# Protein kinases in hormonal regulation of adipocyte metabolism.

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Abstract
Along with liver and muscle tissue, adipose tissue helps maintain normal levels of glucose and lipids in the blood and has a very important role when it comes to storing lipids that can provide whole-body energy. After a meal is ingested, adipocytes take up glucose from the circulation and use it as a substrate for synthesis of new fatty acids (FAs) in a process known as de novo fatty acid synthesis, as well as for synthesis of glycerol. Adipocytes also take up fatty acids from the circulation and incorporate both newly synthesized and imported FAs into triacylglycerides (TAGs), in a process known as lipogenesis. TAGs are stored in large lipid droplets in the cytosol, and during fasting, or in response to physical exercise, they are hydrolysed in a process known as lipolysis, in which FAs are released into the bloodstream for use as energy substrates in other tissues. These cycles of lipogenesis and lipolysis are controlled by the concerted actions of insulin, a hormone that is secreted by the pancreas and catecholamines, hormones that are secreted by the adrenal glands, or derive from the nervous system. Both glucose- and fatty acid uptake, as well as lipid storage and mobilization, are regulated by cellular signaling, and kinases are central enzymatic players in hormone-induced cellular signaling. A dysfunctional adipose tissue can contribute to insulin resistance in many obese individuals. Therefore it is important to elucidate the cellular mechanisms that govern metabolic processes in adipocytes.

Insulin is the hormone that promotes glucose uptake and lipogenesis in adipocytes, and when it induces glucose uptake, insulin exerts it actions through protein kinase B (PKB). Although PKB is known to mediate many effects of insulin, its role in lipogenesis in adipocytes is less clear. We show that PKB is important for the effects of insulin on lipogenesis (de novo and total). We also reveal that PKB can regulate Amp-activated protein kinase (AMPK) in adipocytes by a mechanism previously only seen in heart muscle cells. AMPK is a sensor of cellular energy status and known to inhibit lipogenesis. We speculate that insulin possibly mediates its lipogenic effects via a decrease in AMPK activity accomplished by PKB-phosphorylation of S485 on AMPK.

Furthermore, we find that salt-inducible kinase 3 (SIK3), a kinase that belongs to the AMPK-related family of kinases, and displays structural similarities to AMPK, can be regulated by catecholamines in adipocytes. Catecholamines are hormones that bind to β-adrenergic receptors and act by increasing cellular levels of cAMP, which in turn activates protein kinase A (PKA). We find that in response to such β-adrenergic stimuli, SIK3 is phosphorylated on multiple serine and threonine residues. This regulation coincides with an increase in binding of SIK3 to 14-3-3 molecules. 14-3-3 proteins are cellular scaffolding proteins that can result in cellular re-localization of their binding partners or in their binding to other proteins or lipids. We find that when SIK3 is phosphorylated in response to β-adrenergic stimuli, the kinase does not re-localize, but is partially de-activated. We speculate that SIK3 could potentially have a role in adipocyte metabolism, as it is regulated by catecholamines in this tissue.

Finally, we address the current understanding of the role for AMPK in modulation of the effects of insulin and catecholamines on glucose uptake and lipid metabolism. To this date, it has been suggested that AMPK reduces insulin-induced glucose uptake and lipogenesis, as well as inhibits catecholamine-induced lipolysis in adipocytes. These findings are mainly based on studies performed with AMPK activating agents that act on AMPK in an indirect manner. We have used the allosteric activator A769662, that binds directly to AMPK, and find that AMPK does not appear to modulate hormonally induced glucose uptake, lipolysis or total lipogenesis. However, when we specifically measured the synthesis of new FAs, using acetate as a lipogenic substrate (as opposed to using glucose as a substrate, a molecule which can participate in both FA and glycerol synthesis), we observe that AMPK does indeed reduce insulin-induced de novo fatty acid synthesis.

Collectively, we add novel findings to the available knowledge on key kinases and cellular signaling in adipocyte metabolism. Our findings contribute to the understanding of insulin- and catecholamine-mediated control of lipid storage in adipose tissue, a biological function that, when dysfunctional, is strongly linked to insulin resistance and type 2 diabetes (T2D).

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***For Johan and Julius***

”I only know everything if you ask me the right questions.”

Anonymous


**Shit Academics Say** @AcademicsSay · 21 maj

Smiling on the outside, correcting grammar on the inside.

# Abstract

Along with liver and muscle tissue, adipose tissue helps maintain normal levels of glucose and lipids in the blood and has a very important role when it comes to storing lipids that can provide whole-body energy. After a meal is ingested, adipocytes take up glucose from the circulation and use it as a substrate for synthesis of new fatty acids (FAs) in a process known as de novo fatty acid synthesis, as well as for synthesis of glycerol. Adipocytes also take up fatty acids from the circulation and incorporate both newly synthesized and imported FAs into triacylglycerides (TAGs), in a process known as lipogenesis. TAGs are stored in large lipid droplets in the cytosol, and during fasting, or in response to physical exercise, they are hydrolysed in a process known as lipolysis, in which FAs are released into the bloodstream for use as energy substrates in other tissues. These cycles of lipogenesis and lipolysis are controlled by the concerted actions of insulin, a hormone that is secreted by the pancreas and catecholamines, hormones that are secreted by the adrenal glands, or derive from the nervous system. Both glucose- and fatty acid uptake, as well as lipid storage and mobilization, are regulated by cellular signaling, and kinases are central enzymatic players in hormone-induced cellular signaling. A dysfunctional adipose tissue can contribute to insulin resistance in many obese individuals. Therefore it is important to elucidate the cellular mechanisms that govern metabolic processes in adipocytes.

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regulation coincides with an increase in binding of SIK3 to 14-3-3 molecules. 14-3-3 proteins are cellular scaffolding proteins that can result in cellular re-localization of their binding partners or in their binding to other proteins or lipids. We find that when SIK3 is phosphorylated in response to β-adrenergic stimuli, the kinase does not re-localize, but is partially de-activated. We speculate that SIK3 could potentially have a role in adipocyte metabolism, as it is regulated by catecholamines in this tissue.

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Collectively, we add novel findings to the available knowledge on key kinases and cellular signaling in adipocyte metabolism. Our findings contribute to the understanding of insulin- and catecholamine-mediated control of lipid storage in adipose tissue, a biological function that, when dysfunctional, is strongly linked to insulin resistance and type 2 diabetes (T2D).

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# Populärvetenskaplig Sammanfattning

## Cellers kommunikationssystem

Cellerna i kroppens organ kan kommunicera med varandra och på så sätt styra varandras arbetsuppgifter. Som ett exempel kan cellerna i bukspottkörteln känna av att vårt blodsocker har höjts efter en måltid och utsöndra hormonet insulin till blodbanan. Insulinet färdas sedan i blodet tills det stöter på ett annat organ, till exempel en muskel, som har receptorer för insulinet, en sorts sensorer, på sin yta. Insulinet kan då binda in till receptorerna på muskelcellerna och en signal fortplantar sig i cellerna. Insulinets budskap skickas från molekyl till molekyl i särskilda signalkedjor inne i cellen tills ett budskap har nått fram och muskelcellen har reglerats, det vill säga styrts i något avseende. I fallet då insulin skickats från bukspottkörteln till muskeln är budskapet till muskelcellen att: "vi har socker i blodet, ta vara på det!" Muskelcellen skapar då transportmöjligheter för sockret så att det kan tas upp från blodet och lagras så att vi kan använda det som energi även mellan våra måltider, eller omvandla det till byggstenar för proteiner. När insulinet når vår fettvävnad kan budskapet istället vara: ta inte mer av våra fettlager! Satsa på att lagra vårt fett istället. Vi har socker så det räcker i blodet, vi sparar vårt fett tills vi svälter igen. Ofta går den här sortens kommunikation väldigt snabbt och detaljerna i signaleringen är komplexa. På det här viset kan kroppen hålla blodsockernivåer jämna, förse hjärnan med energi även mellan våra måltider och upprätthålla en balans i uppbyggande och nedbrytande av de lager av fett och socker som finns i kroppen. Förutom insulin så regleras fettceller av katekolaminer, t.ex adrenalin. Medan insulin frisätts efter att vi har ätit en måltid, så utsöndras katekolaminer när vi är hungriga. Katekolaminers främsta uppgift i fettceller är att främja fettnedbrytning av det fett som lagrats i fettdroppar till beståndsdelar som kan utnyttjas som energi.

# Övervikt och diabetes

Övervikt och fetma anses idag vara ett av västvärldens största och mest kostsamma samhällsproblem, och är starkt kopplat till typ 2 diabetes. Medan typ 1 diabetes är en autoimmun sjukdom där bukspottkörteln bryts ner och inte längre kan producera insulin, så verkar utvecklingen av typ 2 diabetes hos en individ bero till viss del på vilka gener individen bär på, men också på vilken livsstil personen har. Övervikt kan orsaka insulinresistens i kroppen, det vill säga cellerna i kroppens organ blir okänsliga för order från bukspottkörteln. Insulinet binder in till cellernas receptorer, men molekylerna som ska föra signalen vidare har satts mer eller mindre ur spel. Insulin kan inte längre styra organen som ska ta upp sockret från blodet och vi får till sist förhöjda blodsockernivåer. Till en början kan kroppen kompensera för den försvagade signalen genom att producera mer insulin i bukspottkörteln, men när bukspottkörteln inte längre kan kompensera har man utvecklat typ 2 diabetes. Man fortfarande öka cellernas insulinkänslighet med motion, vilket har visat sig ha positiva effekter på muskelcellers insulinkänslighet, men när inte heller motion räcker till, måste man ta till läkemedel i tablettform som påverkar de molekyler som ingår i cellernas signaleringskedjor. När inte heller dessa läkemedel förmår att hjälpa kroppens organ att svara på insulinsignalen måste man injicera insulin efter varje måltid för att kompensera för insulinresistensen. Insulinordern måste helt enkelt förtydligas och förstärkas i hopp om att kroppens celler ska lyckas ”höra” den. Förhöjda blodsockernivåer är nämligen farligt för kroppen på lång sikt eftersom de med tiden förstör kroppens minsta blodkärl, kapillärerna, och därmed medför svåra komplikationer, såsom nedsatt syn, amputation och t.o.m njursvikt.

Anledningarna till att övervikt resulterar i att cellerna blir okänsliga mot insulin är många och komplicerade. Ett exempel är att fettceller som normalt lagrar fettsyror i stora fettdroppar i cellen, inte kan upprätthålla en normal fettinlagring. Både frisk fettvävnad och överbelastad fettvävnad utsöndrar egna hormoner, så kallade adipokiner. Frisk fettvävnad ustöndrar faktorer som bland annat skyddar kroppen mot insulinresistens och överbelastad fettvävnad utsöndrar faktorer som har negativa effekter på t.ex insulinkänslighet och som i vissa fall leder till kronisk inflammation i fettvävnaden. Kronisk inflammation medför försämrad fettinlagring och ökad frisättning av fettsyror, som då cirkulerar i blodet i förhöjda nivåer och påverkar molekylerna som ingår i insulinsignaleringen negativt i hormonets målvävnader. Till sist kan de cirkulerande fettsyrorna också lagras i andra vävnader, t.ex i levern eller musklerna, där fett inte hör hemma och har negativa konsekvenser.

Trots det starka sambandet mellan fetma och typ 2 diabetes, är 20 % av typ 2 diabetiker smala och av de som lider av fetma utvecklar ungefär 30 % diabetes. Det finns alltså smala diabetiker och överviktiga människor som inte utvecklar typ 2 diabetes. Detta indikerar att ren överbelastning av fettvävnaden inte orsakar diabetes, utan snarare är det molekylära fel i visa individers förmåga att lagra fett alternativt utsöndra adipokiner som orsakar typ 2 diabetes. Troligtvis har både gener och miljö en inverkan på vem som blir sjuk av övervikt.

## Kinasers roll i cellkommunikation

Kinaser är en speciell sorts protein som kan ”märka” andra proteiner som ingår i signalkedjor med en fosfatgrupp. Detta kan slå av och på andra proteiner, ungefär som en strömbrytare, och på så sätt fortplanta en hormonell signal vidare inuti celler. Strömbrytaren kan bestå i en förändring av målproteinets enzymaktivitet, men fosfatgruppen kan också innebära en märkning som gör att mottagarproteinet förflyttar sig eller att proteiner kan binda till varandra som pusselbitar. Vår arvsmassa innehåller gener för mer än 500 kinaser och de är viktiga mål när man designar nya läkemedel, bland annat för behandling av cancer. Även läkemedlet metformin, som är läkarnas förstahandsval när det gäller diabetesbehandling, ser ut att indirekt aktivera ett kinas.

