999; Lim *et al.*, 2015). The location of adiponectin in close vicinity to the plasma membrane has also been reported in human adipocytes (Halleux *et al.*, 2001). Adiponectin has also been

proposed to be secreted by endosomal pathways (Clarke *et al.*, 2006; Xie *et al.*, 2006; Xie *et al.*, 2008). However, inhibition of the endosomal pathway/ER-Golgi transport vesicles has been shown to only partially block adiponectin secretion, indicating that the release of adiponectin involves other secretory pathways (Xie *et al.*, 2008). In conclusion, while the long-term regulation of adiponectin secretion has been investigated to some extent, there is surprisingly little known about the short-term regulation of adiponectin exocytosis

## **Exocytosis**

Exocytosis is an essential biological process in all eukaryotic cells and enables normal cellular function and intercellular communication by the release of secretory factors. Synthesised proteins in the ER pass the Golgi complex and continue to *trans*-Golgi where they are packed in vesicles destined for secretion. In constitutive exocytosis, secretory vesicles containing peptides or lipids produced by the cell can be released by spontaneous fusion with the plasma membrane. Most neuroendocrine and endocrine cells also have a pathway of regulated exocytosis, where secretory vesicles accumulate in the cytoplasm and undergo fusion with the plasma membrane only upon a triggering signal (Burgoyne & Morgan, 2003) in a process termed *stimulus-secretion coupling*. The terminology was first described in 1962, where Douglas & Poisner showed that the trigger of catecholamine release from chromaffin cells was due to  $\text{Ca}^{2+}$  influx stimulated by extracellular acetylcholine (Douglas & Poisner, 1962). Since then, biophysical methods such as patch-clamp capacitance measurements and optical methods such as total internal reflection fluorescence (TIRF) microscopy has enabled single cell studies of high temporal and spatial resolution, allowing researchers to investigate properties and kinetics of regulated vesicle release (Neher & Marty, 1982; Steyer *et al.*, 1997). Combined, studies in different (neuro)endocrine cell types have led to a rather unified model of the different steps controlling regulated exocytosis (Burgoyne & Morgan, 2003).

### ***Stimulus-secretion coupling in neuroendocrine and endocrine cells***

Secretory vesicles are divided into different functional pools depending on their release competence. A distinction is made between mature vesicles belonging to a

readily releasable pool and immature vesicles belonging to a reserve pool. Readily releasable vesicles can be released directly upon a physiological stimulus. Once the readily releasable pool is depleted, vesicles from the reserve pool need to undergo different functional modifications and/or physical translocation to the plasma membrane in order to be release competent (Burgoyne & Morgan, 2003).

In most endocrine cell types,  $\text{Ca}^{2+}$  is the primary signal that triggers the fusion of readily releasable vesicles with the plasma membrane (Burgoyne & Morgan, 2003).  $\text{Ca}^{2+}$  sources include influx via voltage-gated  $\text{Ca}^{2+}$  channels and/or release from intracellular  $\text{Ca}^{2+}$  stores. Release-ready vesicles can be localised close to  $\text{Ca}^{2+}$  channels, which upon activation expose the vesicles to high local concentrations of  $\text{Ca}^{2+}$  and rapid release (Moser & Neher, 1997; Wisner *et al.*, 1999; Barg *et al.*, 2001; Becherer *et al.*, 2003). Ultimate fusion of secretory vesicles with the plasma membrane involves a complex interplay between several vesicle- and plasma membrane bound proteins termed SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins; Sudhof & Rothman, 2009; Hong & Lev, 2014). Upon  $\text{Ca}^{2+}$  stimulus and activation of vesicular  $\text{Ca}^{2+}$  sensors, release-ready vesicles will be tightened to the plasma membrane by the SNAREs in a zipper-like fashion for ultimate fusion. The conformational changes that occur during the assembly of the SNAREs are thought to provide the energy required for fusion with the plasma membranes (Sudhof & Rizo, 2011; Hong & Lev, 2014). Vesicles that interact with the plasma membrane but are too far away from the SNAREs to assemble are referred to as tethered vesicles, whereas docked vesicles are stationary and morphologically connected to the plasma membrane (Lang *et al.*, 2001; de Wit, 2010). Recent studies have shown that not all vesicles require docking prior to  $\text{Ca}^{2+}$ -dependent exocytosis (Allersma *et al.*, 2004; Toonen *et al.*, 2006; Shibasaki *et al.*, 2007; Kasai *et al.*, 2008). Besides triggering vesicle fusion,  $\text{Ca}^{2+}$  has also been shown to be involved in the physical translocation and docking of vesicles (Becherer *et al.*, 2003). The mechanisms mediating the physical docking of vesicles are not completely understood but seem to involve several exocytotic protein complexes (de Wit, 2010). In order to gain release-competence and refill the readily releasable pool, docked vesicles need to undergo  $\text{Ca}^{2+}$ -, ATP-, cAMP-, time- and temperature-

dependent modifications referred to as priming (Parsons *et al.*, 1995; Eliasson *et al.*, 1997; Renstrom *et al.*, 1997). This was first described in chromaffin cells where  $\text{Ca}^{2+}$ -stimulated secretion in the absence of Mg-ATP occurred only for a short time, and where Mg-ATP only could potentiate secretion only if it preceded  $\text{Ca}^{2+}$ -stimulation (R. W. Holz *et al.*, 1989). In later studies conducted by the same group ATP-dependent priming was shown to be stimulated by low intracellular  $\text{Ca}^{2+}$  levels, and to be preceded by a later rate-limiting temperature-dependent step (Bittner & Holz, 1992a, 1992b). Similarly, ATP has been shown to be required for sustained exocytosis and refilling of the readily releasable pool of vesicles in  $\beta$ -cells (Eliasson *et al.*, 1997). Moreover, a decrease in temperature inhibited the refilling of the readily releasable pool but did not alter  $\text{Ca}^{2+}$ -stimulated exocytosis of mature vesicles, indicating that vesicle replenishment is an energy-dependent process (Renstrom *et al.*, 1996).

### **cAMP-dependent exocytosis**

Intracellular cAMP levels are elevated by various GPCRs and cAMP is a well-known modulator of regulated exocytosis in many secretory cell types. cAMP mediates its actions in several ways involving activation of PKA, exchange proteins directly activated by cAMP (Epac) and/or cyclic nucleotide-gated ion channels (Seino & Shibasaki, 2005).

The PKA-mediated actions of cAMP can be due to phosphorylation of one or several target proteins and have in numerous cell types been shown to be involved in modulations of the vesicle pools rather than in the release process. These effects include increases in vesicle size due to pre-exocytotic vesicle-vesicle fusion (Sikdar *et al.*, 1998; Carabelli *et al.*, 2003) and acceleration of vesicle replenishment of the readily releasable pool (Ammala *et al.*, 1993; Renstrom *et al.*, 1997; Nagy *et al.*, 2004). Part of the PKA-mediated effect can also be due to an amplification of the  $\text{Ca}^{2+}$  signal such as increased  $\text{Ca}^{2+}$  influx by a direct effect on voltage-gated  $\text{Ca}^{2+}$  channels (Ammala *et al.*, 1993; Carabelli *et al.*, 2003; Calejo *et al.*, 2014),  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores (Sedej *et al.*, 2005) or by sensitizing certain vesicle pools to  $\text{Ca}^{2+}$  (Wan *et al.*, 2004; Skelin & Rupnik, 2011).

The role of Epac in the regulation of exocytosis has become increasingly attended to in search of the missing links of cAMP-dependent exocytosis. Epac is a cAMP activated guanine nucleotide exchange factor (cAMP-GEF) and its two isoforms, Epac1 and Epac2, are present in most tissues at various levels (Schmidt *et al.*, 2013). GEFs activate small GTPases by catalysing the release of guanosine diphosphate (GDP) and binding of guanosine triphosphate (GTP). Epac can activate several small GTPases of the Ras superfamily (Schmidt *et al.*, 2013) and the activation of small GTPases of the Ras and Rab family has been shown to be involved in several exocytotic processes (G. G. Holz *et al.*, 2006).

Before the discovery of Epac, a PKA-independent role for potentiation of Ca<sup>2+</sup>-stimulated exocytosis was shown in  $\beta$ -cells (Renstrom *et al.*, 1997). It has later been shown that Epac facilitates the priming of vesicles (Eliasson *et al.*, 2003) but that it also regulates rapid exocytosis of GABA containing synaptic like vesicles that are also secreted by the  $\beta$ -cell (Hatakeyama *et al.*, 2007). Moreover, Epac2 has also been shown to be important for cAMP-induced potentiation of first phase insulin secretion as part of this potentiation was diminished in Rap1 knockdown cells (Shibasaki *et al.*, 2007). The involvement of Epac1 has previously been reported in non-endocrine cell exocytosis. In the exocrine parotid acinar cells, amylase release was shown to be partially mediated by PKA-independent pathways involving Epac1. Furthermore, Epac1 was shown to be present on vesicle membranes and on the plasma membrane (Shimomura *et al.*, 2004). In the sperm acrosomal reaction, Epac1 is crucial for assembly of the fusion machinery and mobilisation of Ca<sup>2+</sup> from intracellular stores, ultimately leading to acrosome exocytosis (Branham *et al.*, 2009; Ruete *et al.*, 2014).

As the activation of different GPCRs can result in different physiological outcomes the importance of GPCR-mediated local regulation of cAMP is evident. cAMP signalling has been shown to be compartmentalised and thus concentrated to specific regions within cells. Compartmentalised signaling can be obtained by differential distribution of diverse ACs or PDEs allowing establishment of cAMP gradients (Cooper, 2003; Houslay & Adams, 2003). In this way, different cAMP

concentrations achieved at distinct cellular locations can differentially regulate specific intracellular actions.

# Aims

The overall aim of this thesis was to investigate the mechanisms and mediators regulating white adipocyte exocytosis and short-term adiponectin secretion.

The specific aims were to:

- 1) Investigate the role of the intracellular mediators  $\text{Ca}^{2+}$ , cAMP and ATP in white adipocyte exocytosis and short-term adiponectin secretion.
- 2) Explore the temperature/energy-dependency of white adipocyte exocytosis and adiponectin secretion.
- 3) Determine the physiological regulation of adiponectin exocytosis and how this regulation might be disturbed in obesity and type 2 diabetes.





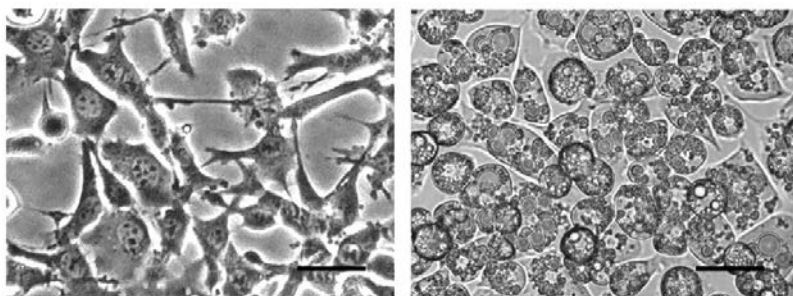
# Methodology

The reader is referred to paper I-III in this thesis for detailed protocols for the methods described in this section.

## 3T3-L1 adipocytes

3T3-L1 adipocytes are an established *in vitro* adipocyte model and widely used in studies of adipocyte differentiation, gene expression and adipokine secretion (Scherer *et al.*, 1995; Fasshauer *et al.*, 2001; Rosen & MacDougald, 2006). The 3T3-L1 preadipocyte cell line is of murine origin and was isolated and cloned 1974 (Green & Meuth, 1974). 3T3-L1 preadipocytes can be differentiated to mature adipocytes by the addition of a differentiation mix consisting of insulin, dexamethasone (dexa) and 3-isobutyl-1-methylxanthine (IBMX). The differentiation mix induces several adipogenic processes (Rosen & MacDougald, 2006; Petersen *et al.*, 2008) including activation of transcriptional activator CREB which induces expression of PPAR $\gamma$ , C/EBP $\alpha$  and C/EBP $\beta$ . The precise mechanisms of adipocyte differentiation are still being elucidated. Our protocol is based on (Kohn *et al.*, 1996) and summarized below.

American Type Culture Collection (ATCC) 3T3-L1 adipocytes were cultured in tissue culture flasks and incubated at 37°C and 5% CO<sub>2</sub> together with high-glucose DMEM containing 1% penicillin-streptomycin and 10% newborn calf serum. At 80% confluency, cells were split from flasks and seeded to plastic or glass-bottom 35 mm dishes or multi-well plates. Cells were then grown to high confluency before the differentiation mix was added (day 0; Fig. 4). The differentiation mix consisted of 1  $\mu$ M dexa, 0.5 mM IBMX and 850 nM insulin in DMEM. After 48 hours the differentiation medium was replaced with medium containing only insulin (day 2). The medium was thereafter replaced every second day, and experiments were conducted between day 8-10 after start of differentiation (Fig. 4).