## Insulinsignalering och proteinkinas B (PKB)

Trots att man länge har känt till de första stegen i den signal som förmedlar insulins budskap i celler, så är de sista stegen i signalen som meddelar fettceller at de ska lagra fett, fortfarande outforskade. Vi visar att proteinkinas B (PKB), ett välkänt kinas och mål för hormonet insulin i andra vävnader, är en nödvändig komponent för att dessa processer ska kunna regleras av insulin (artikel I). Det är viktigt att känna till de exakta signaleringsmekanismer som ligger bakom alla insulins effekter i målceller, dels för att upptäcka möjliga mål för mediciner men också för att veta mer om just hur fettsyror lagras och nybildas, då dessa anses ligga till grund för insulinresistens hos en del överviktiga.

# AMP-aktiverat proteinkinas (AMPK) fungerar som kroppens energisensor

I våra studier har vi också undersökt AMPK-aktiverat proteinkinas (AMPK). Detta kinas fungerar som en energisensor i celler, genom att känna av och upprättahålla nivån av ATP, som är cellens energivaluta. När det råder brist på energi i cellen aktiveras AMPK och styr cellens funktioner mot processer som genererar energi (i form av ATP-molekyler), till exempel nedbrytning av fettsyror, protein och kolhydrater medan den samtidigt förhindrar kroppen från att använda energin till att bygga stora molekyler som fett, protein och kolhydrater. AMPK aktiveras också av diabetesläkemedlet metformin och tros ansvara för en del av de positiva metabola effekterna av detta läkemedel. AMPK-aktivering är en viktigt strategi för diabetesbehandling och är därför väldigt välstuderat i lever och muskel, men inte lika studerat i fettvävnad. Vi har använt en ny aktivator av AMPK och ser att en del av tidigare fynd när det gäller AMPKs roll i fettceller inte riktigt verkar stämma (artikel III). Tidigare har man trott att aktivering av AMPK motverkar hormonella effekter på fettnedbrytning, fettinlagring och på glukosupptag. Med denna nya aktivator utmanar vi dessa resultat och visar att AMPK främst har en sänkande effekt på den nybildning av fettsyror som insulin orsakar i fettceller.

Vi har också kommit fram till att PKB, när det stimulerats av insulin, kan reglera och alltså märka AMPK med en fosfatgrupp, något som gör AMPK mindre aktivt (artikel I). Detta är ett exempel på hur AMPK, förutom som svar på sänkta ATP-nivåer, kan regleras av hormonet insulin och vi spekulerar i om denna inmärkning och deaktivering av AMPK kan kopplas till den ökning av nybildning av fettsyror som insulin orsakar i fettceller (artikel 1 och opublicerad data).

# Salt inducible kinase 3 (SIK3), en outforskad släkting till AMPK, kan regleras hormonellt i fettceller

AMPK är ett evolutionärt mycket gammalt protein som finns i alla organismer, såsom däggdjur, men även lägre modellorganismer som används i forskningssammanhang, som till exempel bananfluga. Hur väl ett protein (i detta fall ett kinas) har bevarats under evolutionens gång säger något om hur viktigt det är för att organismer ska fungera optimalt, och AMPK är tveklöst viktigt för att kroppen ska kunna balansera processer som använder, respektive nybildar energi. Hormonell reglering, såsom till exempel den tidigare nämnda insulinregleringen har tillkommit i efterhand och är ett exempel på hur kroppens regleringssystem har blivit mer och mer sofistikerat för att kunna anpassa sig till en föränderlig miljö.

SIK3 är ett kinas som strukturellt delar många likheter med AMPK. De båda kinaserna är besläktade och man skulle därmed kunna tänka sig att SIK3 också har en viktig roll när det gäller hushållning med energi. Det finns tre stycken SIK-proteiner och SIK3 är det minst studerade; man vet ingenting alls om dess funktion i fettceller trots att det finns i denna celltyp. Vi visar att SIK3 kan regleras av katekolaminer i fettceller och att detta är kopplat till en sänkning av kinasets aktivitet (artikel II). Vi vet fortfarande inget om kinasets funktion i fettceller, men att det kan regleras av katekolaminer, som har en viktig roll i till exempel nedbrytning av fett skickar en tydlig signal om att SIK3 kan ha en viktig roll för ämnesomsättning i denna vävnad.

# List of papers

## Papers included in thesis

**I.** **Protein kinase B activity is required for the effects of insulin on lipid metabolism in adipocytes.** Christine Berggreen, Amélie Gormand, Bilal Omar, Eva Degerman, Olga Göransson. American Journal of Physiology (Endocrinology and metabolism), *2009 American Journal of physiology – endocrinology and metabolism. Apr;296(4):E635-46.*

**II.** **cAMP-elevation mediated by β-adrenergic stimulation inhibits salt-inducible kinase (SIK) 3 activity in adipocytes.** Christine Berggreen, Emma Henriksson, Helena A. Jones, Nicholas Morrice, Olga Göransson. *2012 Cellular Signalling. Sep;24(9):1863-71*

**III.** **Role of AMPK-activated kinase in the regulation of adipocyte metabolism.** Christine Berggreen, Eva Degerman, Olga Göransson. *Manuscript.*

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## Papers not included in thesis

**I.** **Survival of Pancreatic β-cells is partly controlled by a TCF7-L2-p53-p53INP1-dependent pathway.** Yuedan Zhou, Enming Zhang, Christine Berggreen, Xingjun Jing, Peter Osmark, Stefan Lang, Corrado M. Cilio, Olga Göransson, Leif Groop*, Erik Renström, Ola Hansson. 2012 Human molecular Genetics. 1;21(1):196-207*

II. **LKB1 signalling attenuates early events of adipogenesis and responds to adipogenic cues.** Amélie Gormand, Christine Berggreen, Lahouari Amar, Emma Henriksson, Ingrid Lund, Sebastian Albinsson, Olga Göransson. *2014 Journal of molecular endocrinology. Aug;53(1):117-30*

# Abbreviations

| | |
|---|---|
| AC | Adenylate cyclase |
| ACC | Acetyl CoA-carboxylase |
| AICAR | 5-aminoimidazole-4-carboxamide ribonucleoside |
| AMP | adenosine monophosphate |
| AMPK | AMP-activated protein kinase |
| AS160 | Akt substrate of 160 kD |
| ATGL | Adipose triglyceride lipase |
| CaMKK | $Ca^{2+}$calmodulin kinase kinase |
| cAMP | cyclic adenosine monophosphate |
| CRE | cAMP-responsive elements |
| CREB | CRE-binding protein |
| CGI-58 | Coactivator comparative gene identification 58 |
| Co-A | Co-enzyme A |
| CRTC | CREB-regulated transcription co-factor |
| DGAT | diacylglycerol transferase |
| Epac | Exchange protein directly activated by cAMP |
| FA | Fatty acid |
| FAS | Fatty acid synthase |
| GLUT | Glucose transporter |
| HDAC | Histone deacetylase |
| HEK293 | Human embryonic kidney 293 |
| HSL | Hormone sensitive lipase |
| IGF1 | Insulin-like growth factor 1 |
| IR | Insulin receptor |

| | |
|---|---|
| IRS | Insulin receptor substrate |
| KO | knock-out |
| MGAT | Monoacylglycerol transferase |
| MO25 | Mouse protein 25 |
| NLS | Nuclear localization signal |
| PDE | Phosphodiesterase |
| PH | Pleckstrin homology |
| PIP | Phosphatidylinositol monophosphate |
| PI3-kinase | Phosphoinositide 3 kinase |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PP2A | Protein phosphatase 2 A |
| SH | Src homology |
| SIK | Salt-inducible kinase |
| SCD1 | Stearoyl-CoA desaturase 1 |
| TAG | Triacylglyceride |
| T-loop | Activation loop |
| T2D | Type 2 diabetes |
| UBA | Ubiquitin associated |
| WAT | White adipose tissue |
| wt | Wildtype |

# General introduction

Obesity is a rapidly increasing health problem in westernized countries and an escalating phenomenon in certain populations in developing countries [1]. The world health organization (WHO) estimates that by 2015, 2,3 billion adults will be overweight (body mass index > 25 kg/m2), and 700 million obese (BMI > 30 kg/m2) [2]. People who suffer from obesity often show additional signs of what is termed the metabolic syndrome, a condition characterized by high BMI, hypertension and insulin resistance [3]. In healthy individuals insulin has effects on the liver, muscle and adipose tissue following ingestion of a meal, that serve to maintain blood glucose and lipid levels within the normal range. When insulin target tissues become desensitized to the hormonal actions of insulin, Type 2 diabetes (T2D), which is characterized by abnormally high blood glucose levels, and associated complications, ensue [4]. Diabetes is characterized by chronic hyperglyceamia, i.e a fasting blood glucose concentration that is higher than 7,0 mmol/l or a blood glucose level of 11,1 mmol/l after ingestion of 75 g glucose (oral glucose tolerance test). Blood glucose levels of 6,1 mmol/l or higher are considered a sign of impaired glucose tolerance and requires follow-up tests [5]. T2D should not be confused with type 1 diabetes, which is an autoimmune disease in which the body's own antibodies target the insulin-producing pancreas for destruction [6].

The simplified cause of obesity seems to be the dual action of a sedentary lifestyle along with a high-caloric diet, but other contributing factors have been implicated in both obesity and T2D, such as genetic predisposition, age, male gender, urbanization, a stressful lifestyle and sleep deprivation [4, 7-12]. There is a strong causal link between obesity and diabetes as 80 % of type 2 diabetics are obese, yet only 30 % of obese individuals suffer from diabetes [13]. This suggests that in addition to environmental causes, genetic risk factors contribute to the development of the disease, and that some metabolic dysfunction other than a simple excess of lipid accumulation must be present in certain obese individuals in order for diabetes to develop.

Based on the strong link between obesity and T2D, and as a dysfunctional adipose tissue contributes to insulin resistance [14], it is of high importance to study adipose tissue function. Adipocytes primarily store lipids, as an energy reserve for times when energy is required. In mammals, the opposed hormonal actions of insulin and catecholamines govern import of glucose, synthesis and storage of fat, as well as fat mobilization when the organism requires energy [15, 16]. Discovering the exact

signaling mechanisms that regulate these metabolic processes could reveal new ways of treating patients who suffer from obesity and diabetes.

Protein kinases are a powerful group of enzymes that participate in virtually all of these signaling pathways. They regulate the activity, localization and binding ability of other proteins, and hold great potential as targets for pharmacological intervention [17]. As far as insulin signaling is concerned, protein kinase B (PKB), also known as Akt, is a key kinase, mediating many (if not all) effects of insulin on metabolism in target tissues [18]. Amp-activated protein kinase (AMPK) is another kinase that has received vast amounts of attention in the context of energy homeostasis, as its activation has positive effects on metabolism in the context of diabetes [19]. AMPK belongs to the AMPK-related family of kinases, and it shares structural elements with a rather unstudied group of kinases: the salt-inducible kinases (SIKs) [20]. The SIKs are expressed in adipose tissue and could prove important in a metabolic context.

This thesis focuses on the role of key kinases in the regulation of adipocyte metabolism and how they modulate and respond to hormonal stimuli. In order to motivate the particular interest in PKB, AMPK and the SIK isoform SIK3, a background to white adipocyte metabolism, the study of protein kinases, and the structure, function and regulation of each kinase will be provided.

# Scientific background

## White adipose tissue function and its role in the development of insulin resistance

The white adipocyte has a unique morphology when compared to other cell types. It holds a large, single lipid droplet, sometimes comprising as much as 95 % of the adipocyte volume, pushing the nucleus and cytosolic proteins to the edges of the cell membrane [21]. It is believed that in addition to the number of adipocytes principally being established early in life, some new fat cells are generated from progenitor cells in the adult individual [22], following a maturation path that spans pre-adipocytes, immature and mature adipocytes [23]. In essence this means that the adipose tissue in obese individuals undergoes both hypertrophy (increase in cell size) and hyperplasia (increase in cell number) [24]. Besides adipocytes, the white adipose tissue contains supportive cells, vascular cells, nerve cells and immune cells [25-27].