**Fig. 4** 3T3-L1 preadipocytes in the fibroblast-like state before differentiation (left) and differentiated mature adipocytes (right). Scale bar = 50  $\mu$ M

## Isolation of primary adipocytes

Human adipocytes were isolated from subcutaneous tissue biopsies (paper I). Mouse inguinal adipose tissue was isolated from C57BL/6J wild type mice (paper III). Briefly, adipose tissue was minced down and degraded using collagenase. The floating adipocytes were then washed and poured through a nylon mesh to isolate adipocytes in a 50 ml tube. The washing step was repeated twice. Human cells were incubated overnight before adiponectin secretion measurements were performed (paper I). Mouse adipocytes were either directly used for adipokine secretion measurements or frozen at  $-80^{\circ}\text{C}$  for gene expression analysis.

## Adipokine secretion in 3T3-L1 adipocytes

3T3-L1 adipocytes seeded and differentiated on multi-well plates were washed and preincubated for 30 min with glucose-free extracellular solution (EC), in the presence or absence of specified stimulatory/inhibitory agents. The preincubation solution was then replaced with EC containing 5 mM glucose together with test substances. Cells were incubated for 30 min with gentle shaking at  $32^{\circ}\text{C}$  or at indicated temperature. At the end of the incubation, the supernatant was collected and centrifuged (2000 rpm, 5 min) to remove non-adherent cells. The adherent cells were washed and lysed in the presence of a protease inhibitor. All samples were aliquoted and stored at  $-80^{\circ}\text{C}$  for further analysis. Secreted adipokines were measured by ELISA and protein content with Pierce BCA protein kit or Bradford protein assay, according to manufacturer's instructions.

## Patch-clamp

The patch-clamp technique is a powerful method for studying the electrophysiological properties of live single cells. The method was developed by Erwin Neher and Bert Sakmann for which they won the Nobel Prize in Physiology or Medicine in 1991 (Neher *et al.*, 1978).

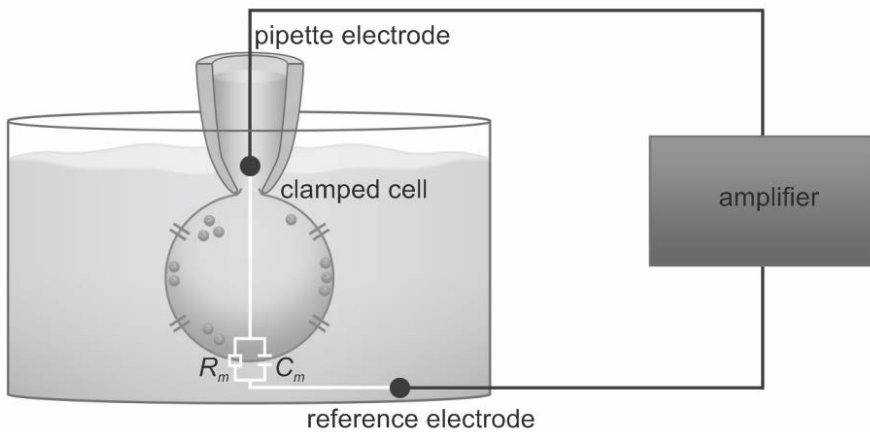
The patch-clamp method can be used to measure the electrical properties over a small membrane patch or over the entire cell membrane. A fire-polished glass pipette is backfilled with an intracellular solution and mounted on an electrode connected to an amplifier. A cell attached to the bottom of a dish is coupled to the electrical circuit by formation of tight seal between the glass pipette and the cell. The seal has a high electrical resistance (typically  $>1\text{ G}\Omega$ ) and is called a “giga-seal”. The cell dish is continuously perfused with an extracellular solution and a constant temperature is maintained by a feedback temperature controller. A reference electrode is situated in the cell bath to complete the electrical circuit.

To obtain an electrical circuit and enable measurements of electrical properties over the entire cell membrane it is necessary to gain access to the cell cytosol. This can be done in two ways. In the *perforated-patch whole-cell configuration*, access to the cytosol is gained by using a membrane pore-forming antibiotic included in the pipette solution. The pores formed are only large enough to permit the flow of monovalent ions and the intracellular environment remains rather intact. To gain full access to the cell interior, gentle suction can be applied to rupture the enclosed membrane to obtain the *standard whole-cell configuration* (Hamill *et al.*, 1981). This configuration has the advantage of permitting control of the intracellular milieu by wash-in of the pipette solution including substances of interest. The standard whole-cell configuration allows application of ions, molecules such as cAMP and ATP, membrane impermeable agonists/antagonist, antibodies etc. to the inside of the cell.

The electrical recordings can be measured in the *current-clamp mode* or the *voltage-clamp mode*. In the *current-clamp mode*, a current is injected by the amplifier through the microelectrode and the membrane potential is measured. In the *voltage-clamp mode* cells are instead clamped at a specific holding potential to measure the

currents. To clamp the membrane potential, any activated currents in this mode are compensated by a negative feedback mechanism; the amplifier injects currents of the opposite sign of that registered from the cell. The voltage-clamp mode further allows recordings of exocytotic events measured as increases in membrane capacitance, the type of electrophysiological recordings applied in this thesis.

The patch-clamp recordings in this thesis were conducted with a HEKA amplifier (EPC9) and PatchMaster software.



**Fig. 5 A simplified representation of a patch-clamp circuit in the standard whole-cell configuration.** Access to the cytosol is gained by disruption of the membrane enclosed by the pipette to allow wash in of the pipette solution. An electrical circuit is completed by the pipette electrode and the reference (bath) electrode. In the voltage-clamp mode the cell is clamped at a specific membrane potential and the sum of membrane currents or capacitance ( $C_m$ ) can be measured.  $R_m$  represents the membrane resistance.

### **Capacitance measurements**

Patch-clamp capacitance measurements allow online registrations of exocytosis measured as the increase in plasma membrane area that arises when secretory vesicles fuses with it (Neher & Marty, 1982). All biological membranes act as capacitors and the specific capacitance of biological membranes is estimated to be  $10\text{fF}/\mu\text{m}^2$  (Hille 1992).

Capacitance ( $C_m$ ; Farad) is measured by the equation:  $C_m = \frac{A \cdot \epsilon}{d}$

where  $A$  denotes the membrane area,  $\epsilon$  is the specific membrane capacitance and  $d$  is the membrane thickness. The thickness of the phospholipid bilayer ( $d$ ) and  $\epsilon$  are constant. Thus, the capacitance is proportional to the membrane area. In exocytosis, the membrane of the secretory vesicle is fused with the plasma membrane of the cell to enable release of the vesicle content. The fusion of the vesicle membrane with the plasma membrane results in an increase in the membrane surface area and can thus be measured as an increase in capacitance.

The most commonly used method to measure alterations of membrane capacitance employs a sine wave voltage stimulus. The amplitude and phase of the resulting sinusoidal current can be resolved using a phase-sensitive detector which is implemented in the software or hardware using a lock-in amplifier.

In this thesis, exocytotic events were instead registered as repetitive Auto C-slow compensations using the CapTrack function of PatchMaster. This procedure applies short trains of square-wave pulses, averages the resulting currents and fits an exponential to deduce the compensation values needed to cancel the current. The number of currents as well as their amplitude can be varied. Here we have applied a train of 10 pulses with an amplitude of 5 mV.

## Fluorescence microscopy

Fluorescence is the outcome of a process where light of a definite wavelength is absorbed by specific molecules called fluorophores or fluorescent dyes. The absorption is followed by emission of light at longer wavelengths.

In this thesis alterations in intracellular concentrations of  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) were measured by dual-wavelength ratiometric  $\text{Ca}^{2+}$  imaging. Cells were loaded with the membrane-permeable fluorescent  $\text{Ca}^{2+}$  dye fura-2 AM (2  $\mu\text{M}$ ) together with 0.02% (wt/vol) Pluronic to facilitate the solubilization of fura-2 AM. The excitation wavelength for fura-2 differs depending on if the dye is bound to  $\text{Ca}^{2+}$  or not. The  $\text{Ca}^{2+}$  unbound form of fura-2 has its excitation maximum at 380 nm and the  $\text{Ca}^{2+}$  bound form at 340 nm. The emitted light is collected at 510 nm. With elevated  $[\text{Ca}^{2+}]_i$  the fluorescence intensity increases at 340 nm while fluorescence decreases at

380 nm for the unbound form. This thus gives a ratio between the  $\text{Ca}^{2+}$ -bound and unbound state of fura-2. Ratiometric measurements have the advantage of eliminating inter-experiment variations in dye loading and declining fluorescence due to photobleaching. We employed a Lambda DG-4 illumination system (Sutter Instrument Company, USA) combined with a Nikon Diaphot 300 inverted microscope (Nikon, Japan) and a QuantEM 512SC CCD camera (Photometrics, USA). Metafluor software was used. The  $[\text{Ca}^{2+}]_i$  was calculated using the equation 5 of (Grynkiewicz *et al.*, 1985) and a  $K_d$  of 224 nM.

Total internal reflection (TIRF) microscopy is an advanced imaging technique that allows visual investigations of events only in the plasma membrane near region (approx. 100-200 nm beneath the plasma membrane; Burchfield *et al.*, 2010). Here we have used a Zeiss TIRF 3 microscope equipped with a Plan-Apochromat objective (100x/1.46 Oil) and a back-illuminated EMCCD digital camera (Evolve 512 Delta, Photometrics).

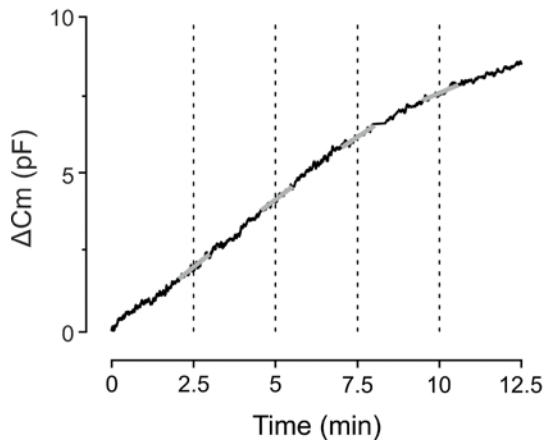
## Quantitative RT-PCR

Real-Time quantitative reverse transcription polymerase chain reaction (Real-Time qRT-PCR) allows the analysis of nucleic acids in a variety of biological applications such as gene expression studies. The technique requires RNA or mRNA to be transcribed to complementary DNA (cDNA) using an enzymatic reaction by reverse transcriptase. In brief, the RNA of interest is chemically extracted and the purity is validated spectrophotometrically by measuring absorbance at 260 and 280 nm. The RNA is then transcribed to its complementary DNA (cDNA) which is the starting material for amplification analysis by thermo cyclers. Amplifications of specific cDNA sequences (amplicon) can be detected due to the presence of fluorescently labelled primers or probes. We have used the fluorescent probe SYBR green which interchelates into newly synthesised double-stranded DNA. The amount of produced amplicon is proportionally correlated with fluorescence produced over time and detected by the cycler. The threshold cycle (Ct value) at which the amplicon is detected during the exponential phase of amplification is inversely proportional to the relative expression of the target gene. Relative gene expression can be determined by

normalising the  $C_t$  value of the gene of interest to the  $C_t$  value of a reference gene known to be stably expressed in the samples.

## Data analysis

The exocytotic rates ( $\Delta C_m/\Delta t$ ; fF/s) were measured by application of linear fits to the capacitance trace at the different indicated time points (Fig. 6). The total capacitance increase reflecting total amount of exocytosis was measured from the initial capacitance value until the end of capacitance increase (plateau) where no further exocytosis was observed, or at indicated time point. Average  $\Delta C_m/\Delta t$  or the total capacitance increase was compared for different series of experiments and statistical significance was calculated using unpaired Student's *t*-test. Free  $[Ca^{2+}]$  for the pipette solution was calculated using MAXCHELATOR downloads (<http://www.stanford.edu/patton/maxc.html>).



**Fig. 6** Example trace with the difference in capacitance ( $\Delta C_m$ ) plotted against time. The rate of exocytosis ( $\Delta C_m/\Delta t$ ) was measured at different time intervals (dotted lines) by application of linear fits (grey) to the indicated data points in the capacitance trace (black).

All data are presented as mean values  $\pm$  SEM. Statistical significance between means was calculated using OriginPro (OriginLab Corporation, USA) and Student's *t*-test, unpaired or paired as appropriate. One-way ANOVA was applied where more than one group is compared to the same control group.





# Results and discussion

## **PKA-independent cAMP stimulation of white adipocyte exocytosis and adipokine secretion: modulations by Ca<sup>2+</sup> and ATP (Paper I)**

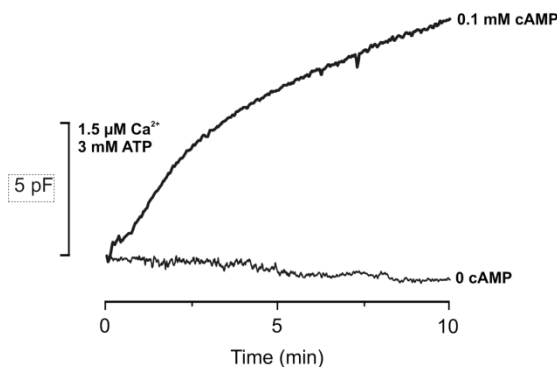
The first paper published on adiponectin 20 years ago suggested the existence of a pool of secretory vesicles containing adiponectin (Scherer *et al.*, 1995). Several investigations have since then been carried out with the aim to elucidate the regulation of adiponectin secretion (Bogan & Lodish, 1999; Halleux *et al.*, 2001; Delporte *et al.*, 2002; Motoshima *et al.*, 2002; Pereira & Draznin, 2005; Cong *et al.*, 2007; Blümer *et al.*, 2008; Xie *et al.*, 2008). However, the majority of those studies have investigated adiponectin secretion during longer time-periods of hours or days. Thus, the mechanisms controlling adiponectin exocytosis are inadequately investigated. Since the introduction of membrane capacitance measurements three decades ago (Neher & Marty, 1982) single cell investigations with high temporal resolution has led to great strides in the research field of regulated exocytosis. However, there are only a handful of studies where the patch-clamp technique has been applied to white adipocytes (Ramirez-Ponce *et al.*, 1990; Ramirez-Ponce *et al.*, 1991; Ramirez-Ponce *et al.*, 1996; Lee & Pappone, 1997; Ramirez-Ponce *et al.*, 1998; Sukumar *et al.*, 2012; Bentley *et al.*, 2014), perhaps due to the methodological complexity combined with the ungainly shape and size of adipocytes. The intracellular messengers Ca<sup>2+</sup>, cAMP and ATP are involved in control of regulated exocytosis in a number of known neuroendocrine and endocrine cell types (Burgoyne & Morgan, 2003). In this study we define the mechanisms controlling exocytosis and adipokine secretion in white adipocytes using a combination of membrane capacitance recordings and biochemical measurements of released adipokines.

## **The role of cAMP in white adipocyte exocytosis and adipokine secretion**

### **3T3-L1 adipocyte exocytosis is stimulated by cAMP**

Being an endocrine cell, white adipocyte exocytosis was hypothesised to be regulated similarly to how exocytosis is regulated in other (neuro)endocrine cell types. To investigate the mechanisms and mediators involved in stimulation of adipocyte exocytosis, we infused cells with a pipette solution containing a high concentration of  $\text{Ca}^{2+}$  (9 mM  $\text{CaCl}_2$  buffered with 10 mM EGTA resulting in a free  $[\text{Ca}^{2+}]$  of  $\sim 1.5 \mu\text{M}$ ) together with 0.1 mM cAMP and 3 mM ATP. This composition of intracellular solution has previously been shown to potently stimulate exocytosis in the pancreatic  $\beta$ -cell (Barg *et al.*, 2002; Olofsson *et al.*, 2009) and in chromaffin cells (Neher & Augustine, 1992). As shown in Fig. 7 the solution stimulated exocytosis and the exocytotic rate ( $\Delta C_m/\Delta t$ ) measured during the second minute after entering the whole-cell configuration averaged  $\sim 25 \text{ fF/s}$ . It is interesting that similar rates of exocytosis have been reported in  $\beta$ -cells ( $\sim 30\text{--}40 \text{ fF/s}$ ) (Proks *et al.*, 1996; Barg *et al.*, 2002; Olofsson *et al.*, 2009) despite the  $\sim 10$ -fold larger size of adipocytes. This may indicate that the large size of adipocytes is more related to their role as lipid storing cells than to their endocrine function.

We next infused cells with the same solution lacking cAMP. Interestingly, exocytosis could not be triggered when cAMP was omitted from the pipette solution although  $1.5 \mu\text{M Ca}^{2+}$  was still present (Fig. 7).



**Fig. 7 Adipocyte exocytosis is triggered by cAMP.** Representative capacitance traces of cells infused with  $1.5 \mu\text{M}$  free  $\text{Ca}^{2+}$  and 3 mM ATP, in the presence or absence of 0.1 mM cAMP.

To investigate if cAMP could stimulate exocytosis under  $\text{Ca}^{2+}$ -free conditions we included cAMP together with the fast  $\text{Ca}^{2+}$  chelator BAPTA (10 mM) in the pipette solution (ATP still present). Indeed, cAMP alone was capable of triggering exocytosis, although at a ~40% lower rate compared to in the presence of  $\text{Ca}^{2+}$  (measured during the 2<sup>nd</sup> minute of infusion).

We thus concluded that adipocyte exocytosis can be triggered by cAMP in a  $\text{Ca}^{2+}$ -independent manner. The observation that cAMP can stimulate exocytosis under  $\text{Ca}^{2+}$ -free conditions and that  $\text{Ca}^{2+}$  alone was not sufficient to trigger exocytosis show that white adipocyte exocytosis is regulated in a way quite different from how endocrine cell exocytosis is usually controlled.

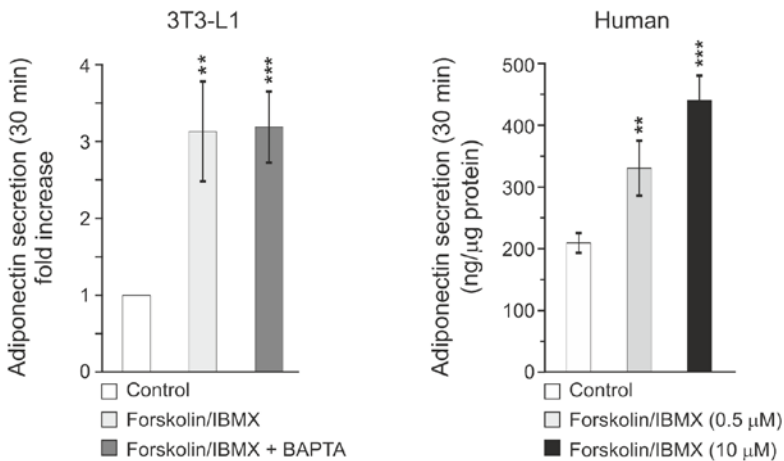
### **cAMP-stimulated exocytosis correlates with adiponectin secretion in 3T3-L1 and primary human subcutaneous adipocytes**

Capacitance measurements provide mechanistic information of vesicle fusion with the plasma membrane but give no information about the secretory product. We thus investigated if elevated cAMP levels were able to stimulate the secretion of adiponectin, adipisin, apelin, leptin or resistin, all adipocyte peptide/protein hormones suggested to be released by regulated exocytosis (Bogan & Lodish, 1999; Bradley & Cheatham, 1999; Roh *et al.*, 2000; Zhong *et al.*, 2002; Xie *et al.*, 2008; Ye *et al.*, 2010). Adipokine levels were analysed in medium from adipocytes incubated for 30 min in the presence of cAMP-elevating agents. The incubation time was short enough for correlations with capacitance measurements, yet long enough to allow for measurable adipokine levels. We used the AC activator forskolin in combination with the PDE inhibitor 3-Isobutyl-1-methylxanthine (forsk-IBMX) to elevate intracellular cAMP levels. An elevation of cAMP stimulated adiponectin secretion >3-fold compared to control (the same solution lacking forsk-IBMX). Forsk-IBMX did not stimulate the release of Adipsin. Apelin and leptin was detected at very low levels. Resistin was secreted upon stimulation with forsk-IBMX but at a 17-fold lower level than adiponectin. We thus focused our secretion studies on adiponectin.

Next, we investigated if adiponectin secretion, similar to adipocyte exocytosis, could be stimulated by elevations of cytoplasmic cAMP under  $\text{Ca}^{2+}$ -free conditions.

For this purpose, adipocytes were pre-treated with the membrane-permeable  $\text{Ca}^{2+}$  chelator BAPTA-AM (30 min) and thereafter exposed to forsk-IBMX. Measurements of intracellular cAMP levels confirmed that the cAMP-elevating properties of forsk-IBMX were unaltered under  $\text{Ca}^{2+}$ -free conditions. In agreement with the capacitance measurements, adiponectin secretion could indeed be stimulated in the absence of  $\text{Ca}^{2+}$  and the secretory response was equally potent as that measured in cells not exposed to BAPTA (Fig. 8).

To verify the physiological importance of our findings we investigated if adiponectin secretion could be stimulated by cAMP elevation in human subcutaneous adipocytes. Forsk-IBMX stimulated adiponectin secretion >2-fold compared to untreated cells (Fig 8).

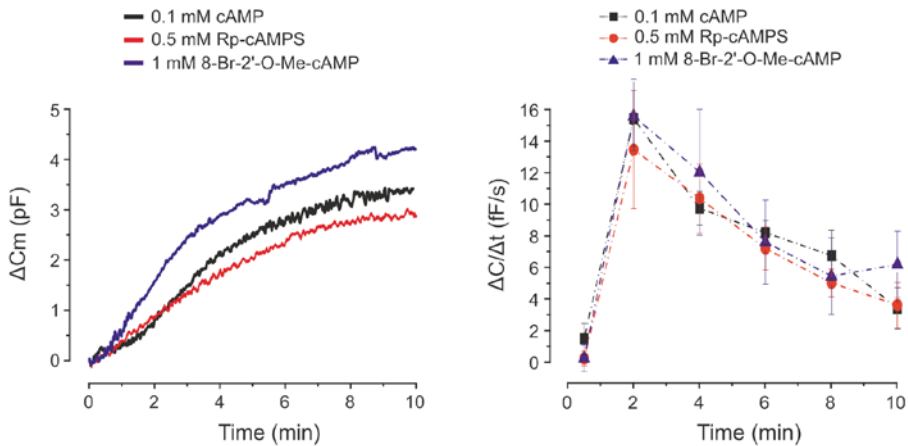


**Fig. 8 Elevations of intracellular cAMP stimulate adiponectin secretion.** 3T3-L1 adipocytes (left) were treated with forsk-IBMX under normal or  $\text{Ca}^{2+}$ -free conditions. Adiponectin secretion was also stimulated at lower concentrations of forsk-IBMX in human adipocytes. Data are mean values  $\pm$  SEM of 14 experiments in 3T3-L1 adipocytes and isolated adipocytes from 4 patients. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

### **cAMP stimulates white adipocyte exocytosis and adiponectin secretion via activation of Epac**

The effect of cAMP in regulated exocytosis has in other cell types been shown to usually be due to activation of PKA, sometimes with contribution of activated Epac (Seino & Shibasaki, 2005). To elucidate the pathway involved in regulation of

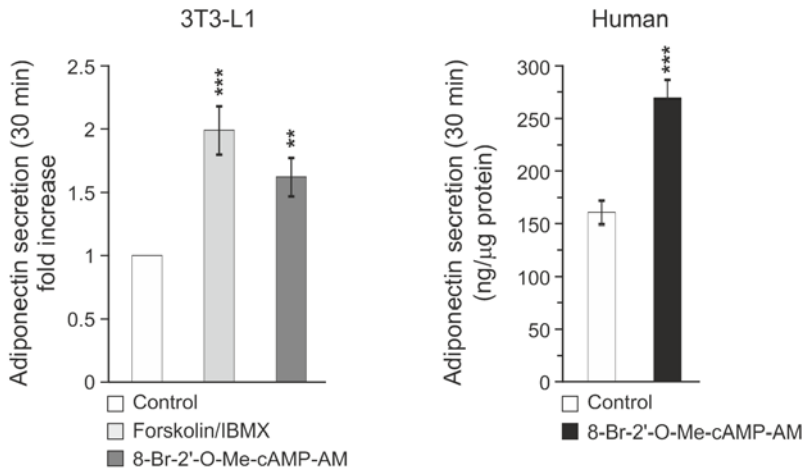
adipocyte exocytosis, we included the specific PKA-inhibitor Rp-8-Br-cAMPS together with cAMP in the patch-pipette solution. Somewhat to our surprise, cAMP-stimulated exocytosis was completely unaffected by PKA inhibition and  $\Delta C_m/\Delta t$  was equal to that in experiments where exocytosis was stimulated by cAMP (Fig. 9). To further elucidate the mechanisms of PKA-independent exocytosis, we investigated if exocytosis could be triggered by activation of Epac in the absence of cAMP. For this purpose we infused cells with the same  $\text{Ca}^{2+}$ -free pipette solution including the specific Epac activator 8-Br-2'-O-Me-cAMP. Epac activation stimulated exocytosis as potently as cAMP and  $\Delta C_m/\Delta t$  was similar to the rate measured upon cAMP-triggered exocytosis at all time points (Fig. 9). Taken together, our results indicate that white adipocyte exocytosis is triggered by cAMP-dependent activation of Epac.