The major role for white adipose tissue is to store dietary lipids as triacylglycerides (TAGs) in the lipid droplet, but also to de novo synthesize some fatty acids (FAs) from glucose. Energy storage in adipocytes is principally governed by two hormonal influences: insulin and catecholamines (adrenalin and noradrenalin) [22, 28]. In adipocytes, insulin promotes uptake of glucose and fatty acids after a meal has been ingested, while simultaneously promoting de novo fatty acid synthesis as well as esterification of FAs into triacylglycerides (TAGs) in a process known as lipogenesis [22]. In the same tissue, catecholamines released from the adrenal glands mediate the hydrolysis of these high-energy TAGs into FAs and glycerol between meals, so that the FAs can be transported to other tissues, oxidized and thus ultimately used to generate ATP [28]. Once another meal is ingested, insulin exerts an anti-lipolytic effect, returning lipolysis to basal levels, and once more causing utilization of glucose and FAs for TAG storage [16, 22].

Different adipose tissue depots exhibit different properties, and the depot that is considered to be the most relevant in a diabetic context is the visceral fat, which more or less coats the internal organs [29, 30]. This particular white adipose tissue acts as an endocrine organ, secreting hormonal factors known as adipokines. Adipokines can be secreted from adipocytes or from immune cells within the tissue, and they can exert positive influences (for example accomplished by leptin and adiponectin) or negative influences (for example attributed to resistin and TNF-α) on other metabolic

organs [30, 31]. As an example, hypertrophic adipocytes secrete factors that attract immune cells and low-grade inflammation is actually common in obese individuals [2]. Immune cells in turn secrete TNFα that cause decreased glucose uptake as well as increased release of FAs from adipocytes through several mechanisms. Abnormally high levels of circulating FAs result in ectopic fat accumulation in muscle and liver and can cause cell dysfunction and cell death in the insulin-producing pancreas (lipotoxicity) [14, 32, 33]. In addition to the release of excessive amounts of FAs, other adipose-derived lipid derivates (such as for axample ceramides) play a direct role in attenuation of insulin signaling, contributing to insulin resistance [14, 33]. In contrast, healthy adipose tissue secretes the adipokine adiponectin, which activates AMPK, an activation that has positive effects on insulin sensitivity in insulin target tissues [34]. Taken together, these are examples of how unhealthy adipose tissue can contribute to insulin resistance and how healthy adipose tissue can serve a protective role. Lately, subcutaneous fat has received a high degree of attention, and recent findings suggest that when this adipose depot fails to adequately store fat, this leads to storage of lipids in the omental depot as well as in muscle and liver, and subsequent development of insulin resistance [29, 35].

Studies of adipose tissue in healthy and obese individuals, storage of triglycerides and its regulation by hormonal signaling, as well as the role for kinases in these processes are of great importance as there are direct causal relationships between adipose tissue dysfunction and the development of insulin resistance.

## Protein kinases

Signaling pathways that control metabolic processes, such as lipolysis, anti-lipolysis, glucose uptake or fatty acid synthesis always involve one or more kinases and these enzymes are often a point of cross-talk between signaling pathways. The study of kinases in metabolism therefore constitutes a strategy when searching for pharmacological ways to treat obesity and diabetes.

During the 90s, sequencing of the human kinome was a major ongoing mission, culminating in the finalization of the project in the beginning of the 21st century. Quickly after its mapping, the details concerning the genes encoding all human kinases were revealed, and the subset of genes were referred to as the human kinome. Not counting the non-functional pseudogenes that once encoded kinases, there are 500 plus kinases encoded by the human genome, approximately 70 of which had not been discovered before the human genome project. It is a large number; kinases comprise 1,7 % of the human genome, and it is estimated that one third of all cellular proteins are phosphorylated at any given time [17].

Most kinases exhibit similar catalytic domains, with a substrate-binding pocket where monophosphate can be transferred to an amino acid residue on a protein substrate from ATP molecules. Some protein kinases specifically phosphorylate serine and threonine residues on substrates, whereas others are tyrosine or histidine specific kinases. Some kinases even exhibit dual specificity, for example phosphorylating both serine and threonine residues as well as tyrosine residues [36, 37].

Phosphorylation can result in activation or de-activation of other kinases or enzymes, target substrates for a new subcellular location or create/expose binding sites on substrates for other regulatory proteins or lipids. The study of protein kinases in cells can prove challenging, as there is a certain degree of shared substrate preference, redundancy and cross-talk.

Many kinases are kept relatively inactive by their own structure in an auto-inhibitory manner, until another kinase or regulating agent causes a conformational change that renders the kinase fully active [38].

The phosphorylation process governed by kinases is counteracted and balanced by the actions of protein phosphatases that dephosphorylate protein substrates, so that no phosphorylation is entirely permanent [38]. The concerted actions of kinases and phosphatases allow for cellular signaling that can be highly specialized and fine-tuned. Perhaps this is why kinases are often considered suitable drug targets.

Kinases play a direct role in the signaling that underlies the synthesis and release of lipids. In addition to this role, some kinases participate in attenuation of the insulin signal, while some exert a positive influence (for example AMPK), thereby enhancing insulin sensitivity.

# Structure, expression and regulation of PKB, AMPK and SIK3

## Protein kinase B (PKB)

PKB, also known as Akt, is a conserved member of the AGC group of kinases (after PK*A*, PK*G* and PK*C*) [39]. It was discovered in the 70s in a tumorigenic virus, and characterized as an oncogene [40]. After years of intense research it has been shown that PKB participates in the control of a diverse array of cellular processes, such as proliferation, survival, cell growth and energy metabolism and that it can be regulated by growth factors, such as insulin and insulin-like growth factor 1 (IGF1) [41, 42].

Three distinct genes on different chromosomes encode three isoforms of PKB; PKBα (Akt1), PKBβ (Akt2) and PKBγ (Akt3). These isoforms exhibit a degree of homology

that is higher than 80 % [18, 43], yet they seem to elicit highly specific cellular responses in different tissues and even within the same cell type [44]. PKBα is ubiquitously expressed, PKBβ primarily expressed in insulin-sensitive tissues whereas PKBγ is mostly expressed in brain and testis [45, 46]. The specific expression pattern and function of PKB isoforms is substantiated by isoform-specific knock-out (KO) mice, as targeted deletion of PKBα results in impaired fetal growth [47], deletion of PKBβ results in hyperglycemia, impaired insulin response in skeletal muscle, fat and liver and underdeveloped fat mass [48, 49], and targeted deletion of PKBγ results in impaired brain development [45].

PKB consists of an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal domain. The C-terminal domain contains a hydrophobic motif that is common to all AGC kinases [18]. In order to achieve full activation of PKB, two residues, S473 and T308 (in PKBα), must be phosphorylated. S473 is located in the C-terminal domain and T308 in the catalytic domain. The kinase responsible for S473 phosphorylation is the rapamycin-insensitive companion of mTOR (Rictor)-complex mTORC2, whereas T308 phosphorylation can be attributed to phosphoinositide-dependent kinase 1 (PDK-1) [50]. Phosphorylation of S473 is considered more crucial as it stabilizes PKB in an active conformation that resembles the constitutively phosphorylated and activated catalytic subunit of fellow AGC kinase PKA [43].

## Amp-activated protein kinase (AMPK)

AMPK is a protein kinase that senses changes in cytoplasmic energy levels, directing the cell towards energy generating processes, while at the same time halting energy consuming processes [51]. AMPK is conserved in virtually all eukaryote organisms [52].

Structurally AMPK functions as a heterotrimeric complex consisting of α-,β- and γ-subunits, each isoform encoded by several genes, namely α1 and α2, β1 and β2 and γ1, γ2 and γ3. Considering the different isoforms, as well as the possiblity of alternative splice variants, a wide array of AMPK combinations are possible and the expression of these do indeed vary between species and same-species tissues [53]. The α- and β-subunits are the most conserved among species, whereas the γ-subunit displays a greater degree of variation [52].

The α-subunit, also known as the catalytic subunit, contains a classical serine/threonine kinase domain and an activation loop with a particular threonine residue, T172, hat can be phosphorylated by the constitutively active upstream kinase LKB1 when it is complexed to STE20-related adaptor (STRAD) and mouse protein 25 (MO25) [53, 54]. The regulatory β-subunit contains a carbohydrate-binding motif (CBM) that enables AMPK to bind to glycogen [51]. The γ-subunit, also known as the regulatory subunit, contains four tandem repeats of a CBS motif

(named after cystathionine beta synthase, the protein where it was discovered) that can bind adenine nucleotides. The first and third CBS motif can bind AMP, ADP and ATP, whereas the second remains unoccupied at all times and the fourth can only bind AMP tightly (this may have a structural role) [52]. In adipocytes, AMPKα1β1-containing complexes appear to be the dominating complexes [55-57].

In addition to the constitutive activation by LKB1, activation of AMPK is greatly enhanced by binding of AMP to the γ-subunit, which causes a conformational change that promotes T172 phosphorylation (and hence AMPK activation), prevents T172 dephosphorylation (and hence AMPK de-activation) as well as yielding an allosteric activation of AMPK, in addition to the activation caused by phosphorylation. ADP can also compete with ATP for binding to the γ-subunit in a similar manner to AMP, but this binding only elicits the two first mechanisms of AMPK activation, not adding any further allosteric activation of AMPK [52]. AMPK can also be activated by Ca2+/calmodulin-dependent protein kinase β (CAMKKβ) [52, 55]. As Ca2+ influx often results in energy-consuming processes, this may be a way for the cell to anticipate a requirement for ATP [52].

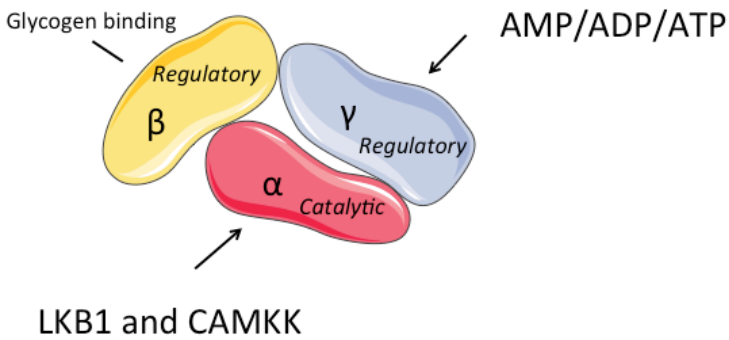


**Figure 1. Schematic drawing of AMPK subunits and their regulation**
The α-subunit harbors the T172 site which is phosphorylated and constitutively activated by LKB1 and in response to Ca2+ ions by CAMKK. The β-subunit contains a carbohydrate-binding motif which can bind glycogen. The γ-subunit contains four tandem CBS motifs that are either structurally important or can bind AMP, ADP and ATP, the first two resulting in increased AMPK kinase activity.

Several physiological conditions, pharmaceutical agents and antioxidants can activate AMPK. Among these are muscle contraction and stressors such as hypoxia and glucose deprivation [58, 59]. Drugs that activate AMPK are metformin and its predecessor phenformin, as well as thiazolidindiones, all of which are current or past treatments of type 2 diabetes [60]. As for antioxidants, resveratrol found in red wine, has been found to have an activating impact on AMPK [61].

Since its discovery in the early 70s [62, 63], there has been continous interest in discovering ways in which hormonal stimuli can regulate AMPK, and there are some examples: AMPK is rendered more active by the adipokine adiponectin and by the gut-derived hormone ghrelin [64, 65]. Additionally, AMPK can be activated by catecholamines in adipocytes [66, 67]. Another example is the recent discovery that

AMPK can be inhibited by insulin. This mechanism of deactivation occurs in heart muscle cells, where insulin can deactivate AMPK both in normoxic and ischemic conditions [68, 69]. Since the discovery of this regulation, it has been shown that the deactivation of AMPK that is seen in heart muscle cells in response to insulin depends on PKB phosphorylation of S485 on AMPK [69]. In addition, a similar means of regulation of AMPK seems to be in play in vascular smooth cells [70].

AMPK has been implicated in the regulation of adipocyte metabolism, findings that will be descirbed in the section that covers hormonal regulation of lipid metabolism.