**Fig. 9 cAMP stimulates exocytosis via activation of Epac.** Left: Example capacitance traces of cells infused with cAMP (black), cAMP in combination with the PKA inhibitor Rp-cAMPS (red) or with the Epac agonist 8-Br-2'-O-Me-cAMP (blue). Right: The average rates of exocytosis ( $\Delta C/\Delta t$ ) at different time points. Data are mean values  $\pm$  SEM of 7-9 recordings.

We next explored the cAMP signalling pathway involved in short-term adiponectin secretion. The involvement of PKA was investigated in adipocytes pretreated with membrane permeable Rp-8-Br-cAMPS (to inhibit PKA) and subsequently exposed to forsk-IBMX (in the continued presence of the PKA inhibitor). Forsk-IBMX remained capable of stimulating adiponectin secretion in the presence of the PKA inhibitor, of a magnitude similar to that measured in cells exposed to forsk-IBMX alone. We further investigated if the membrane-permeable version of the Epac agonist

8-Br-2'-O-Me-cAMP-AM stimulated adiponectin secretion in 3T3-L1 and human primary subcutaneous adipocytes. In agreement with the Epac-dependent capacitance increases recorded in 3T3-L1 adipocytes, adiponectin secretion was stimulated by the Epac agonist in both 3T3-L1 and human adipocytes (Fig. 10). Our secretion data thus strongly indicate that the capacitance changes recorded in 3T3-L1 adipocytes reflect secretion of adiponectin-containing vesicles. Adiponectin release measurements further show a similar regulation of adiponectin exocytosis in cultured and primary subcutaneous human adipocytes.



**Fig. 10 Adiponectin secretion is stimulated via Epac activation.** The membrane-permeable Epac agonist 8-Br-2'-O-Me-cAMP-AM stimulated adiponectin secretion during 30 min incubations in both 3T3-L1 (left) and human (right) adipocytes. Data are mean values  $\pm$  SEM of 11 (3T3-L1 adipocytes) and human adipocytes from three patients. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

## ***The role of Ca<sup>2+</sup> and ATP in white adipocyte exocytosis***

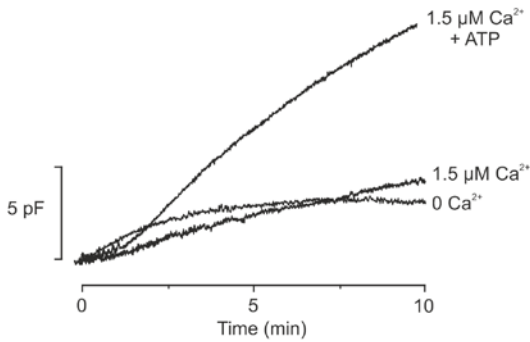
### **A combination of Ca<sup>2+</sup> and ATP augments cAMP-triggered adiponectin exocytosis**

To in detail study the role of Ca<sup>2+</sup> in cAMP-stimulated exocytosis, we investigated the exocytotic response in cells infused with different concentrations of Ca<sup>2+</sup> corresponding to 1.5, 0.7 and 0.25 μM free Ca<sup>2+</sup>. cAMP and ATP were included in the pipette solution. As described above, the rate of cAMP-stimulated exocytosis in the complete absence of Ca<sup>2+</sup> was ~40% lower compared to when 1.5 μM Ca<sup>2+</sup> was included in the pipette solution. An interesting observation was that the cAMP-stimulated capacitance increase reached a steady-state plateau after ~8 minutes where after no further exocytosis was observed. The plateau value indicates that no more vesicles are available for release and that cAMP alone is insufficient to sustain the exocytotic response during protracted time-periods. In the presence of Ca<sup>2+</sup> exocytosis did not plateau but continued for >10 min and throughout the duration of the experiments (some lasting as long as 20 min). The lack of capacitance plateaus upon infusion of a Ca<sup>2+</sup>-containing solution indicates the presence of a Ca<sup>2+</sup>-dependent continuous replenishment of vesicles to the pool stimulated for release by cAMP.

Infusion of cells with 0.7 μM Ca<sup>2+</sup> also stimulated exocytosis, although at a rate that tended to be decreased compared to 1.5 μM Ca<sup>2+</sup> (P=0.07). Exocytosis was continuous also in the presence of this lower [Ca<sup>2+</sup>] as shown by the absence of an exocytotic plateau in experiments lasting >10 min. Interestingly, a lower [Ca<sup>2+</sup>] of 0.25 μM was not sufficient to potentiate exocytosis triggered by cAMP and plateau values were again reached, signifying the depletion of readily releasable vesicles. The rate of exocytosis using 0.25 μM Ca<sup>2+</sup> was equal to  $\Delta C_m/\Delta t$  in the complete absence of Ca<sup>2+</sup>.

In order to investigate the role of ATP in cAMP-stimulated exocytosis we infused cells with 1.5 μM Ca<sup>2+</sup> and cAMP in the absence of ATP. Interestingly, the augmenting effect of Ca<sup>2+</sup> at early time points ( $\leq 6$  min) was abolished upon ATP

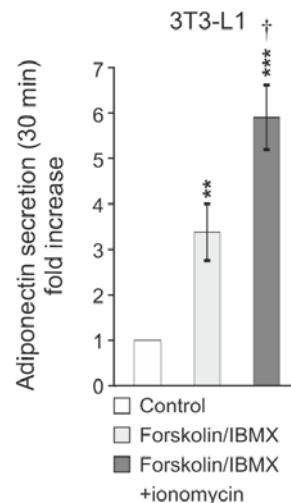
depletion (Fig. 11). However, vesicle replenishment at later time points was unaffected.



**Fig. 11** Typical capacitance traces of cells dialyzed with cAMP alone, cAMP together with 1.5  $\mu\text{M}$   $\text{Ca}^{2+}$  or cAMP in combination with 1.5  $\mu\text{M}$   $\text{Ca}^{2+}$  and 3 mM ATP, as indicated.

We next investigated the role of  $\text{Ca}^{2+}$  in cAMP-stimulated adiponectin secretion. 3T3-L1 adipocytes were incubated with forsk-IBMX alone or in combination with the  $\text{Ca}^{2+}$  ionophore ionomycin (1  $\mu\text{M}$ ). Again, forsk-IBMX stimulated adiponectin secretion >3-fold compared to control cells (Fig. 12). The  $\text{Ca}^{2+}$  elevation induced by ionomycin augmented forsk-IBMX-stimulated adiponectin secretion 2-fold over that produced by forsk-IBMX alone. Thus, the  $\text{Ca}^{2+}$ -potentiating effect observed in the secretion measurements is in agreement with the augmenting effect of  $\text{Ca}^{2+}$  in the capacitance recordings.

Our results indicate that  $\text{Ca}^{2+}$  and ATP are involved in potentiation of cAMP-stimulated adiponectin vesicle release. The augmenting effect involves a direct effect on exocytosis as shown by a  $\text{Ca}^{2+}$ - and ATP-dependent increase of the exocytotic rate at early time-points (already during the 2<sup>nd</sup> minute after start of experiment).



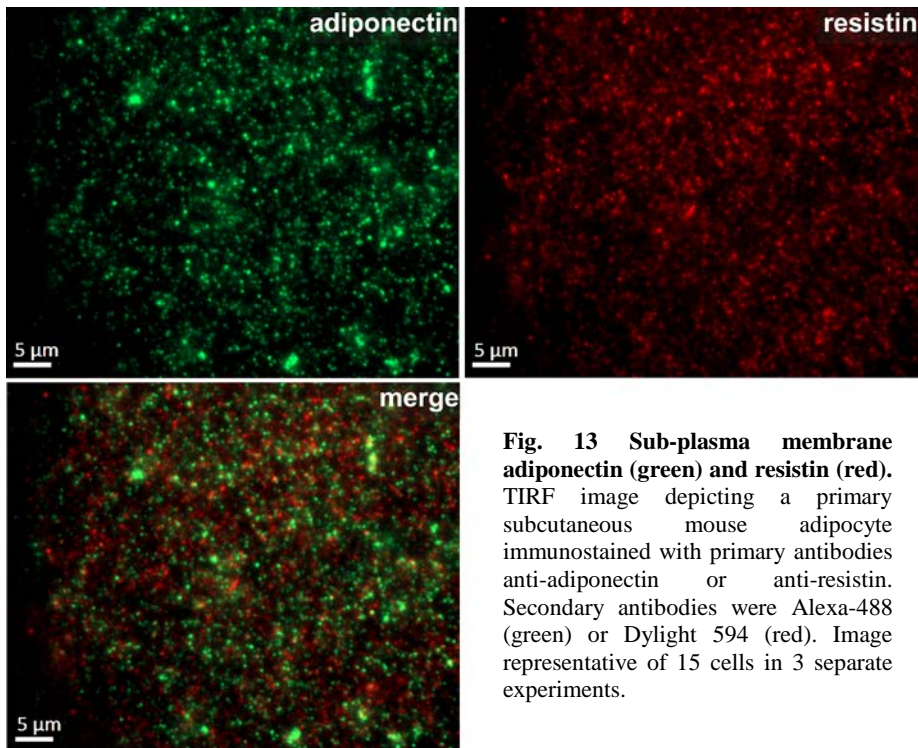
**Fig. 12**  $\text{Ca}^{2+}$ -elevation potentiates adiponectin secretion stimulated by cAMP. 3T3-L1 adipocytes were treated with forsk-IBMX in the absence or presence of ionomycin. Data are mean values  $\pm$  SEM of 8 experiments. \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .



Ca<sup>2+</sup> further mediates the recruitment of new releasable vesicles from a reserve pool in an ATP-independent manner as demonstrated by the continuous exocytosis in cells infused with a Ca<sup>2+</sup>-containing solution lacking ATP.

### ***Adiponectin vesicles appear as plasma membrane-located punctuate structures (unpublished)***

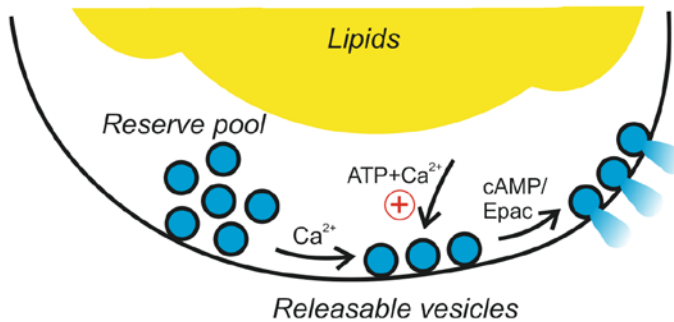
The ultrastructural characteristics as well as the intracellular location of adiponectin-containing vesicles remain inadequately investigated. However, own unpublished results in primary mouse and rat subcutaneous adipocytes immunostained for adiponectin and resistin studied with TIRF microscopy shows the abundant existence of distinct adiponectin- or resistin-containing vesicles in the sub-plasma membrane region (Fig. 13). Since adiponectin and resistin are both released by cAMP elevation (described above) it is feasible that the adipokines would be co-released from the same vesicle. However, our TIRF data suggests that adiponectin and resistin are accommodated in separate vesicle populations.



**Fig. 13 Sub-plasma membrane adiponectin (green) and resistin (red).** TIRF image depicting a primary subcutaneous mouse adipocyte immunostained with primary antibodies anti-adiponectin or anti-resistin. Secondary antibodies were Alexa-488 (green) or Dylight 594 (red). Image representative of 15 cells in 3 separate experiments.

Moreover, recent studies from the laboratory of Dr Weiping Han demonstrate punctate vesicular structures in the TIRF zone in 3T3-L1 adipocytes transfected with adiponectin-venus (Lim *et al.*, 2015) or leptin-venus (Y. Wang *et al.*, 2014). Thus, it appears as if white adipocytes contain several populations of plasma membrane-associated adipokine-containing vesicles.

## Conclusions paper I



**Fig. 14 Model of regulation of white adipocyte adiponectin exocytosis. See text for details.**

We suggest that white adipocyte adiponectin exocytosis is regulated as described in the model in Fig. 14. A pool of readily releasable adiponectin containing vesicles is secreted upon cAMP stimulation via activation of Epac, in a PKA-independent manner. The readily releasable pool is refilled by new vesicles residing in a reserve pool in a Ca<sup>2+</sup>-dependent manner. Thus, in the absence of Ca<sup>2+</sup> the readily releasable pool is depleted and exocytosis can not continue during longer time-periods (seen as a plateau in our capacitance measurements). Further, Ca<sup>2+</sup> in combination with ATP potentiates the cAMP-triggered adiponectin release at early time points (2-6 min). It is interesting that adiponectin exocytosis in the white adipocyte, in contrast to the Ca<sup>2+</sup>-stimulated exocytosis in classical (neuro)endocrine cell types (Burgoyne & Morgan, 2003), is triggered by cAMP. Pancreatic  $\beta$ -cell exocytosis can be triggered by Ca<sup>2+</sup> in the absence of cAMP (at a lower rate than in combination with the cyclic nucleotide) but cAMP alone is incapable of triggering  $\beta$ -cell exocytosis under intracellular Ca<sup>2+</sup>-free conditions (Renstrom *et al.*, 1997; Eliasson *et al.*, 2003).

The regulation of exocytosis in the adipocyte instead shares some similarities with how exocytosis is controlled in several exocrine cell types (Seino & Shibasaki, 2005; Szaszak *et al.*, 2008). Capacitance measurements in pancreatic acinar cells have revealed that cAMP can trigger exocytosis in these cells even under  $\text{Ca}^{2+}$ -free conditions (Sato *et al.*, 2002). Under physiological conditions, the release of amylase from pancreatic acinar cells is regulated by an elevation of intracellular cAMP (Seino & Shibasaki, 2005). Furthermore, the downstream regulation of acinar exocytosis involves the activation of Epac (Sabbatini *et al.*, 2008).

A role of  $\text{Ca}^{2+}$  in adiponectin secretion has been suggested previously (Bogan & Lodish, 1999). Elevations of intracellular  $\text{Ca}^{2+}$  were shown to marginally increase adiponectin secretion measured between 30-90 minutes, whereas a significant stimulation was observed past 90 minutes. This is in reasonable agreement with our observation of the potentiating effect of  $\text{Ca}^{2+}$  on adiponectin secretion. The earlier augmenting effect of  $\text{Ca}^{2+}$  observed in our studies is likely due to the concomitant elevation of cAMP (and the inclusion of ATP in our electrophysiological recordings) making more adiponectin vesicles available for the potentiation by  $\text{Ca}^{2+}$ .

Our study is the first to characterize the regulation of adiponectin exocytosis. However, controlling mechanisms as well as adiponectin vesicle dynamics and maturation steps need to be further investigated.

## **Cooling reduces cAMP-stimulated exocytosis and adiponectin secretion at a $\text{Ca}^{2+}$ -dependent step in 3T3-L1 adipocytes (Paper II)**

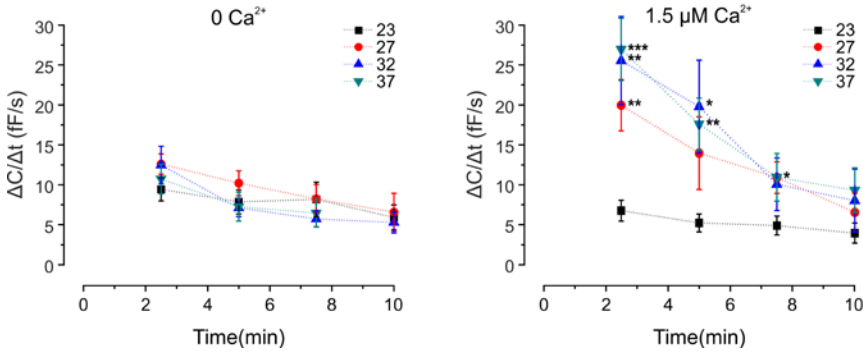
Most biological processes show rate-temperature dependency. A decrease in temperature affects several physicochemical properties and ultimately affects biological processes such as enzymatic activity and protein stability (Elias *et al.*, 2014). Therefore, regulated exocytosis in several (neuro)endocrine cell types show sensitivity to alterations in temperature (Bittner & Holz, 1992b; Thomas *et al.*, 1993; Renstrom *et al.*, 1996). In this study we explore temperature/energy-dependent steps in adiponectin exocytosis.

### ***$\text{Ca}^{2+}$ -dependent potentiation of cAMP-stimulated exocytosis is diminished by cooling***

To investigate the temperature sensitivity of adiponectin exocytosis we utilized capacitance measurements at 23°C, 27°C, 32°C and 37°C. We normally conduct the capacitance measurements at 32°C as it is very difficult to obtain a high resistance seal at higher temperatures. The chosen temperature of 32°C is therefore a compromise between an increased experimental success rate and the physiological temperature of 37 °C.

As shown in Fig. 15, infusion of 3T3-L1 adipocytes with a pipette solution containing 1.5  $\mu\text{M}$  free  $\text{Ca}^{2+}$ , 0.1 mM cAMP and 3 mM ATP at 32°C stimulated exocytosis at a rate similar to that reported in paper I.  $\Delta C_m/\Delta t$  (measured during the 2<sup>nd</sup> minute after start of infusion) was not significantly altered by a temperature increase to 37°C or by decreasing the temperature to 27°C (although the rate of exocytosis tended to be reduced at this temperature;  $P=0.07$ ). However, a decrease of temperature to 23°C significantly lowered the exocytotic rate. At 2.5 minutes,  $\Delta C_m/\Delta t$  at 23°C was decreased by ~70% compared to exocytosis at higher temperatures. We next infused cells with the same solution lacking  $\text{Ca}^{2+}$  (10 mM EGTA included; Fig. 15) The  $\text{Ca}^{2+}$ -depleted solution stimulated exocytosis at 32°C at a ~50% lower rate than in the presence of  $\text{Ca}^{2+}$ , thus again in agreement with results in Paper I. Cooling did not affect the exocytotic rate at any investigated temperature.

In fact, the rates were at all time points similar to that measured at 23°C with the Ca<sup>2+</sup>-containing pipette solution.



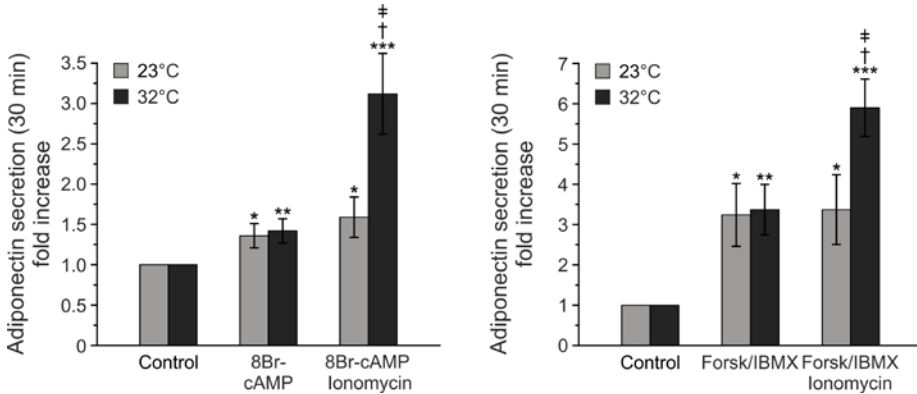
**Fig. 15 Cooling is without effect on cAMP-stimulated exocytosis but diminishes the Ca<sup>2+</sup>-dependent potentiation.** Exocytotic rates at different time points and temperatures for 3T3-L1 adipocytes infused with cAMP and ATP, in the absence or presence of Ca<sup>2+</sup>. \*P<0.5; \*\*P<0.01; \*\*\*P<0.001.

Our results indicate that cooling interferes with the exocytotic process at a step upstream of the cAMP-triggered fusion of readily releasable vesicles. This is in agreement with a study in pancreatic  $\beta$ -cells by Renström and colleagues. In this study it was shown that cooling abolishes cAMP- and ATP-potentiated insulin granule replenishment to the same extent as that caused by omitting the nucleotides from the pipette solution (Renstrom *et al.*, 1996).

### **Ca<sup>2+</sup>-potentiated adiponectin secretion is diminished by cooling**

In order to investigate the temperature sensitivity of adiponectin secretion, 3T3-L1 adipocytes were incubated for 30 min at 23°C or 32°C and exposed to the membrane permeable cAMP analogue 8-Br-cAMP alone or in combination with the Ca<sup>2+</sup> ionophore ionomycin. 8-Br-cAMP stimulated adiponectin secretion ~1.5-fold over control and this effect was equal at both investigated temperatures (Fig. 16). At 32°C the combination of 8-Br-cAMP and ionomycin doubled adiponectin secretion compared to that stimulated by 8-Br-cAMP alone. However, the ionomycin-induced potentiation was completely abolished at 23°C. The effect of temperature was similar when adiponectin secretion was instead stimulated by forsk-IBMX. Adiponectin

secretion was >3-fold stimulated by forsk-IBMX alone and ionomycin produced a >2-fold augmentation of that response at 32°C (Fig. 16). The Ca<sup>2+</sup>-dependent augmentation was again abolished at 23°C. Thus, our secretion measurements are in agreement with the electrophysiological recordings and we conclude that Ca<sup>2+</sup>-dependent potentiation of adiponectin exocytosis is a temperature-sensitive step.



**Fig. 16 Ca<sup>2+</sup>-potentiated adiponectin secretion is abolished by cooling.** Cells were stimulated with 8Br-cAMP (left) or Forsk/IBMX (right) in the presence or absence of ionomycin at 23°C or 32°C. Data are mean values ± S.E.M. of 11 experiments at each temperature in the left and 7 (23°C) and 8 (32°C) in the right histogram. \**P*<0.05; \*\**P*<0.01; \*\*\**P*<0.001 vs. control at corresponding temperature. †*P*<0.01 vs 8-Br-cAMP or forsk/IBMX alone at 32°C; ‡*P*<0.05 vs. 8-Br-cAMP + ionomycin or forsk/IBMX + ionomycin at 23°C.

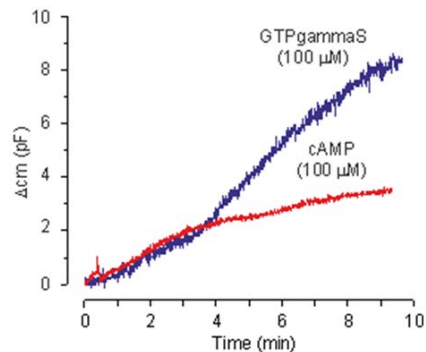
### ***The Q<sub>10</sub> values of Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent adiponectin exocytosis***

The temperature dependence of a biological process such as vesicle replenishment can be calculated by the temperature coefficient Q<sub>10</sub>. The Q<sub>10</sub> coefficient value is the factor of which the rate of a process changes when the temperature is increased by 10°C (Elias *et al.*, 2014). Q<sub>10</sub> was estimated to 3.3 and 1.4 for Ca<sup>2+</sup>-dependent and Ca<sup>2+</sup>-independent adiponectin exocytosis respectively. Processes involving enzymatic reactions normally display Q<sub>10</sub> values of ≥2 (Elias *et al.*, 2014). Thus, our results indicate that the activity of enzymatic proteins involved in the maturation of adiponectin vesicles is diminished by cooling, whereas the fusion process itself is insensitive to temperature changes.

To investigate the temperature dependence of adiponectin exocytosis in more detail we constructed Arrhenius plots to calculate the energy of activation ( $E_A$ ) for cAMP-triggered and  $Ca^{2+}$ -augmented exocytosis. The energy of activation for exocytosis in the absence of  $Ca^{2+}$  amounted to 5.7 kJ/mol whereas  $Ca^{2+}$ -dependent exocytosis required an  $E_A$  of 53 kJ/mol. The larger  $E_A$  in the presence of  $Ca^{2+}$  indicates that the maturation of vesicles, making them ready for release, is a considerably more energy requiring step than the fusion of release ready vesicles.