## Salt-inducible kinase 3 (SIK3)

AMPK is an evolutionarily conserved kinase, with a crucial and ancient role in cellular energy metabolism [52]. It is a member of the AMPK-related kinases, a family of kinases that have evolved along the same evolutionary pathway [20]. One could imagine metabolic roles for these kinases as they share similar structural elements with AMPK.

The Salt-inducible kinases (SIKs) constitute a relatively unexplored branch in the AMPK-related kinase family tree. SIK1 was discovered in the adrenal glands of rats that had been fed a high-salt diet [71], and two additional SIK isoforms, SIK2 (also known as QIK) and SIK3 (also known as QSK) were discovered in subsequent homology searches [72].

In a similar manner to AMPK, all SIKs require phosphorylation by the constitutively active kinase LKB1, on a T-loop residue that corresponds to T172 in AMPK (T221 in SIK3), in order to be rendered active [73]. For SIK1 and SIK3, this phosphorylation also mediates binding of 14-3-3 proteins, a phenomenon that appears to be important for their activity as well as for the punctate cytosolic distribution of SIK3 [74]. 14-3-3 proteins are scaffolding proteins that bind to phosphorylated residues on a multitude of cellular targets. The main result of such an interaction is a change in the subcellular localization of the target protein or facilitated interaction with other proteins [75].

Structurally, in addition to their N-terminally located catalytic domain, the SIKs contain a ubiquitin associated (UBA) domain. This domain likely plays a structural role as it is necessary for LKB1 phosphorylation [76]. The SIKs do not bind ubiquitin molecules in vitro [76], however recent findings suggest that SIK1 can bind ubiquitinated proteins and localize to ubiquitin clusters [77] and it is known that mutation of residues in this domain results in loss of the punctate nuclear distribution of SIK1 [76]. SIK1 contains a C-terminal nuclear localization signal (NLS), whereas SIK2 and SIK3 do not [78, 79]. SIK1 is the smallest isoform (approximately 85 kDa, 783 aa), SIK2 being slightly larger (approximately 104 kDa, 926 aa) while SIK3 is the

largest (150 kDa, 1368 aa). The SIKs display great sequence homology in their kinase- and UBA domain, whereas their C-terminal regions differ to a higher degree.

SIK1 is highly expressed in adrenal glands [78, 80] and SIK2 in adipocytes [81, 82], whereas SIK3 exhibits ubiquitous expression [20]. However, tissue-specific expression levels do not necessarily reflect the function and relative importance of a kinase in the particular tissue.

As is often the case when new proteins are being explored, the reports on regulation and role for the SIKs derive from a wide array of fields. Apart from several reports of roles for SIKs in cancer (both causal and protective roles) [83-87], SIK1 has been implicated in steroidogenesis and sodium transport [78, 80, 88-90]. Altered expression levels of SIK2 has been demonstrated in mouse models of diabetes and obesity, and this isoform has been implicated in brown adipocyte thermogenesis and in the regulation of gluconeogenesis and insulin secretion [82, 91-94]. SIK3 in turn has been suggested to play a role in cartilage formation [95]. A recent mouse model deficient in SIK3 also suggests a role for the kinase in glucose- and lipid metabolism in the liver as well as a role in cholesterol and bile acid homeostasis [96]. SNPs in the gene encoding SIK3 also appear to correlate with reduced clearance of blood TAGs in humans [97].

Most studies have failed to demonstrate any regulation of SIKs by AMP [73, 81, 98], and LKB1 activates these kinases in a constitutive manner, which suggests that some other mechanism must be responsible for modulation of SIK kinase activity. Initially there was no known example of an external stimuli that could regulate SIK3. SIK1 was however reported to be regulated by adrenergic stimuli in adrenal glands, a regulation resulting in PKA-mediated phosphorylation of a particular serine residue, (murine) S577, localized in the nuclear localization signal in the kinase. As predicted, this means of regulation of SIK1 results in its shuttling from the nucleus to the cytosol [78]. As the cyclic AMP (cAMP)-regulated serine residue on SIK1 is conserved in all SIKs (S551 SIK3) and as cAMP has a paramount role in lipid metabolism in adipocytes [16], it would be of interest to examine a possible regulation of SIK3 by adrenergic stimuli in adipocytes. For SIK2, three other sites, S343, S358 and T484, appear to be regulated by cAMP/PKA (in addition to S587, which corresponds to S577 in SIK1) [98]. Additionally, a SIK-equivalent kinase in drosophila was shown to be regulated by insulin, controlling the energy balance of the fly [99].

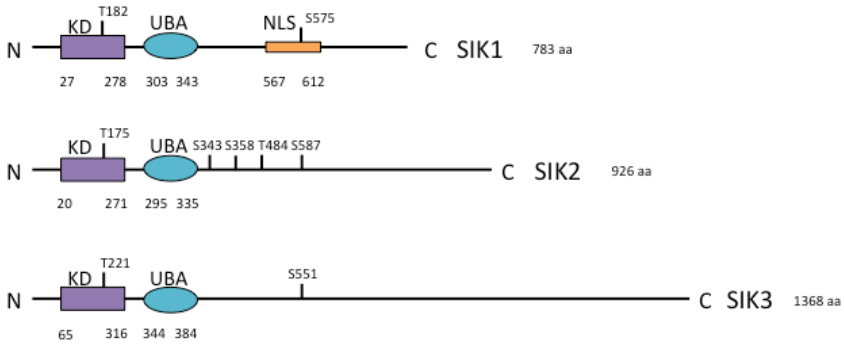


**Figure 2. Structure of human SIK isoforms**
The N-terminal kinase domain harbors the activation loop (T-loop) of the kinases with the critical residue controlled by LKB1. All kinases also have a UBA domain, important for the structural integrity of the kinase and shown to bind ubiquitin molecules (SIK1). SIK1 also contains a nuclear localization signal. S575 (SIK1), S587 (SIK2) and S551 (SIK3) are conserved sites and the S575 in SIK1 is phsophorylated in response to elevated cAMP levels and mediates nucleocytoplasmic shuttling. For SIK2, three additional sites, S343, S358 and S484, appear to be regulated by cAMP.

Common suggested substrates for the SIKs are proteins that belong to the class II histone deacetylase (HDAC) and CREB-regulated transcription co-activator (CRTC) protein families. In unstimulated conditions, CRTCs are phosphorylated by SIKs, promoting their binding to 14-3-3 proteins and causing their retention in the cytosol [100, 101]. One hypothesis regarding cAMP-mediated regulation of SIKs, is that they become less active towards CRTC substrates, so that these co-activators are free to enter the nucleus and to participate in the regulation of CREB target gene transcription [100, 102]. A similar mechanism of cytosolic retention by the SIKs is suggested for the class II HDACs [103, 104].

# Hormonal regulation of lipid storage in adipocytes

## Insulin-induced glucose uptake in white adipocytes

Insulin binds to tetrameric insulin receptors in target tissues. Binding of insulin to the α-subunits of the receptor will cause a rapid conformational change that stimulates the intrinsic tyrosine kinase activity of the two β-subunits. Autophosphorylation of the insulin receptor takes place and the insulin signal is propagated, as insulin receptor substrate (IRS) proteins are recruited to the phospho-tyrosine motifs on the receptor, and phosphorylated [105]. There are several IRS proteins, but from a metabolic perspective IRS1 and -2 are the most important, IRS1 being the main isoform in adipocytes and muscle, and IRS2 being the predominant isoform in the liver [106]. Tyrosine phosphorylation of IRS proteins creates binding sites for signaling molecules that contain src homology (SH2) domains, such as phosphoinositide 3 kinase (PI3K). This kinase in turn transmits the insulin signal by phosphorylating membrane-bound lipids. Its preferred substrate is phosphatidylinositol 4,5 bis-phosphate (PIP2), which once phosphorylated is converted to phosphatidylinositol 3,4,5 tris-phosphate (PIP3), that can bind to proteins containing a pleckstrin homology (PH) domain, such as 3-phosphoinositide-dependant kinase 1 (PDK1) and its downstream target PKB [50]. PKB and PDK1 are recruited to the membrane where PIP3-binding causes a conformational change in PKB that allows for full activation of PKB by upstream kinases by phosphorylation on S473 and T308 [107]. T308 phosphorylation induces a catalytically active conformation, which S473 stabilizes [50].

Even though the target tissues are many and the biological responses to insulin signaling are diverse, the insulin signaling pathway is quite conserved up until the point of PKB activation. This is the critical node in the pathway, where one single hormone can accomplish a multitude of cellular responses.

The fact that cells can take up glucose from the bloodstream, thus clearing the blood of high levels of glucose after a meal, is made possible by the glucose transporter (GLUT) family of hexose transporters. The ubiquitously expressed GLUT1 serves as a basal glucose transporter that can be found at the plasma membrane and in intracellular endosomes [108]. GLUT1 can be regulated allosterically by ATP [109]. Hepatocytes and β-cells also express GLUT2, with a relatively low affinity for glucose. GLUT4 is another GLUT isoform that is expressed in muscle and adipocytes and this particular transporter is considered the major insulin sensitive glucose transporter in the body, with a high affinity for glucose [108]. Insulin promotes glucose uptake via GLUT4 by increasing the number of GLUT4 molecules at the plasma membrane by translocation of GLUT4 from intracellular vesicles to the plasma membrane [110]. There is always some degree of general protein recycling between endosomes and the plasma membrane [111]. However, GLUT4 translocation is mediated by additional

mechanisms, as a large number of GLUT4 molecules are sequestered in GLUT4-containing small vesicles (GSVs) until insulin causes their fusion with the membrane in a manner similar to exocytosis [112]. Once glucose has been taken up into cells, such as muscle cells and adipocytes, hexokinase rapidly phosphorylates the glucose molecule, in order to ensure a low intracellular glucose level, and a gradient that favors glucose influx [110].

In adipocytes and in muscle, insulin-mediated regulation of glucose uptake via GLUT4 molecules is mediated by PKB. The specific mechanisms of the regulation remain elusive, but one PKB substrate, TBC1D4, also known as Akt substrate of 160 kDa (AS160), seems to play a part. It is believed that TBC1D4, which contains a GTPase activating domain (GAP domain), negatively regulates an unknown member of the Rab GTPase family in unstimulated conditions, causing retention of GSVs in the cytosol [113]. Once phosphorylated by PKB, TBC1D4 interacts with 14-3-3 and is rendered less active and the GLUT4-containing vesicles can fuse with the plasma membrane [114-117]. TBC1D4 has been found to co-localize with GLUT4 in GSVs [113].

Interestingly, in muscle, AMPK might also be involved in GLUT4 translocation by phosphorylation of TBC1D isoforms (but perhaps most likely the TBC1D1 isoform), as AMPK has been implicated in increased TBC1D phosphorylation and increased glucose uptake [118][119]. In other words, two distinct pathways could possibly converge on a mutual target substrate in muscle [120]. It would be valuable in the context of insulin resistance, if the machinery responsible for translocating GLUT4 to the membrane remained intact in the pathological state.

Contrary to findings in muscle, AMPK does not appear to increase glucose uptake in adipocytes. In fact, the evidence to date points in the opposite direction, as AICAR-mediated activation of AMPK seems to result in decreased insulin-induced TBC1D4 phosphorylation as well as glucose uptake in both 3T3L1 adipocytes and in primary rat adipocytes [121, 122].

Even though muscle is the main site for insulin-stimulated glucose uptake, the glucose uptake that takes place in adipocytes appears to be important for maintenance of normal whole-body glucose homeostasis, because reduction of GLUT4 expression specifically in adipose tissue results in insulin resistance in muscle and liver, as well as glucose intolerance [123].

## Insulin-induced de novo fatty acid synthesis and lipogenesis in white adipocytes

Insulin is the most important hormone when it comes to regulation of synthesis of new FAs (known as de novo fatty acid synthesis) in the liver and adipose tissue, and the synthesis of TAGs from synthesized and imported FAs, and glycerol (known as lipogenesis) in adipocytes [22].