### ***The involvement of small G-proteins in adiponectin exocytosis (unpublished results)***

Epac is known to activate several small G-proteins involved in exocytosis (Schmidt *et al.*, 2013). In particular the small G-proteins of the Ras and Rab subfamilies are known regulators of endocrine cell secretion (G. G. Holz *et al.*, 2006). Moreover, small G-protein activity has been shown to be controlled by  $Ca^{2+}$  (reviewed in Aspenstrom, 2004) and energy in the form of ATP has been proposed to be required in order to achieve robust stimulation of

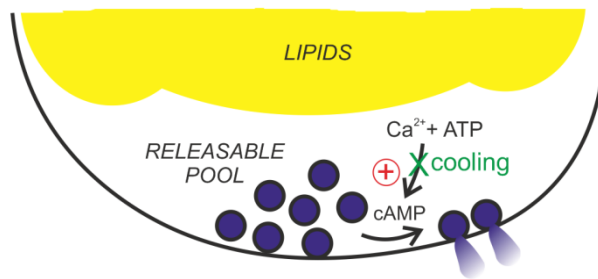


**Fig. 17** GTPgammaS stimulates adipocyte exocytosis. Examples of change in cell capacitance ( $\Delta C_m$ ) upon stimulation with GTPgammaS ( $n=10$ ) or cAMP ( $n=6$ ), as indicated.

exocytosis by GTPgammaS (non-hydrolysable form of GTP; Okano *et al.*, 1993; Proks *et al.*, Eliasson *et al.*, 1997). We thus investigated if GTPgammaS was capable of stimulating exocytosis in 3T3-L1 adipocytes. Cells were infused with a non-stimulatory cAMP-deprived intracellular solution (paper I) lacking  $Ca^{2+}$  (10 mM BAPTA included) and supplemented with 3 mM ATP and 100  $\mu$ M GTPgammaS. As shown in Fig. 17, GTPgammaS triggered exocytosis and the rate ( $\Delta C_m/\Delta t$ ) measured during the 2<sup>nd</sup> minute after pipette break-in averaged  $16\pm 4$  fF/s, comparable to that upon stimulation by dialyzing cAMP ( $\Delta C_m/\Delta t=16\pm 6$  fF/s). Interestingly, while adipocyte exocytosis triggered by cAMP in the absence of intracellular  $Ca^{2+}$  reaches

a plateau within ~10 min (signifying depletion of a readily releasable vesicle pool: paper I) GTP $\gamma$ S-stimulated exocytosis continued for an extended time-period and  $\Delta C_m/\Delta t$  at t=10 min averaged  $13\pm 4$  fF/s (n=8). Our results indicate, in analogy to what has been shown in other secretory cell types (Okano *et al.*, 1993; Proks *et al.*, 1996), an involvement of a GTP-dependent step in white adipocyte exocytosis. GTP-dependent mechanisms appear to regulate both the release process itself as well as the recruitment of new releasable vesicles. Further, GTP $\gamma$ S still triggered exocytosis when ATP was excluded from the pipette filling solution but at a lower rate ( $\Delta C_m/\Delta t = 8\pm 2$  fF/s; n=6) confirming, in analogy to findings in other secreting cell types (Okano *et al.*, 1993; Proks *et al.*, 1996; Eliasson *et al.*, 1997), the energy/ATP requirement for a robust GTP $\gamma$ S stimulation.

## Conclusions paper II



**Fig. 18 Model of the effect of cooling on white adipocyte adiponectin exocytosis. See text for details.**

The temperature-dependence of adiponectin exocytosis shown in our study clarifies regulatory mechanisms involved in vesicle recruitment/maturation needed for augmentation and long-term maintenance of adiponectin release. In agreement with the model in Fig. 18, cAMP-stimulated exocytosis of release ready adiponectin vesicles is unaffected by cooling while the Ca<sup>2+</sup>/ATP-dependent augmentation of secretion is an energy-dependent process affected by a temperature drop. Exocytosis in several secreting cell types requires ATP hydrolysis, for vesicle replenishment and for several other steps prior to the fusion process (Burgoyne & Morgan, 2003). We thus suggest that the temperature-dependence of adiponectin exocytosis mainly is



due to the need of ATP hydrolysis to generate energy for recruitment/maturation of new releasable vesicles. The effect of ATP may involve phosphorylation of exocytotic proteins.

The  $E_A$  of 53 kJ/mol for  $Ca^{2+}$ -dependent adiponectin exocytosis is lower than what has been reported for sustained insulin secretion (145 kJ/mol; Renstrom *et al.*, 1996). However, vesicle replenishment in the  $\beta$ -cell is a highly energy-dependent process due to its rather small pool of readily releasable insulin granules.

Our infusion experiments demonstrate that GTPgammaS stimulates adipocyte exocytosis and that ATP is required to achieve a potent GTPgammaS stimulation. We propose that  $Ca^{2+}$ - and ATP-dependent activation of small G-proteins is part of the signalling pathway downstream of Epac involved in stimulation of adiponectin exocytosis. Interestingly, reduced levels of small G-proteins have been correlated to diabetes in rodent models (Fukuda, 2008).

## **White adipocyte adiponectin exocytosis is stimulated via $\beta$ 3 adrenergic signalling and activation of Epac1 – catecholamine resistance in obesity and type 2 diabetes (Paper III)**

The central roles of cAMP and  $\text{Ca}^{2+}$  in adiponectin exocytosis propose adrenergic signalling as a likely physiological regulator of acute adiponectin secretion. Although insulin has been shown to induce adiponectin release (Bogan & Lodish, 1999; Motoshima *et al.*, 2002; Pereira & Draznin, 2005; Cong *et al.*, 2007; Blümer *et al.*, 2008; Li *et al.*, 2013; Lim *et al.*, 2015) the physiological regulator of adiponectin exocytosis has not yet been established. In this study we investigate the hypothesis that adrenergic stimulation is the physiological trigger of adiponectin exocytosis/secretion. We further explore how this regulation might be disturbed in obesity and type 2 diabetes.

### ***White adipocyte exocytosis is stimulated via adrenergic pathways and activation of Epac1***

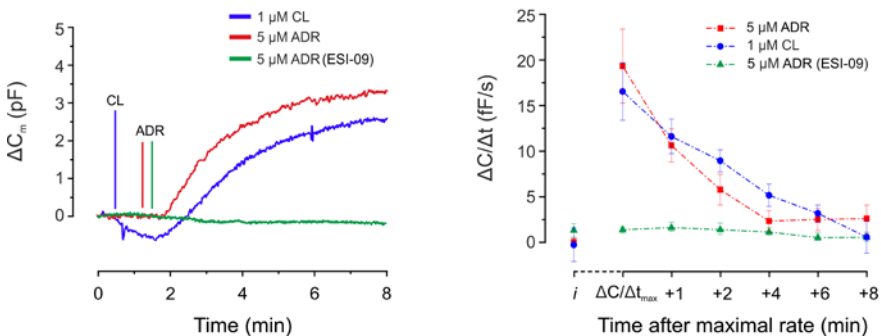
We analyzed the relative gene expression of the five ARs and the two Epac isoforms (Epac1 and Epac2) in undifferentiated and differentiated 3T3-L1 adipocytes. The  $\beta$ 3-AR was highly expressed in mature adipocytes and lower expression levels could be detected for  $\alpha$ 1d-,  $\beta$ 2- and the  $\beta$ 1-ARs. The two isoforms of Epac have tissue-dependent expression (reviewed in Schmidt *et al.*, 2013). In agreement with what has previously been described (Petersen *et al.*, 2008) we found the Epac1 isoform to be expressed in 3T3-L1 adipocytes whereas Epac2 was undetectable. In accordance with the shown role for Epac1 in adipocyte differentiation (Petersen *et al.*, 2008; Jia *et al.*, 2012), the cAMP-GEF was highly expressed in undifferentiated cells. Epac1 was still readily expressed in mature adipocytes, although at a reduced level (~60% lower than in undifferentiated cells).

To investigate the involvement of ARs in 3T3-L1 adipocyte exocytosis, cells were infused with a non-stimulatory pipette solution lacking cAMP and  $\text{Ca}^{2+}$  (10 mM EGTA and 3 mM ATP included). Adrenaline (5  $\mu\text{M}$ ) was added in the extracellular solution perfusing the cell dish ~4 min after establishment of the standard whole-cell

configuration. At the time of catecholamine addition no capacitance increase (exocytosis) could be observed, thus consistent with previous results using this cAMP-depleted solution (Paper I). As shown in Fig.19, adrenaline stimulated exocytosis potently and the maximum rate of exocytosis ( $\Delta C/\Delta t_{\max}$ ) was  $\sim 19$  fF/s. Interestingly, this rate was not significantly different from when exocytosis was stimulated with a pipette solution containing cAMP ( $\sim 15$  fF/s).

Due to the high abundance of  $\beta 3$ -ARs we investigated the contribution of this receptor to the exocytotic response evoked by adrenaline. Cells were again infused with the non-stimulatory pipette solution and the  $\beta 3$  agonist CL 316,243 ( $1 \mu\text{M}$ ; CL) was added  $\sim 4$  min after start of the recording. Exocytosis was stimulated by the  $\beta 3$ -agonist and the rate of exocytosis was similar to the rate induced by adrenaline at all measured time points (Fig. 19).

To investigate the role of Epac in adrenergic stimulation of adipocyte exocytosis, we pre-treated 3T3-L1 adipocytes with the Epac inhibitor ESI-09 ( $10 \mu\text{M}$ ) for 30 min. Cells were infused with the non-stimulatory pipette solution and adrenaline was again added extracellularly. Epac inhibition completely abolished adrenaline stimulated exocytosis at all measured time points (Fig. 19).



**Fig. 19 Adrenaline or CL stimulates exocytosis via  $\beta 3$ -AR mediated activation of Epac.** Left: Example capacitance traces of cells dialyzed with a non-stimulatory pipette solution lacking cAMP with extracellular addition of CL or adrenaline as indicated by arrows. Green trace depicts cells pre-treated for 30 min with the membrane permeable Epac agonist ESI-09. Right: Exocytotic rates at different time points after  $\Delta C/\Delta t_{\max}$ .  $i$  is the initial rate of exocytosis prior to addition of ADR or CL. Data are mean values  $\pm$  SEM of 6 (ADR), 7 (ADR+ESI-09) and 8 (CL) recordings.

## ***Role of Ca<sup>2+</sup> in adrenergically stimulated adipocyte exocytosis***

Adrenaline is known to elevate intracellular Ca<sup>2+</sup> levels by activation of the  $\alpha 1d$ -AR and gene expression data indicated the presence of this receptor in 3T3-L1 adipocytes, although at low levels. To investigate how adrenaline affects [Ca<sup>2+</sup>]<sub>i</sub> levels in 3T3-L1 adipocytes, the catecholamine was added extracellularly and [Ca<sup>2+</sup>]<sub>i</sub> levels were measured by ratiometric Ca<sup>2+</sup> imaging. Adrenaline stimulated elevations of [Ca<sup>2+</sup>]<sub>i</sub> in ~60% of the 3T3-L1 adipocytes while [Ca<sup>2+</sup>]<sub>i</sub> was unaffected by catecholamine addition in the remaining 40% of cells. Elevations in [Ca<sup>2+</sup>]<sub>i</sub> were characterised by high amplitude oscillations, one or several peaks or a slow increase over several minutes. The different response patterns may depend on an uneven inter-cellular distribution of the AR subtypes and similar observations have previously been reported in human adipocytes exposed to noradrenaline (Seydoux *et al.*, 1996).

To investigate the contribution of [Ca<sup>2+</sup>]<sub>i</sub> to catecholamine-stimulated adipocyte exocytosis, we added adrenaline to cells infused with a solution containing a lower [EGTA] of 50  $\mu$ M to allow for fluctuations of [Ca<sup>2+</sup>]<sub>i</sub>. The exocytotic response was not augmented under those conditions and the maximal rate of exocytosis was if anything smaller when [Ca<sup>2+</sup>]<sub>i</sub> was allowed to vary compared to when Ca<sup>2+</sup> was chelated by EGTA ( $\Delta C/\Delta t_{\max} = 11 \pm 2.5$  fF/s at 50  $\mu$ M EGTA vs.  $19 \pm 4.1$  fF/s;  $P=0.1$ ). To investigate if CL-triggered exocytosis, in accordance with our model of adiponectin exocytosis presented in paper I, could be potentiated by higher intracellular Ca<sup>2+</sup> levels 3T3-L1 adipocytes were infused with a pipette solution lacking cAMP and containing ~1.5  $\mu$ M free Ca<sup>2+</sup>. In agreement with previous results (paper I), Ca<sup>2+</sup> alone did not trigger exocytosis. CL, added ~3.5 min after start of the recording, stimulated exocytosis and the maximal rate achieved was ~60% higher than under Ca<sup>2+</sup>-free conditions although this increased rate did not quite reach statistical significance ( $\Delta C/\Delta t_{\max} = 31 \pm 8$  fF/s vs.  $17 \pm 3.2$  fF/s;  $P=0.08$ ).

## ***Adrenergic stimulation triggers adiponectin secretion mainly via $\beta$ 3-ARs***

Next we investigated the effect of adrenergic stimulation on short-term adiponectin secretion. 3T3-L1 adipocytes were incubated for 30 minutes in the presence of 5  $\mu$ M adrenaline or 1  $\mu$ M CL. Adrenaline stimulated adiponectin secretion ~1.8-fold compared to control conditions. The stimulation was equally potent in cells stimulated with CL, suggesting that adrenergic stimulation of adiponectin exocytosis occurs chiefly via activation of  $\beta$ 3-ARs with little involvement of signalling via other ARs. To investigate the role of  $\text{Ca}^{2+}$  in adrenergically stimulated adiponectin secretion, 3T3-L1 adipocytes were pre-treated with the membrane permeable  $\text{Ca}^{2+}$ -chelator BAPTA-AM and thereafter stimulated with adrenaline or CL. Adrenaline and CL stimulated adiponectin secretion of a similar magnitude under  $\text{Ca}^{2+}$ -free conditions and in cells not exposed to BAPTA.