The TAGs that make up the lipid droplet in adipocytes are synthesized from FAs of multiple origin. Either these FAs derive from synthesis in the liver, where they are packaged to proteins to form very low density lipo-proteins (VLDL) [124]. These VLDL-particles are hydrolyzed by lipoprotein lipases (LPL) at the adipocyte cell surface before FA uptake [125]. The FAs can also derive from the diet (chylomicron delivery) or be synthesised in adipocytes in a process that begins with acetyl Co-A molecules, originating from glucose [22, 126]. Glucose enters the glycolytic pathway and the resulting product, pyruvate, in turn enters the citric acid cycle in the mitochondrion [127]. When energy (ATP) levels are low, pyruvate continues through the citric acid cycle in order to generate ATP. However, in times of energy (ATP) abundance, isocitrate dehydrogenase, one of the enzymes participating in the citric acid cycle, is inhibited by ATP. Citrate exits the mitochondrion and is cleaved by ATP citrate lyase (ACL) to form acetyl-CoA and oxaloacetate [22].

FA synthesis involves step-by-step incorporation of carbons from acetyl-CoA molecules into a growing FA chain. The first and rate-limiting step in this process is the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, a reaction catalysed by acetyl-CoA carboxylase (ACC) [128]. There are two isoforms of ACC; ACC1 and ACC2, which generate two separate pools of malonyl-CoA. ACC1 is the isoform that is responsible for generating malonyl-CoA destined for continued de novo FA synthesis [129]. The growing FA chain is elongated by fatty acid synthase (FAS) until it reaches its end product, the 16-carbon FA palmitate [130]. As palmitate is a saturated FA, and the key substrates for TAG synthesis are unsaturated, palmitate is converted to an unsaturated FA by stearoyl-CoA desaturase 1 (SCD1) [131].

The synthesis of TAGs occurs in the cytosol and involves esterification of FAs to glycerol-3-phosphate by the enzymes monoacylglycerol acyltransferase (MGAT) and diacylglycerol acyltransferase (DGAT) [132]. The glycerol component of TAGs is generated from the first steps of glycolysis, which means that glucose is a substrate for both FA synthesis and for glycerol synthesis [133].

Insulin is an anabolic hormone and it exerts positive influences on de novo FA synthesis and lipogenesis in many ways. First of all, it stimulates glucose uptake, providing an ample supply of substrate for both FAs and glycerol. Insulin signaling also results in increased expression of FAS and ACC1, key enzymes involved in lipogenesis, and in a net dephosphorylation of ACC1 on S79 [22, 121, 134, 135].

What is also known about the regulation of ACC1 is that it is phosphorylated by AMPK on S79, a fact that results in deactivation of the enzyme [136]. Insulin results in dephosphorylation of ACC1 on S79, however the underlying mechanism for this hormone-mediated regulation remains to be discovered.

The main site for de novo FA synthesis is the liver. In humans, the contribution of adipose tissue is considered to be smaller than in rodents, only amounting to 2 % of whole-body de novo FA synthesis [22]. However, one study suggests that 20 % of FAs in newly stored TAGs derive from de novo FA synthesis [137]. This amount is far too large to be attributed solely to the liver, as only 2-10 % of FAs in VLDL derive from synthesis in the liver [22]. The residual newly synthesized FAs that are stored in newly-formed TAGs likely derive from adipocytes. In other words, the contribution of de novo FAs synthesized in adipose tissue to whole body de novo FA synthesis in humans does not seem negligible.

## Catecholamine signaling, lipolysis, and the anti-lipolytic role of insulin in white adipocytes

The hydrolysis of stored TAGs in the adipocyte lipid droplet is stimlated by catecholamines (such as adrenalin and noradrenalin), which are released from the adrenal glands between meals [138].

Catecholamines bind to β-adrenergic receptors on the plasma membrane of adipocytes (primarily β3-receptors in rodents, and β1- and β2-receptors in humans) [16] and elicit activation of adenylate cyclase (AC) molecules through a mechanism that involves a G-stimulatory protein. Activated adenylate cyclase subsequently gives rise to a large amount of the second messenger cAMP)[21]. cAMP molecules can bind to the two regulatory subunits of protein kinase A (PKA). This causes a conformational change that allows the regulatory subunits to dissociate from the two catalytic subunits, resulting in a dimeric regulatory unit and two active and separate catalytic subunits [38].

PKA proceeds to phosphorylate two key proteins in the lipolytic pathway, perilipin 1 and hormone sensitive lipase (HSL) on multiple sites [16]. Lipid droplets are coated with what can almost be described as a protective “skin”, which is made up of group of PAT (perilipin, adipophilin/adipocyte differentiation-related protein (ADRP), and tail-interacting protein of 47 kDa (TIP47)) proteins [16], and perilipin proteins are important members of this group of proteins. Perilipin 1 is the pre-dominant isoform in white adipocytes and it limits the access of cytosolic lipases, such as HSL, to TAGs, by participating in the protective coating of the lipid droplet [21].

Coactivator comparative gene identification 58 (CGI-58) is a protein that binds tightly to perilipin 1 in basal and unstimulated conditions. When perilipin 1 is

phosphorylated by PKA, it causes exposure of the lipid droplet to lipases. In addition, PKA-mediated regulation of perilipin 1 results in the release of CGI-58 to the cytosol where it can bind to adipose triglyceride lipase (ATGL), acting as a cofactor. Recently, it has been shown that PKA can phosphorylate ATGL in a direct manner, but the physiological relevance of this regulation remains to be substantiated [139]. ATGL-CGI-58-complexes localize to the lipid droplet along with HSL, which translocates to the lipid droplet upon the direct phosphorylation by PKA [140]. ATGL and HSL proceed to hydrolyse triacylglycerides into diacylglyceride (DAG) and one FA, and monoacylglyceride (MAG) and one FA, respectively. These two lipases are considered the most important for lipolysis, accounting for approximately 95 % of lipolysis. However, an additional lipase, monoacylglyceride lipase (MAG), hydrolyses the final FA from monoacylglyceride, releasing a free glycerol moiety. Fatty acid binding protein 4 (FATB4), which binds to exposed FAs associate with HSL at the lipid droplet surface, participates in the transport of FAs from the lipid droplet to the plasma membrane [16]

FAs are transported to the bloodstream by fatty acid transporters and used as energy substrate in target tissues [16], and the glycerol molecule leaves the adipocyte through members of the aquaporin family of transporters [141] and is primarily used in the liver for gluconeogenesis [21].

Interestingly, HSL can be phosphorylated by AMPK and CAMKK on S565, a site believed to be mutually exclusive to the phosphorylation achieved by PKA, resulting in an anti-lipolytic effect [16].

In adipocytes, insulin exerts a counteracting, anti-lipolytic action on catecholamine-induced lipolysis in the post-prandial state. This is accomplished by phosphorylation and activation of the main phosphodiesterase expressed in adipocytes, phosphodiesterase 3 B (PDE3B). PDE3B hydrolyses cAMP, resulting in reduced activity of PKA, HSL and renewed tight binding of perilipin to the lipid droplet, allowing the lipolytic process to revert to basal levels [16]. Insulin also phosphorylates and activates PP-1, the phosphatase that mediates dephosphorylation of HSL, and mediates down-regulation of ATGL and HSL expression.

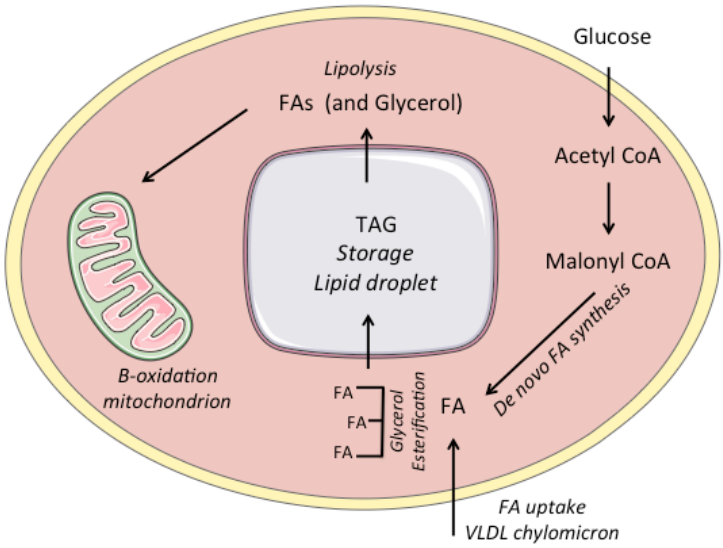


**Figure 3. Overview of lipid storage in adipocytes**
When glucose is abundant in the blood, glucose is taken up by adipocytes and used as a substrate for de novo FA synthesis (and for glycerol synthesis). FAs can also be taken up from the blood and along with FAs synthesized in the adipocyte are esterified to glycerol units that can harbour three FAs, creating high-energy TAGs. Between meals, TAGs are hydrolyzed into FAs and glycerol and the FAs can enter the mitochondrion for β-oxidation.

# Aims

The general aim of the thesis has been to analyze the role for PKB and AMPK in adipocyte metabolism as well as the ability of AMPK and its relative SIK3 to respond to hormonal signals in adipocytes. The following bullets describe the more particular aims:

- To determine whether PKB activity is required for the effects of insulin on lipid metabolism in adipocytes.
- To investigate whether AMPK is under the influence of insulin regulation in adipocytes
- To investigate the role for AMPK in glucose- and lipid metabolism in adipocytes using a direct pharmacological activator
- To investigate the regulation of SIK3 in response to extracellular signals in adipocytes

# Models, tools and methods

## Primary cells and cultured cell lines

Adipose tissue was dissected from the epididymal fat pads of 6-8 week old male Sprague-Dawley rats and digested for a carefully monitored amount of time in a collagenase solution, followed by adipocyte isolation. Isolation was accomplished by gentle centrifugation of the tissue digest, as fat cells are light and even have the ability to float. We used a buffer that contained adenosine, which exerts a negative influence on lipolysis, and BSA that bind excess FAs, as large amounts of FAs are known to have a toxic effect on adipocytes.

3T3L1 cells are pre-adipocytes derived from mouse embryonic cells that under a defined protocol will differentiate into adipocyte-like cells [142]. One morphological difference between this cell-line compared to primary adipocytes from rats or humans is that as the cells start to accumulate lipids, several lipid droplets are formed, as opposed to the singular droplet seen in primary adipocytes [140]. However, the advantage of this cell-line is their ability to be kept in culture for several days. Primary cells must be used within the first day or so, as they become insulin resistant during extended culture.

HEK293 cells, a cell-line derived from human embryonic kidney cells, were used for overexpression experiments for SIK3. HEK293 is a commonly used model cell-line that is easily cultured and transfected in comparison to both primary- and cultured adipocytes.

## In vitro protein kinase activity assay

In vitro protein kinase assay is a valuable and quantitative method for analysis of changes in the intrinsic enzyme activity of a specific kinase in response to extracellular stimuli, inhibitors, activators or mutations of the kinase. The kinase in question is immunoisolated from cell lysates with a specific antibody, and allowed to react for a fixed amount of time with radioactivelly labelled ATP and a peptide- or protein substrate. Different amounts of protein kinase and antibody were tested during the initial stages of experiments, to ensure that the amount of kinase was the only

parameter limiting the rate of phosphorylation. Phosphorylated substrate peptides were stopped by application on cation exchange paper and immersion in phosphoric acid (in order to wash away non-specific phosphate binding) and the emission of radioactivity was measured by liquid scintillation counting.

## Autoradiography

In order to further substantiate changes in SIK3 activity in response to adrenergic stimuli, we analysed SIK3 activity towards a full-length substrate (CRTC2) expressed and purified from E.Coli by autoradiography (paper II). In this method SIK3 was allowed to react in vitro with the full-length substrate and radioactivelly labelled ATP. The combined reaction was subjected to polyacrylamide gel electrophoresis, colloidal coomassie staining, subsequent fixing and drying and finally developed using an image plate that captures the radioactive emission of β-particles from the bands on the gel using a phosphorimager. When SIK3 phosphorylates a substrate that is in its native conformation, the reaction more closely resembles the actual cellular event. This method is sensitive to the possibility that some structural component of CRTC2 could be important for its regulation by SIK3.

## Localization studies

As the activity of several AMPK-related kinases are regulated by re-localization within the cell, we speculated whether such a mechanism of regulation applied to SIK3. Analyzing changes in subcellular localization is made difficult in primary adipocytes, as the cytoplasm and organelles are forced to the plasma membrane by the large drop of stored TAGs. In order to fully evaluate the possibility of a subcellular translocation of SIK3 in response to cAMP-induction and subsequent 14-3-3 binding (paper II), we expressed wt and mutant versions of HA-tagged human SIK3 in HEK293 cells, which were stimulated with forskolin. We then used antibodies detecting the HA-tag on SIK3 combined with a fluorescent secondary antibody in order to visualize the subcellular localization of SIK3 in a confocal microscope. Precautions were taken to make sure that these antibodies did not result in visualization of unspecific protein binding. SIK1 localization was analyzed in a similar manner, as this kinase is known to undergo nucleocytoplasmic shuttling in response to forskolin.

## Phosphorylation site analysis

In order to establish the particular phosphorylated residues on SIK3 in response to β-adrenergic stimulation, human HA-SIK3 was expressed in HEK293 cells, which were stimulated with forskolin or left untreated. HA-SIK3 was immunoprecipitated and the resulting immunoprecipitates were loaded on a polyacrylamide gel, which was stained and the bands excised and trypsinized. The tryptic digest was analyzed by LC-MS on a LTQ-Orbitrap mass spectrometer system. This allowed us to determine which particular residue was phosphorylated. We could also determine which 14-3-3 isoforms that could bind to SIK3, by analyzing excised bands of co-immunoprecipitated 14-3-3 protein.

As a means of separating proteins in a cell lysate or detecting immunoisolated proteins we performed western blotting, where whole-cell lysates or immunoprecipitates of proteins were subjected to polyacrylamide gel electrophoresis (separating proteins of different sizes) and subsequent electrotransfer to a nitrocellulose membrane. Protein bands were detected with antibodies that were raised against phosphorylated or non-phosphorylated peptide sequences from the protein of interest. All antibodies were tested for efficiency and specificity. We also employed antibodies that could recognize consensus motifs for protein kinases. Such consensus antibodies can sometimes exhibit unspecific binding, as kinases belonging to the same family of kinases may phosphorylate similar consensus motifs. Also, consensus antibodies can display varying binding affinities for different phosphorylated sites, depending on how well theses sites correspond to the consensus motif that the antibodies were raised against. Proteins bands were visualized by treatment with enhanced chemiluminescence (ECL) reagent. In some figures that concern 14-3-3, the interaction of specific proteins with 14-3-3 was studied by incubating the membrane with recombinant GST-14-3-3 protein, followed by detection of 14-3-3 with anti-GST antibodies.

## Glucose- and lipid metabolism in adipocytes

Fatty acids and glycerol are synthesized from glucose. The resulting FAs are incorporated into TAG molecules by esterification to glycerol molecules. When measuring lipid synthesis we used radioactively labeled glucose or acetate. When using glucose, one should keep in mind that glucose is the substrate for both FAs and glycerol (that make up TAGs along with FAs). Thus, using glucose in this assay means measuring both incorporation of both newly synthesized fatty acids into TAGs, and it has in fact been shown that the majority of glucose is incorporated in the glycerol moiety [133]. This assay is therefore not a sensitive method for measuring de novo FA synthesis, but rather reflects the rate of glucose uptake into the cells. Using acetate as a substrate for lipid synthesis, means that all the detected

radioactivity originates from newly synthesized FAs, as the acetate can only be incorporated into Acetyl-CoA molecules that form the starting point of synthesis of new fatty acids. The radioactivity was measured by liquid scintillation counting. Using a toluene-based scintillation liquid enabled separation of the lipid phase (containing TAGs or FAs) and the aquatic phase (containing radioactive substrates that had not been incorporated into lipids) [143].

Basal and catecholamine-induced lipolysis was measured in a quantitative manner with an enzyme-based assay. After stimulation of the adipocytes with a β-adrenergic stimulus, the cell medium was removed and the amount of glycerol released into the cell medium was measured in an enzymatic assay [144].

In adipocytes, GLUT1 is the glucose transporter responsible for basal glucose uptake, whereas GLUT4 accounts for the insulin-stimulated glucose uptake. In the glucose uptake assay used in paper III, radioactively labeled glucose was used to measure the uptake of glucose. The radioactivity was measured by liquid scintillation counting. Cytochalasin B, a poison that competes for the glucose-binding site on glucose transporters, thus blocking glucose transport, was used to assess the amount of radioactive glucose found in the cell fraction, but which has not entered the cells. This value was subtracted from all other values.

## PDE3B activity assay

The enzymatic activity of PDE3 acitivity with radiolabelled cAMP as a substrate [145]. Radioactive AMP, resulting from the hydrolysis of radioactive cAMP was measured using scintillation counting.

## Pharmacological manipulation of protein kinases

Manipulating the activity of a kinase with pharmacological agents is difficult as many kinases can phosphorylate similar substrates, and as they all use ATP in order to phosphorylate substrates and often have the ability to compensate for each other's lack of action. However, when used in an experimentally appropriate setting with adequate controls, an inhibitor or activating agent can provide valuable information as to the role for a particular kinase in metabolism. Furthermore, pharmacological manipulation of kinases is advantegous over genetic approaches as the latter are often introduced during embryogenesis and can produce phenotypes that are indirectly caused by the genetic manipulation. An inhibitor or activator should ideally bind directly and with high affinity to the kinase in question, act on said kinase in a specific manner and exhibit the ability to permeate cells [146].

## 5 aminoimidazole-4-carboxamide ribonucleoside (AICAR)

AMPK remains an interesting target for drug development [147]. 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) is an adenosine analog that is taken up by cells by adenosine transporters and converted into ZMP. ZMP mimics the AMPK-activating actions of AMP, by binding to the gamma subunit and promoting T172 phosphorylation, preventing T172 dephosphorylation and by activating the kinase in an allosteric manner, albeit in a much less potent manner [148].

## Phenformin

Phenformin is the precursor drug of Metformin, the current first drug of choice for treatment of type 2 diabetes. Phenformin was used for treatment of diabetic patients until it was withdrawn in the late 70s following the discovery that it causes lactic acidosis. It is more potent than metformin when it comes to activation of AMPK and it is still used in an experimental setting. Phenformin (and metformin) activates AMPK in a non-specific manner by inhibiting complex I of the respiratory chain, causing an intracellular increase in AMP to ATP ratio [149, 150]. Metformin has poor plasma membrane permeability, but its uptake is promoted by the organic cation OTC1 transporter OCT1, whereas phenformin is less dependent on this transporter [151].

## A769662

A769662 is a small molecule belonging to the thienopyridine family [152]. It activates AMPK in an allosteric, reversible manner, by binding to an (as of yet) unknown residue on the β1-subunit (thereby only activating AMPKβ1-containing complexes) and by protecting AMPK from T172 dephosphorylation [153-155]. As this activator acts in a manner that is independent from the activating actions of AICAR, these two activators can act in a synergistic manner [156].

## Akti

Akti is an irreversible inhibitor of PKBα and PKBβ that almost completely abolishes the activity of PKBα and PKBβ, and exhibits a 10- to 100-fold selectivity towards these PKB isoforms when compared to a large panel of other kinases, as well as upstream kinases involved in the insulin signaling pathway [157, 158]. In primary rat adipocytes and 3T3L1 cells, Akti inhibited the majority of, but was unable to abolish, the activity of PKBγ at similar concentrations as those required for PKBα and PKBβ inhibition. However, PKBγ is not the primary PKB isoform expressed in these cell

types. Akti requires the pleckstrin homology domain, as it prevents the conformational change that is induced by PIP3 in this domain and thereby the phosphorylation and activation of PKB by upstream kinases PDK1 and mTORC2 [157, 158]. Since this paper was published, a new oral allosteric PKB inhibitor has emerged, MK-2206 [159].

### H89 (N-2(-(bromocinnamylamino)ethyl)-5-isoquionlinesulfonamide)

H89 is a successor to previous H8 PKA inhibitors prouced by Calbiochem, only more potent. It is more selective for inhibition of cAMP-mediated activation of PKA than for cGMP-mediated inhibition [146]. It exhibits some degree of unspecificity [160], which is why analysis of other pathways involving kinases that phosphorylate similar consensus motifs as PKA were included in paper II.

### PD 0325901

PD 0325901 is a non-competitive and highly selective inhibitor of MAPK kinase-1, also known as MEK1, which is a Raf substrate and a target of anti-cancer drugs. It is more potent than its predecessors and even more potent when used in cell experiments than when used in vitro [161].

### Wortmannin

Wortmannin is a fungal metabolite that is relatively specific towards PI3K. It has some effect on PI4K, another member of the PI3K superfamily, as well as on myosine light chain kinase and polo-like kinase [162, 163], but as these kinases are not reported to be involved in insulin signaling and as Akti ½ has proven to be a very specific PKB inhibitor, this does not affect results presented in paper I.

# Main findings

## Paper I

- Akti is an effective and specific tool for PKB inhibition in primary rat adipocytes that does not affect upstream signaling components in the insulin signaling cascade.
- Insulin-induced activation of PDE3B in primary rat adipocytes and 3T3L1 adipocytes as well as insulin-induced anti-lipolysis in primary rat adipocytes are PKB-dependent processes, as they are reversed with increasing doses of Akti.
- Insulin induces a dephosphorylation of S79 in ACC, the rate-limiting enzyme in de novo lipogenesis. This event is PKB-dependent, as it can be reversed by pre-treating cells with Akti.
- Insulin induces an increase in lipogenesis, an event that is PKB-dependent, as it can be reversed by pre-treating cells with Akti.
- Insulin induces a phosphorylation of AMPK on S485, a modification that is associated with a decrease in the activity of AMPK. Both these effects are PKB-dependent, as they can be reversed with Akti.

## Paper II

- SIK3 is regulated by the β-adrenergic receptor agonist CL 316,243 in primary rat adipocytes. This causes an increase in PKA-mediated phosphorylation of SIK3 as well as increased binding of SIK3 to 14-3-3.
- Regulation of SIK3 by the β-adrenergic agonist CL 316,243 in primary rat adipocytes causes a decrease in SIK3 kinase activity.
- Phosphopeptide mapping of human SIK3 expressed in HEK293 cells revealed that SIK3 is phosphorylated by PKA on multiple residues.

- Expression of human SIK3 in HEK293 revealed no change in subcellular localization as a result of cAMP induction in response to forskolin treatment.

## Paper III

- A769662 is a useful tool for activation of AMPK in primary rat adipocytes.
- In contrast to results obtained with AICAR, A769662 has no effect on catecholamine-induced lipolysis or on insulin-induced lipogenesis and glucose uptake in primary rat adipocytes.
- A769662 inhibits insulin-induced fatty acid synthesis in primary rat adipocytes.

# Results and discussion

## Role for PKB in the hormonal regulation AMPK activity and of lipid metabolism in adipocytes (paper I and unpublished data)

In paper I, we examined the role for PKB in lipid metabolism with a specific PKB-inhibitor. Previous findings regarding the role for PKB in adipocytes have relied on overexpression of active PKB, a manipulation that resulted in increased lipogenesis [134, 164-166]. However, overexpression of constitutively active PKB could produce artifacts in the sense that it may trigger the phosphorylation of proteins that are normally substrates of other AGC kinases and does not determine absolute requirement. Similarly, overexpression of dominant negative PKB might affect other AGC kinases that are also activated by PDK1. The advent of a specific PKB-inhibitor allowed us to target PKB in a direct manner in adipocytes. We were also curious as to whether PKB could regulate AMPK in this tissue, a mechanism of regulation that is present in heart muscle cells, and that could potentially explain the acute effect of insulin on ACC and de novo fatty acid synthesis in adipocytes.

We started the project by establishing whether the PKB-inhibitor Akti, which inhibits PKB in a not fully characterized, yet non ATP- or substrate-competitive manner, is a useful tool in our models (primary adipocytes and 3T3L1 cells). Indeed, Akti did effectively inhibit PKB α- and β-isoforms in these models at quite low concentrations, as seen both at the level of the two activating PKB-sites S473 and T308, and at the level of PKB kinase activity. Even though Akti could not completely eradicate PKBγ activity (especially in 3T3L1 cells), and higher concentrations were necessary to inhibit this isoform, the residual PKBγ activity could not be detected in the form of T308 or S473 phosphorylation, suggesting that this isoform is only present to a minor extent in adipocytes.