Measurements of intracellular cAMP content in cells stimulated with adrenaline or  $\beta$ 3 agonist showed a >7.5-fold and 3-fold elevation respectively compared to unstimulated cells. Under  $\text{Ca}^{2+}$ -free conditions (BAPTA pre-treatment) adrenaline-induced cAMP production was decreased to levels similar to that produced by CL whereas CL-mediated cAMP levels were unaffected by  $\text{Ca}^{2+}$  chelation. Our cAMP measurements indicate a role for  $\text{Ca}^{2+}$  in the adrenaline-induced elevation of cAMP, likely by activation of  $\text{Ca}^{2+}$ -dependent AC activity (Halls & Cooper, 2011). However, the 3-fold increase of cAMP levels generated by CL stimulated adiponectin secretion as potently as adrenaline that elevates cAMP >7-fold. Thus, our results indicate that the  $\text{Ca}^{2+}$  elevation induced by adrenaline has a minor role in adiponectin exocytosis.

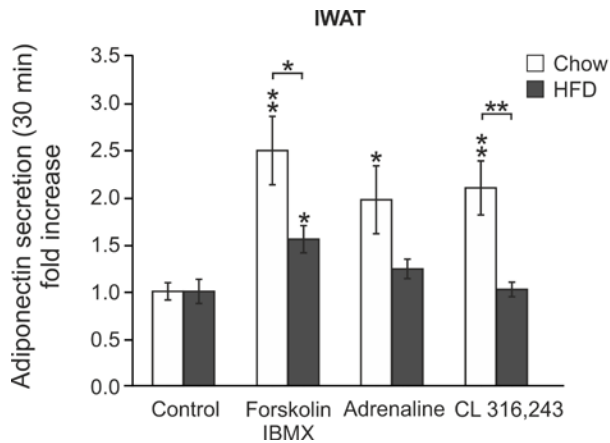
In order to verify the physiological importance of our findings using 3T3-L1 adipocytes, we investigated the effect of adrenaline and CL on adiponectin secretion under normal or  $\text{Ca}^{2+}$ -free conditions in primary subcutaneous mouse adipocytes. In 30 minute incubations adrenaline and CL stimulated adiponectin secretion ~2-fold and the stimulation was unaffected by BAPTA pre-treatment. Based on our electrophysiological findings of the Epac-dependence of adrenergically stimulated

exocytosis, we investigated adiponectin secretion in primary adipocytes pre-treated with ESI-09. Epac inhibition abolished adiponectin secretion stimulated by both adrenaline and CL. Our data show that adrenaline and CL stimulates adiponectin secretion by activation of Epac in both 3T3-L1 and primary mouse adipocytes. The results further demonstrate that the stimulation is independent of  $Ca^{2+}$ .

### ***Adiponectin secretion is disturbed in adipocytes from obese and diabetic mice***

To investigate how the physiological regulation of adiponectin secretion might be disturbed in obesity and type 2 diabetes, subcutaneous adipocytes were isolated from mice fed a chow or high fat diet (HFD) for 8 weeks. The HFD animals were obese and displayed a diabetic phenotype as shown by elevated plasma glucose ( $307 \pm 14$  mg/dl in HFD vs.  $234 \pm 11$  mg/dl in chow;  $P < 0.001$ ) and insulin ( $4.0 \pm 0.7$  ng/ml in HFD vs.  $1.5 \pm 0.1$  ng/ml in chow;  $P < 0.01$ ). Adipocytes from chow or HFD animals were stimulated with forsk-IBMX, adrenaline or CL during 30 min incubations. In agreement with a previous study (Lin *et al.*, 2013), basal adiponectin secretion was  $>2$ -fold elevated in HFD adipocytes compared to adipocytes from chow-fed animals. Forsk-IBMX stimulated adiponectin secretion 2.5-fold in adipocytes from chow-fed animals and adrenaline and CL stimulated adiponectin secretion  $\sim 2$ -fold, thus to a similar extent as in 3T3-L1 adipocytes. Adiponectin secretion could be induced by forsk-IBMX also in HFD adipocytes, although of a magnitude significantly lower than that observed in adipocytes isolated from chow-fed mice ( $\sim 1.5$ -fold;  $P < 0.05$  vs. chow adipocytes). Interestingly, adrenaline- and CL-stimulated adiponectin secretion was abolished in HFD adipocytes (Fig. 20).

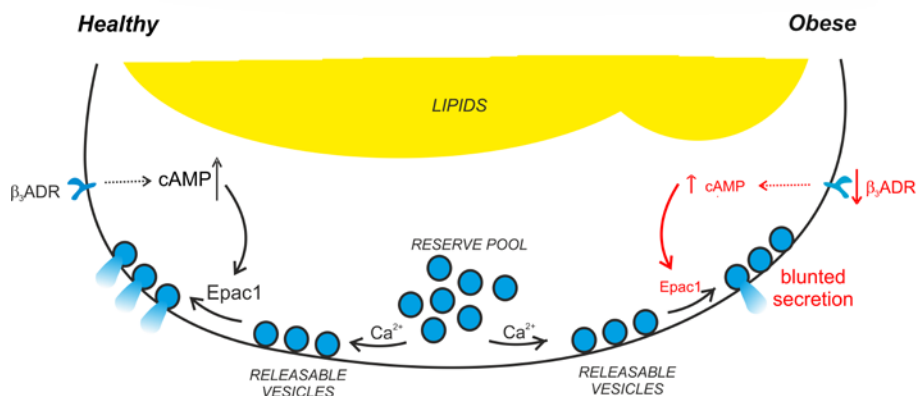
To investigate if the diminished adiponectin levels were due to decreased cellular adiponectin content we measured total adiponectin in adipocytes from chow- and HFD-fed animals. The total adiponectin content was not significantly decreased in HFD adipocytes ( $\sim 30\%$  reduced,  $P = 0.1$ ) thus indicating other underlying causes for the secretory disturbance.



**Fig. 20 Adrenergically stimulated adiponectin secretion is blunted in adipocytes from HFD mice.** Primary subcutaneous adipocytes from animals fed chow or HFD were stimulated with forsk-IBMX, adrenaline or CL during 30 min incubations. Data are mean values  $\pm$  SEM of 9 (chow) and 13 (HFD) animals. \* $P$ <0.05; \*\* $P$ <0.01

Gene expression analysis showed that adipocytes from chow-fed animals expressed high levels of  $\beta$ 3-ARs and that  $\alpha$ 1d-,  $\beta$ 2- and  $\beta$ 1-ARs were expressed at lower levels. Moreover, the Epac1 isoform was present whereas Epac2 was undetectable. Thus, expression data from primary adipocytes were very similar to results in 3T3-L1 adipocytes. Interestingly, the  $\beta$ 3-ARs were 5-fold down-regulated in adipocytes from HFD-fed mice and mRNA levels for the  $\beta$ 1-receptor were reduced 3.5-fold. Expression of Epac1 was reduced by 40% in HFD adipocytes compared to chow adipocytes.

## Conclusions paper III



**Fig. 21 Model of adiponectin exocytosis in health (left part) and in metabolic disease (right part). See text for details.**

Our results show that adiponectin exocytosis/short-term secretion is stimulated by the catecholamine adrenaline as well as by the  $\beta_3$  agonist CL. We further demonstrate that this stimulation is dependent on Epac. Our findings are in accordance with results in Paper I showing that cAMP stimulates adiponectin exocytosis via activation of Epac. Moreover, gene expression analysis identified Epac1 as the isoform present in both cultured and primary adipocytes and thus the Epac isoform involved in regulation of adiponectin exocytosis. In agreement with the left part of the model in Fig. 21, we propose that catecholamines stimulate adiponectin exocytosis mainly by activation of  $\beta_3$ -ARs resulting in an elevation of cAMP and activation of Epac1.

Signalling via  $\alpha_1$ -ARs appears to be of minor importance in adiponectin exocytosis, as shown by the lack of effect of  $Ca^{2+}$  chelation on adrenaline stimulated adiponectin secretion. The effects of adrenaline on adipocyte  $[Ca^{2+}]_i$  are small and 40% of the cells did not respond at all with a change in  $[Ca^{2+}]_i$  upon adrenaline exposure. Nonetheless, measurements of cAMP levels in the presence and absence of  $Ca^{2+}$  indicate that  $Ca^{2+}$ -dependent ACs are activated upon adrenaline stimulation. This finding may seem contradictory to the inability of adrenaline to stimulate adiponectin



exocytosis beyond that triggered by CL. However, we propose that the 3-fold elevation of cAMP produced by CL is sufficient to maximally stimulate adiponectin exocytosis. Due to cAMP compartmentalization (Stefan *et al.*, 2007), the additional cAMP produced by activation of Ca<sup>2+</sup>-dependent ACs may be involved in other cellular processes such as regulation of lipolysis (Ohsalo, 1980; Izawa & Komabayashi, 1994). In adiponectin secretion the cAMP signalling may be further localized to specific sub-cellular regions due to restricted distribution of Epac1 (Schmidt *et al.*, 2013). We conclude that the effect of adrenergically elevated Ca<sup>2+</sup> on adiponectin exocytosis is of little importance. However, the role of Ca<sup>2+</sup> to potentiate cAMP-stimulated adiponectin exocytosis is clear (Paper I and II). We suggest that other pathways leading to an elevation of [Ca<sup>2+</sup>]<sub>i</sub> are more important for this augmenting effect.

In contrast to the stimulatory effect of adrenergic signaling demonstrated here, a few studies have shown that long-term (several hours or days) adrenergic receptor activation leads to reduced expression and/or secretion of adiponectin (Fasshauer *et al.*, 2001; Delporte *et al.*, 2002; Cong *et al.*, 2007; Fu *et al.*, 2007). In one of these studies, the strongest inhibitory effect on adiponectin secretion was observed when β<sub>3</sub>-ARs were targeted specifically (Delporte *et al.*, 2002). It is feasible that this long-term detrimental effect of catecholamines on adiponectin secretion is attributable to depletion of adiponectin-containing vesicles, similar to how pancreatic β-cells exposed to extended stimulation of insulin release are exhausted (Robertson *et al.*, 2003). Further, sustained exocytosis of adiponectin is a Ca<sup>2+</sup>- and energy-dependent step requiring the presence of ATP (Paper II). It has been shown that β-adrenergic stimulation of adipocytes with consequent activation of ACs leads to ATP depletion (the substrate for cAMP production; Chung *et al.*, 1985). Thus, long-term adrenergic stimulation of adiponectin secretion in the absence of necessary substrates for vesicle replenishment can be envisaged to keep the adipocyte in a state where replenishment of releasable vesicles is blocked.

Our investigations further demonstrate that adrenergically stimulated adiponectin exocytosis is disrupted in adipocytes isolated from obese/diabetic mice. As shown in the right part of the model in Fig. 21, we propose that the blunted catecholamine

stimulation of adiponectin exocytosis is mainly due to a marked lower expression of  $\beta$ 3-ARs. The ability of forsk-IBMX to still stimulate adiponectin secretion in adipocytes from obese mice, although at a lower level, indicates that post-receptor signalling is partly intact. However, as shown in the model Fig. 21, we suggest that the demonstrated reduction of Epac1 contributes to the disturbed secretion. It is interesting that the magnitude of forsk-IBMX-stimulated adiponectin secretion is reduced by a similar magnitude as Epac1 expression (~40%), indicating that the disturbed post-receptor effect on adiponectin exocytosis is chiefly due to the lower abundance of the cAMP-GEF. The combined marked reduction of  $\beta$ 3-ARs and a down-regulation of Epac1 results in blunted catecholamine-stimulated adiponectin exocytosis. It is interesting that the basal secretion of adiponectin is 2-fold higher in adipocytes isolated from obese mice compared to lean littermates. It is possible that basal (unstimulated) secretion is elevated to compensate for the reduced stimulated adiponectin release.

## Pathophysiological implications and future directions of our studies

The discovery of white adipose tissue as an endocrine organ was a big leap forward in understanding the importance of this tissue in metabolic health and longevity. There is not a single organ or cell in the body that is not directly or indirectly affected by the adipose tissue. Our studies elucidate both the mediators and mechanisms regulating adiponectin exocytosis under normal physiological conditions as well as how those mechanisms are disturbed in obesity and metabolic disease in a condition we characterise as *catecholamine resistance*. The group of Prof Arner first described the occurrence of catecholamine resistance in human obesity in the early 90s when they showed that lipolytic noradrenaline insensitivity arose as a result of a low density of  $\beta$ 2-ARs (Lonnqvist *et al.*, 1992; Reynisdottir *et al.*, 1994).