When we employed Akti in primary rat- and 3T3L1 adipocytes, and analyzed effects on lipid metabolism, we could confirm previous data suggesting that the anti-lipolytic action of insulin in adipocytes is mediated through PKB. This was apparent both at the level of HSL phosphorylation and at the level of glycerol release, as increasing concentrations of Akti could reverse both the decrease in HSL phosphorylation and the decrease in glycerol release normally seen with insulin in both adipocyte models.

The anti-lipolytic action of insulin is known to depend on the hydrolysis of cAMP by PDE3B and when we assayed PDE3B activity, we could see that PKB is required for the activation of PDE3B by insulin in both primary rat adipocytes and 3T3L1 adipocytes, a fact previously only supported by overexpression or co-localization experiments.

We also used the Akti inhibitor to analyze the effects of PKB activity ablation on lipogenesis. The mechanism by which insulin promotes activation of ACC probably involves changes in S79 phosphorylation of ACC. The phosphorylation of this site by AMPK is known to lead to inhibition of ACC activity, and we (and others) observe that S79 is dephosphorylated in response to insulin. It was apparent that PKB was necessary for the insulin-induced increase in lipogenesis as inhibition of PKB with Akti decreased both basal and insulin-induced lipogenesis significantly, if not entirely, in primary rat adipocytes and 3T3L1 adipocytes. The differences seen in the ability of Akti to inhibit insulin-induced lipogenesis in primary rat adipocytes and 3T3L1 could potentially be explained by slightly higher expression of the PKBγ-subunit in this cell type.

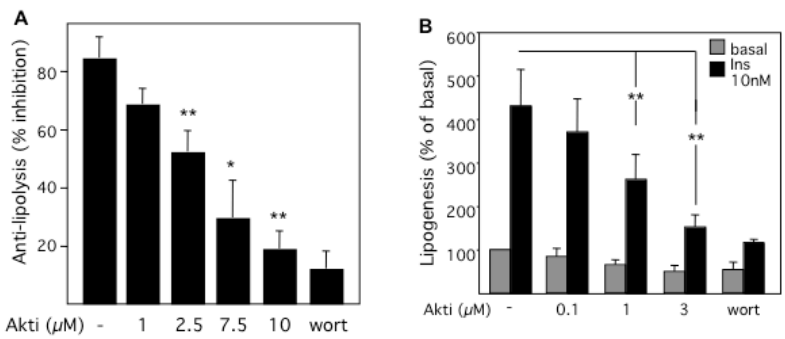


**Figure 4. PKB is required for the anti-lipolytic action of insulin**
Primary rat adiopocytes were pre-incubated with increasing concetrations of Akti and left untreated, and stimulated with isoproterenol and insulin (a) or just insulin (b). The anti-lipolysis seen with insulin treatment was abolished when the cells were pre-treated with Akti, showing that PKB is required for the anti-lipolytic action of insulin (a). In a similar manner, the lipogenesis seen with insulin treatment was

abolished when the cells were pre-treated with Akti, showing that PKB is required for the lipogenic action of insulin

As mentioned, AMPK is the kinase known to negatively regulate ACC, the rate-limiting enzyme in the lipogenic pathway (by phosphorylation of S79) and we set out to investigate whether PKB can regulate AMPK in adipocytes. A known pathway for PKB-regulation of AMPK is S485-phosphorylation, a mechanism of regulation that is present in heart muscle cells. We found that PKB was required for regulation of AMPK on the S485 site in adipocytes and that this regulation was associated with a 25 % reduction of AMPK activity in primary rat adipocytes. Similarly, PKB was responsible for a decrease in ACC phosphorylation of approximately 40 % in primary rat adipocytes.

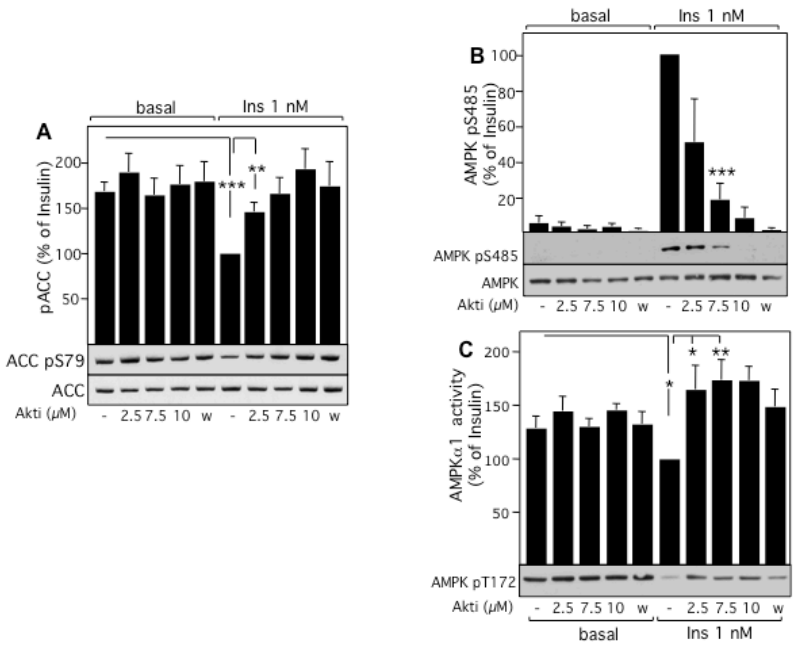


**Figure 5. Insulin negatively regulates AMPK activity.**
Primary rat adipocytes were pre-incubated with increasing concentrations of Akti or left untreated, and stimulated with insulin. Insulin resulted in increased S485 phosphorylation of AMPK (b) which was accompanied by a coresponding decrease in AMPK activity (c). Insulin also resulted in decreased S79 phosphorylation of ACC, the rate-limiting enzyme in lipogenesis, and a known AMPK target.

These data are in line with a hypothesis wherein the insulin-mediated increase in lipogenesis could be caused by PKB-mediated inhibition of AMPK and subsequent activation of ACC in this cell type. Other possible mechanisms include the potential direct regulation of ACC by PKB, a regulation that could possibly prevent S79 phosphorylation, or PKB-regulation of a phosphatase that acts on ACC. Another, less likely, pathway could be that the PDE3B-mediated hydrolysis of cAMP caused by PKB activation is the underlying cause of insulin-induced inhibition of AMPK. cAMP is a known activator of AMPK because the resulting increase in lipolysis causes a rise in the AMP/ATP ratio due to re-esterification of FAs, a process that consumes ATP. However, as PKB-regulation of AMPK on S485 is also present in heart muscle, this pathway seems unlikely, as insulin-sensitive PDE-isoforms are not expressed in this tissue and lipolysis is not a major metabolic pathway in heart (a tissue which expresses very low levels of HSL [167].

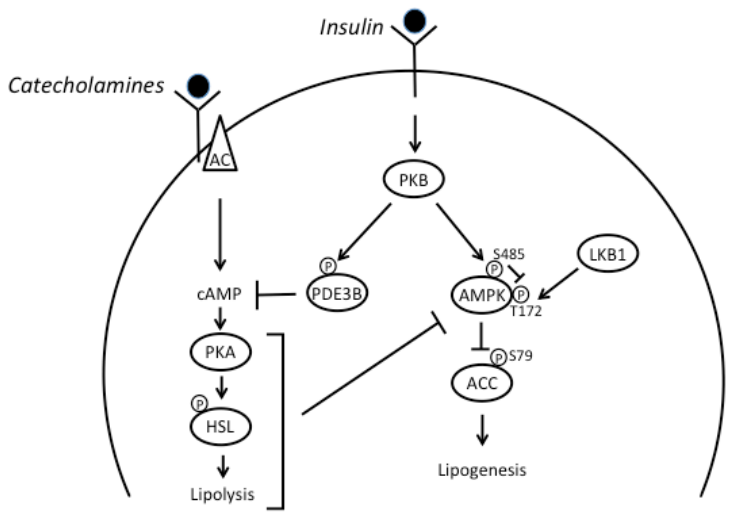


**Figure 6. Possible pathways for the effect on insulin on de novo lipogenesis**
We speculate that the regulation of S485 on AMPK by PKB results in decreased AMPK activity. This decrease in AMPK activity could be responsible for the reduction of ACC phosphorylation seen on S79 in response to insulin, and the decrease in lipogenesis seen with insulin treatment. As AMPK can be activated by cAMP and increased lipolys in adipocytes, due to the indirect increase in AMP levels that arise when FAs undergo ATP-consuming re-esterification, another possible mechanism could be indirect activation of AMPK through PKB-mediated activation of PDE3B.

Since our findings, regulation of AMPK by PKB on S485 has been seen in another cell type, vascular smooth cells [70]. Interestingly, this site has been found to be regulated by p70S6 kinase in the hypothalamus, inhibiting AMPKα2 activity in a similar manner. It is suggested that PKB, rather than directly phosphorylating this site, acts as an upstream kinase of p70S6 kinase, and that this regulation inhibits CAMKKβ-mediated, not LKB1-mediated, activation of AMPK, [168]. However, recently, Hawley et al reported that the AMPKα2-site that is equivalent to S485 on AMPKα1 is not a very good PKB-substrate (in contrast to S485 on AMPKα1). They also find that the regulation of S485 in AMPKα1 is p70S6 kinase-independent, suggesting that PKB-activating agents, such as insulin and IGF-1 perhaps only inhibit the activity of AMPKα1-containing complexes. Furthermore, they show that PKB-mediated regulation of S485 in AMPKα1 accomplished a decrease in both CAMKKβ-mediated and LKB1-mediated activation of AMPK [169].

Collectively, these data solidify a role for PKB in the impact insulin exerts on adipocyte metabolism and prove that AMPK is inhibited by insulin in adipocytes. Whether the latter is responsible for the effects of insulin on lipogenesis remains to be established.

# Role for AMPK in the hormonal regulation of carbohydrate- and lipid metabolism in adipocytes (paper III)

Many drugs that are currently being prescribed for diabetes treatment appear to achieve their effects through AMPK activation. For example, a widely used drug, whose positive effects on gluconeogenesis used to be attributed to AMPK, is metformin. Recently, it was discovered that the effects of metformin on gluconeogenesis are AMPK-independent – at least in mice [170]. Although the inhibitory effect of metformin on gluconeogenesis does not involve AMPK, the positive effects that the drug exerts on glucose uptake and insulin sensitivity are still attributed to AMPK. In another study it was revealed that the ability of AICAR to inhibit gluconeogenesis was also not dependent on the presence of AMPK [171]. Collectively, these data questions the specificity of metformin, phenformin

(metformin analogue) and AICAR, and their use to evaluate the role AMPK in various biological processes.

Previous papers regarding the role for AMPK in adipocyte metabolism describe how AICAR-mediated activation of AMPK also inhibited insulin-induced lipogenesis in primary rat adipocytes, and that both AICAR- and phenformin-induced activation of AMPK reduced isoproterenol (an adrenalin analogue)-induced lipolysis in primary rat adipocytes. These findings are also supported by data generated through adenoviral overexpression of constitutively active or dominant-negative AMPK in rat and mouse adipocytes [121, 122, 172, 173].

Since what is currently known about the role of AMPK in the regulation of adipocyte metabolism is based mainly on studies using phenformin or AICAR we saw a reason for re-addressing the role of AMPK in adipocytes using a more specific tool. In paper III, we therefore investigated the ability of a newer AMPK activator, A769662, to modulate hormonal effects on carbohydrate- and lipid metabolism in adipocytes, and compared it with that of AICAR and phenformin.

Even though muscle is the major organ for glucose-disposal (70-90 % of blood glucose is taken up by muscle tissue and 80 % of carbohydrates are stored in muscle [41], glucose uptake into adipocytes is important for several reasons. First, since tissue-specific KO of GLUT4 transporters in mouse adipose tissue results in insulin resistance in liver and muscle, a preserved glucose uptake in adipocytes appears to be critical, albeit indirectly, for glucose homeostasis [123]. Also, the uptake of glucose into adipocytes is very important for the formation of glycerol and thus synthesis of TAGs.

Previous research show that activation of AMPK by AICAR resulted in dramatically reduced insulin-induced glucose uptake in both 3T3L1 adipocytes and primary adipocytes. In muscle the opposite scenario applies, as AMPK is known to increase glucose uptake [119].

As in previous studies, we observed that AICAR and phenformin markedly reduced insulin-induced glucose uptake and lipogenesis, as well as isoproterenol-induced lipolysis. However, activation of AMPK in response to A769662 had no effect on these processes.