The elevated basal secretion of adiponectin observed in adipocytes isolated from obese mice is somewhat difficult to reconcile with the reported decreased plasma adiponectin levels in obesity/type 2 diabetes. Again, this elevated adiponectin release is perhaps a transient condition and reduced basal secretion may occur at later stages of metabolic disease development. Serum adiponectin levels measured after 4 weeks have in a previous study been shown to be higher in HFD compared to chow fed mice but this elevation was reversed after 35 weeks of HFD (Lin *et al.*, 2013). In line with this observation, our preliminary results indicate that basal adiponectin release is distinctly reduced in adipocytes isolated from animals fed HFD for 20 weeks (Musovic, Komai, Olofsson, unpublished). It should also be noted that the ratio between HMW and total adiponectin rather than the total adiponectin levels has been suggested to be affected in obesity/diabetes (Pajvani *et al.*, 2004; Murdolo *et al.*, 2009).

Insulin has in several studies been suggested to be the physiological regulator of adiponectin secretion (Scherer *et al.*, 1995; Bogan & Lodish, 1999; Motoshima *et al.*, 2002; Pereira & Draznin, 2005; Blümer *et al.*, 2008). Furthermore, the reported inhibitory effect of elevated cAMP on adiponectin secretion (Delporte *et al.*, 2002; Cong *et al.*, 2007; Fu *et al.*, 2007) has been reported to be reversed by insulin

(Cong *et al.*, 2007). However, a recent study shows that insulin-stimulated adiponectin secretion in 3T3-L1 adipocytes is evident first at time-points around 30 min (Lim *et al.*, 2015). Those studies are in agreement with own unpublished time-course secretion studies in cultured and primary adipocytes showing that forsk-IBMX stimulates adiponectin secretion after ~5 min while the stimulatory effect of insulin is evident after 20-30 minutes (Musovic, Brännmark, Komai, Olofsson, unpublished). Thus, based on published results and our own preliminary data we propose that insulin is not a physiological regulator of white adipocyte adiponectin exocytosis. We instead suggest that insulin affects adiponectin release in the longer term, perhaps by an elevation of “constitutive-like” adiponectin release involving an endosomal pathway (Clarke *et al.*, 2006; Xie *et al.*, 2008), functionally different from adrenergically triggered adiponectin vesicle release. Further studies are merited in this field to clarify the mechanisms of insulin action on adiponectin release.

The observations that an elevation of adiponectin levels decreases the risk of developing metabolic disease (Spranger *et al.*, 2003) indicate that adiponectin may be an interesting pharmacological target. Recombinant adiponectin is difficult and expensive to produce due to complex post-translational modifications. An agonist of adiponectin receptors (AdipoRon) has recently been developed (Okada-Iwabu *et al.*, 2013) but further investigations are required regarding the specificity and effects of this compound. In addition, efforts should be aimed at elevating endogenous adiponectin secretion. In order to correct secretory defects the mechanisms regulating adiponectin exocytosis as well as how the regulation is disturbed in obesity/type 2 diabetes need to be established. The results presented in this thesis moves us one step closer to resolving obesity-related defects of adiponectin secretion.

## Concluding remarks

In this thesis we have, for the first time, characterized the intracellular mediators and mechanisms involved in stimulation of white adipocyte adiponectin exocytosis. We have further determined adrenergic signalling as a physiological trigger of adiponectin exocytosis/secretion. Finally, we have shown that catecholamine stimulation of adiponectin exocytosis is disturbed in obesity and type 2 diabetes due to a malfunctioning adrenergic signalling pathway. The following conclusions were reached:

- 1) White adipocyte exocytosis is stimulated by cAMP and activation of Epac, in a  $\text{Ca}^{2+}$ - and PKA-independent manner. The cAMP-triggered exocytosis can however be greatly augmented by a combination of  $\text{Ca}^{2+}$  and ATP via a direct effect on the release process as well as via recruitment of vesicles residing in a reserve pool. The exocytotic responses (increases in membrane capacitance) can be largely correlated to release of adiponectin-containing vesicles.
- 2) The cAMP-triggered adiponectin exocytosis is unaffected by cooling while the  $\text{Ca}^{2+}$ /ATP-dependent augmentation of release is largely reduced by a temperature decrease. We propose that cooling affects adipocyte exocytosis/adiponectin secretion at a  $\text{Ca}^{2+}$ -dependent step, likely involving ATP-dependent processes, essential for amplification of cAMP-stimulated adiponectin secretion.
- 3) Adrenergic signalling is the physiological trigger for adiponectin exocytosis. Catecholamines stimulate the release of adiponectin-containing vesicles mainly via binding to  $\beta$ 3-ARs and activation of Epac1. Adrenergically stimulated adiponectin secretion is disturbed in obesity/type 2 diabetes due to a marked lower abundance of  $\beta$ 3-ARs and reduced expression of Epac1 in a state we define as *catecholamine resistance*.



# Populärvetenskaplig sammanfattning

Dagens fetmaepidemi är ett faktum. Idag dör fler människor av överviktsrelaterade sjukdomar än undervikt. Övervikt kan leda till en rad olika sjukdomar och tillstånd, bland annat hjärt- och kärlsjukdomar, stroke, inflammation och alltmer vanligt typ-2 diabetes. Övervikt karaktäriseras av en ökad storlek av kroppens vita fettväv. I den vita fettväven finns flera olika celltyper, men den största delen består av vita adipocyter (fettceller) som är kroppens enda celltyp som är specialiserad för att lagra fett och överskotts kalorier vi får i oss från födan. Historiskt sett har den vita fettväven endast betraktats som ett passivt förvaringsorgan för fett, men på senare år har forskning visat att fettväven är ett ytterst komplext endokrint organ. Från fettväven frisätts ett flertal olika biologiskt aktiva protein-molekyler och hormoner som kallas för adipokiner. Adipokiner frisätts direkt in i blodet och fettväven kan på detta sätt direkt eller indirekt kommunicera med och påverka hela kroppen. Adiponektin är ett hormon som frisätts från den vita adipocyten och har sedan dess upptäckt för 20 år sedan fått väldigt stor uppmärksamhet på grund av dess positiva effekter. Adiponektin kallas för ett anti-diabetiskt hormon och har visats kunna öka bland annat insulin-känslighet, glukosupptag och fettförbränning. Höga adiponektinnivåer kan därför skydda mot utveckling av typ-2 diabetes. Adiponektin har också anti-inflammatoriska egenskaper och kan också ha en skyddande roll i hjärt- och kärlsjukdomar. Till skillnad från flertalet andra kända adipokiner så minskar paradoxalt nog frisättningen av adiponektin med ökad fettmassa och nivåer är därför lägre i patienter med övervikt och typ-2 diabetes. Trots all forskning om de hälsofrämjande effekterna av adiponektin och de negativa konsekvenserna av minskade adiponektinnivåer, är mekanismerna som reglerar fettcellernas frisättning av detta hormon fortfarande i stort okända.

Hormonfrisättande celler har oftast en färdig depå av hormoner lagrade i små "blåsor" som kallas för vesiklar och som kan frisättas från cellen som svar på en fysiologisk stimulering. När de frisättningsbara vesiklarna är slut kan nya hormoninnehållande vesiklar snabbt genomgå ett eller flera mognadssteg för att kunna frisättas och därmed upprätthålla hormonfrisättningen. Frisättningen av mogna

vesiklar stimuleras vanligen av kalcium-joner ( $\text{Ca}^{2+}$ ), och vesikelmognaden involverar bland annat de intracellulära mediatorerna adenosintrifosfat (ATP) och cykliskt-AMP (cAMP). Vid frisättningsprocessen, som kallas för exocytos, smälter den hormoninnehållande vesikeln ihop med cellens plasmamembran och vesikelinnehållet släpps ut utanför cellen. Exocytos leder till att cellmembranets area ökar och detta kan experimentellt mätas på enskilda celler med patch-clamp tekniken som en ökning av en elektrisk egenskap som heter kapacitans. I detta avhandlingsarbete har jag, med en kombination av kapacitansmätningar och biokemiska mätningar av frisatt adiponektin, karaktäriserat mekanismer och signalvägar som styr exocytos av adiponektin i vita adipocyter.

Vår forskning visar, för första gången, att exocytos i den vita adipocyten stimuleras av cAMP och att exocytosen huvudsakligen motsvarar frisättning av adiponektin. Denna cAMP-stimulerade exocytos skiljer alltså adipocyten från flertalet hormonfrisättande celler där exocytosen är  $\text{Ca}^{2+}$ -stimulerad. Dock kan den cAMP-triggade exocytosen förstärkas av en kombinerad närvaro av ATP och  $\text{Ca}^{2+}$  som har en essentiell roll i mognadsprocessen av nya frisättningsbara vesiklar. Den  $\text{Ca}^{2+}$  och ATP-beroende mognaden/påfyllnaden av frisättningsbara vesiklar kräver energi i form av ATP. Våra studier visar vidare att den cAMP-stimulerade exocytosen motsvarar frisättningen av vesiklar innehållandes adiponektin. cAMP kan signalera antingen via aktivering av enzymet protein kinas A (PKA) eller via aktivering av det cAMP-specifika proteinet Epac. Ännu ett intressant och viktigt fynd i vårt arbete är att exocytos av adiponektin verkar stimuleras enbart via Epac, helt oberoende av PKA. Detta skulle kunna vara ett sätt för adipocyten att skilja mellan cAMP-stimulerad adiponektinfrisättning och fettförbränning (som stimuleras via PKA).

Våra studier visar också att adiponektinfrisättning fysiologiskt stimuleras via aktivering av membranbundna adrenerga receptorer. Det finns flera olika adrenerga receptorer (uppdelade i alfa och beta) som bland annat binder till och stimuleras av katekolaminet adrenalin, ett hormon som utsöndras från binjurebarken. När beta-adrenerga receptorer aktiveras leder det till en ökning av cAMP inuti cellerna. Våra resultat demonstrerar att adrenalin-medierad aktivering av adrenerga beta-3 receptorer är viktigast för adiponektinexocytosen och att den påföljande ökningen av



cAMP stimulerar exocytosen via Epac1. Vi visar även att signalleringen via adrenerga beta-3 receptorer är störd i adipocyter isolerade från överviktiga och diabetiska möss och att detta beror på en nedreglering av beta-3 receptorn och Epac1 i de feta djuren.

För effektiva behandlingar av fetma och alla dess följsjukdomar krävs en grundlig förståelse för hur fettcellen fungerar. Betydelsen av en hälsosam fettväv och intakt frisättningen av adiponektin för en god hälsa är uppenbar. Våra studier utgör därmed en viktig pusselbit i vår strävan att förstå de mekanismer som reglerar frisättningen av adiponektin i den vita fettcellen.



# Acknowledgements

First and foremost, I would like to thank my supervisor Charlotta Olofsson. Words are insufficient to express my gratitude for your continuous support, generosity and the many things you have taught me. Fat has been a constant in my life, and I am grateful for the opportunity you have given me to further research it through my new found passion of exocytosis. I could not have wished for a better supervisor and I want you to know that you are a true inspiration. Again, thank you.

I also wish to express my gratitude to:

My co-supervisors Patrik Rorsman and Eric Hanse: For motivational words and their support.

Ingrid Wernstedt-Asterholm: For scientific input and your effort to promote science for future generations.

My colleagues Mickaël and Saliha. Mike, for all our interesting discussions and our memories. You have left an empty space behind, but I wish you the best of luck at your new workplace. Life is beautiful, no? Saliha, for your kindness and for always being there for me. For all the times you remember things that I forget.

My coworkers at the department of Metabolic Physiology for always being helpful, supportive and encouraging. Every single one of you has contributed to making the 4<sup>th</sup> floor a truly amazing work place.

Our lab-technicians Birgit and Ann-Marie, BLAMA. You two are the sunshine's of the 4<sup>th</sup> floor. Not much would work without you. Thank you for all the help and for making me happy, always.

Seid, Kosrat and Abtin. For all the late night experiments and experimental support in our projects.

Ahmed, for staying late nights and keeping me motivated/caffeinated.

My former coworkers of the Department of Physiology (3<sup>rd</sup> floor) and CBR for making me feel welcome and for all the help since I started here five years ago.

My contemporary fellow PhD students Reza, Andreas, Rozita and Giulia. For their support during these years and for sharing this experience with me.

Habib, for helping out with the graphics and everything else.

A special thank you to friends and family outside the lab. No one mentioned, no one forgotten.

Last but not least, to my hero and role model, mom:

مادر عزیزم

برای همه زحمات و برای اینکه در همه دوران زندگی در کنارم بودی از صمیم قلب قدردانم

عزیزم بدان که هیچ وقت محبتت را فراموش نمیکنم

ایسرکتب را تقدیم به شما میکنم

فرزندت

علی

Financial support was obtained from Ollie and Elof Elofssons Foundation, the Åke Wiberg Foundation, the Magnus Bergvall Foundation, Jeanssons Stiftelse, Knut och Alice Wallenbergs Foundation, IngaBritt och Arne Lundbergs Foundation, Diabetesfonden, the Novo Nordisk Foundation and the Swedish Medical Research Council

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