As de novo fatty acid synthesis is the step in lipogenesis that is perhaps the most interesting in relation to AMPK, we also chose to measure FA synthesis, using radioactive acetate, and indeed observed a decrease in insulin-induced de novo fatty acid synthesis. This decrease of approximately 50 % was less than the decrease seen with AICAR and phenformin.

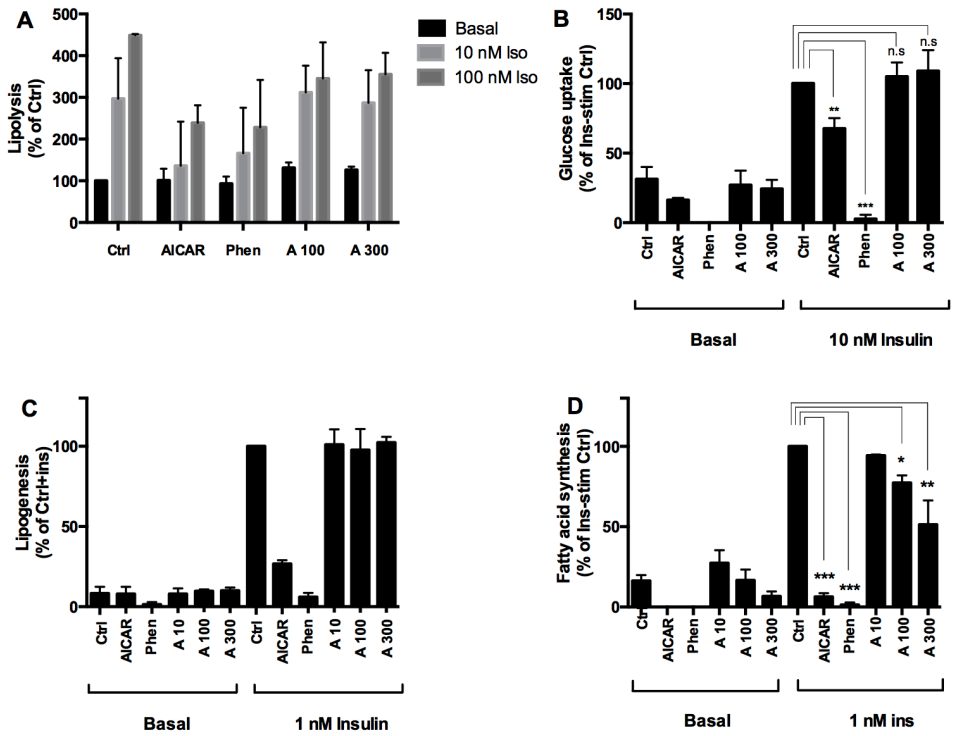


**Figure 7. Effect of AMPK activation on lipid metabolism**
Primary rat adipocytes were pre-treated with AMPK-activators AICAR, phenformin and A769662 and subsequently stimulated with hormones. The resulting alterations in lipolysis, glucose uptake and lipogenesis were compared to the non-pretreated state.

In summary, activation of AMPK by A769662 elicits different effects on adipocyte metabolism than those seen upon activation of AMPK by AICAR or phenformin. Even though A769662 results in a similar, or higher, level of phosphorylation of downstream AMPK substrates ACC and Raptor when compared to effects seen with AICAR and phenformin, AICAR and phenformin resulted in a much larger degree of inhibition of de novo fatty acid synthesis. This is likely explained by the fact that AICAR and phenformin have a large impact on glucose uptake, whereas A769662 does not. The substantial inhibition of glucose uptake caused by the two indirect AMPK activators, likely limits glycolysis and the availability of acetyl-CoA for fatty acid synthesis to an extent that is visible in the de novo fatty acid synthesis assay. We conclude that the differences seen in carbohydrate- and lipid metabolism with AICAR and phenformin and A769662 could be due to AMPK-independent effects of AICAR and phenformin. Another quite possible mechanism involves the fact that A769662 only activates β1-subunit containing AMPK complexes. In this light, β1-containing complexes could be important for regulation of de novo lipogenesis, whereas β2-containing complexes might be important for the regulation of glucose uptake and

lipolysis. Another quite possible mechanism involves the fact that A769662 only activates β1-subunit containing AMPK complexes. In this light, β1-containing complexes could be important for regulation of de novo lipogenesis, whereas β2-containing complexes might be important for the regulation of glucose uptake and lipolysis. The notion that β1- and β2-containing complexes can have different substrates is supported by the fact that KO mouse models of the two isoforms have different phenotypes. However, the lack of noticeable changes in AMPK activity in adipose tissue after whole-body KO of the β2-subunit [56], suggests that β1 is the dominating isoform in this tissue and renders the latter explanation less likely.

## Hormonal regulation of SIK3 activity in adipocytes (paper II)

The fact that SIK1 has been shown to be regulated by cAMP in adrenal cells [78] and the fact that a drosophila SIK-homologue can be regulated by insulin [99], prompted us to explore whether SIK3 could be regulated by these hormones in adipocytes. The fact that LKB1, the upstream regulator of SIK3, is constitutively active [73], makes it interesting to explore alternative ways in which SIK3 responds to extracellular stimuli.

In paper II, we used primary rat adipocytes to evaluate the possibility of hormonal regulation of SIK3 in this tissue. We employed antibodies recognizing PKA and PKB consensus motifs as an initial step towards detection of cAMP-mediated and insulin-mediated regulation of SIK3, respectively. There was no increase in phosphorylation of SIK3 with either antibody in response to insulin- or IGF1 treatment in primary rat adipocytes and HEK293-cells (overexpressed SIK3), suggesting that insulin likely does not regulate SIK3 in this tissue. However, in response to treatment with CL 316,243, a pharmacological activator of β3-adrenergic receptors, there was an increase in phosphorylation on PKA consensus sites on SIK3. The same increase in phosphorylation was observed with the PKB consensus motif-recognizing antibody, but the lack of signal seen in response to insulin suggests that this was merely a result of antibody cross-reactivity (PKA and PKB recognize similar consensus motifs).

When immunoprecipitated SIK3 was analyzed with purified 14-3-3 proteins in a far western approach, it became apparent that the increase in phosphorylation coincided with an increase in 14-3-3 protein binding to SIK3. It is known that non-stimulated SIK3 can bind 14-3-3 via T221, the activity-controlling (LKB1) site of SIK3, a fact that results in a punctate cytosolic distribution in HeLa cells [74].

We used the PKA-inhibitor H89, to investigate whether the regulation of SIK3 by cAMP-elevating agents was dependent on PKA and excluded other possible pathways.

We found that the results seen on SIK3 in terms of cAMP-induced phosphorylation and 14-3-3 binding were indeed PKA-dependent.

We proceeded to uncover residue(s) responsible for the cAMP-mediated regulation of SIK3. In order to accomplish this, we expressed human SIK3 constructs in HEK293-cells, which are easily transfected and therefore a good tool for generation of material for phosphopeptide mapping. We found that SIK3 is a heavily phosphorylated kinase even in a non-stimulated state and this basal phosphorylation could not be the result of autophosphorylation, as a kinase inactive version of SIK3 displayed the same degree of phosphorylation. Four peptides generated particularly high peaks, three of which exhibited substantially increased phosphorylation in response to forskolin treatment. The sites phosphorylated in these peptides; T469, S551 and S674, all appeared to qualify as 14-3-3 binding sites. We proceeded to construct single-site mutants of SIK3 in which the three aforementioned sites as well as the activity-controlling site were mutated. We also constructed an SIK3 mutant, which lacked the ability to be phosphorylated at all four sites (quadr.). When expressing these mutants in HEK293 cells and subjecting the cells to forskolin treatment, we saw that all mutated sites contributed to the cAMP-mediated increase in phosphorylation and 14-3-3 binding, T469 to a larger extent and S551 (the site equivalent to the cAMP-regulated site on SIK1) and S674 to a more intermediate and modest extent, respectively. Mutation of T221 on SIK3 resulted in an expected decrease in basal 14-3-3 binding, as this site is known to bind 14-3-3 in non-stimulated cells. However, T221 also seemed to partially contribute to the cAMP-mediated increase in phosphorylation, as mutation of this site resulted in a decrease in cAMP-mediated phosphorylation. This was surprising since this site was found to be unresponsive to forskolin treatment, as determined by western blotting and phosphopeptide mapping. Perhaps this result is a case of cross-reactivity of the PKA consensus motif-recognizing antibody, as T221 does not undergo any change in response to forskolin treatment. To conclude, no single site proved solely responsible for the increased phosphorylation and 14-3-3 binding seen in response to cAMP-elevation. However, mutating all four sites resulted in the return of both phosphorylation and 14-3-3 binding to basal levels.

In order to unveil the functional consequence of cAMP-mediated regulation and 14-3-3 binding of SIK3 in adipocytes, we decided to address potential changes in the kinase activity and subcellular localization of SIK3. The fact that SIK1 undergoes nucleocytoplasmic shuttling in response to cAMP-elevation in adrenal cells, along with the increase in PKA-mediated phosphorylation of SIK3 and increase in 14-3-3 binding seen in response to cAMP-elevation, made us curious as to whether this regulation controls subcellular localization of SIK3. However, when expressed in HEK293 cells and analyzed with a confocal microscope, both wildtype and the quadruple mutant version of SIK3 localized to the cytosol in non-stimulated cells, a localization that did not change in response to forskolin treatment. We did however find that β-adrenergic elevation of cAMP-levels in adipocytes resulted in a decreased

kinase activity of SIK3 of approximately 30 % when measured with an in vitro kinase assay. Even though the site that is responsible for re-localization of SIK1 in adrenal cells, S577, is conserved in SIK3 at the S551 position, the lack of SIK3 re-localization was not a completely unexpected finding, as SIK1 contains a nuclear localization signal (NLS) [78, 79], whereas SIK3 and SIK2 do not. For SIK2, another site, S358, seems to be responsible for cAMP-mediated regulation and 14-3-3 binding and this does not have any effect on the kinase activity of SIK2 [98], neither does cAMP-mediated regulation of SIK1 [78]. This suggests diverging mechanisms for how these kinases are regulated, at least in adipose tissue, which likely reflects their structural differences. Whether or not the PKA-mediated phosphorylation of SIK3 is directly responsible for decreased SIK3 activity remains to be investigated.

To summarize, we identify cAMP/PKA as a novel pathway for SIK3 regulation and an example of how this kinase can respond to a hormonal stimulus in adipocytes. As we could not replicate the insulin-mediated regulation of an SIK-isoform seen in drosophila [99], this constitutes the first example of SIK3-regulation in mammalian cells. The physiological relevance of this regulatory mechanism remains to be discovered, although it is apparent that the kinase activity of SIK3 is modulated by elevated cAMP - whether or not this is a direct or indirect effect of PKA-regulation. It is a known fact that cAMP is often released and sequestered in localized pools in the cell, thereby resulting in more specialized cellular effects [174]. As the decrease in SIK3 activity is modest, yet consistent, and in spite of the appearance of an unchanged localization of SIK3 in response to this regulation, we speculate that perhaps a smaller subset of SIK3 molecules re-localize due to regulation by certain subcellular pools of cAMP.

The regulation of SIK3 by β-adrenergic stimuli could also involve binding of A-kinase anchoring proteins (AKAP), which are scaffolding proteins with a tissue-specific expression that anchor proteins together with cAMP-regulated/regulating signaling components, such as PKA, phosphodiesterases, phosphatases or Epac's (molecules that participate in PKA-independent cAMP-mediated regulation) in multiprotein complexes [175]. The slightly punctate visible structure of SIK3 in the cytosol could warrant such speculations, as could the hypothesis that only a part of SIK3-molecules are regulated by cAMP. AKAP's constitute one of several mechanisms by which cAMP-signaling can be tailored to target particular cellular events [175].

The exact role for SIK3 in adipocytes remains undiscovered, but SIK3 was affected in dual ways by adrenergic regulation. cAMP elevation did not result in any subcellular trans-localization for SIK3 (as for SIK1), however it did result in an increase in binding of SIK3 to 14-3-3 proteins. 14-3-3 are scaffolding molecules that homo- or heterodimerize and interact with phosphorylated serine and threonine residues on proteins, for example mediating subcellular translocations or complex formation. In addition to this increased 14-3-3 binding, SIK3 also became less enzymatically active in response to cAMP regulation.

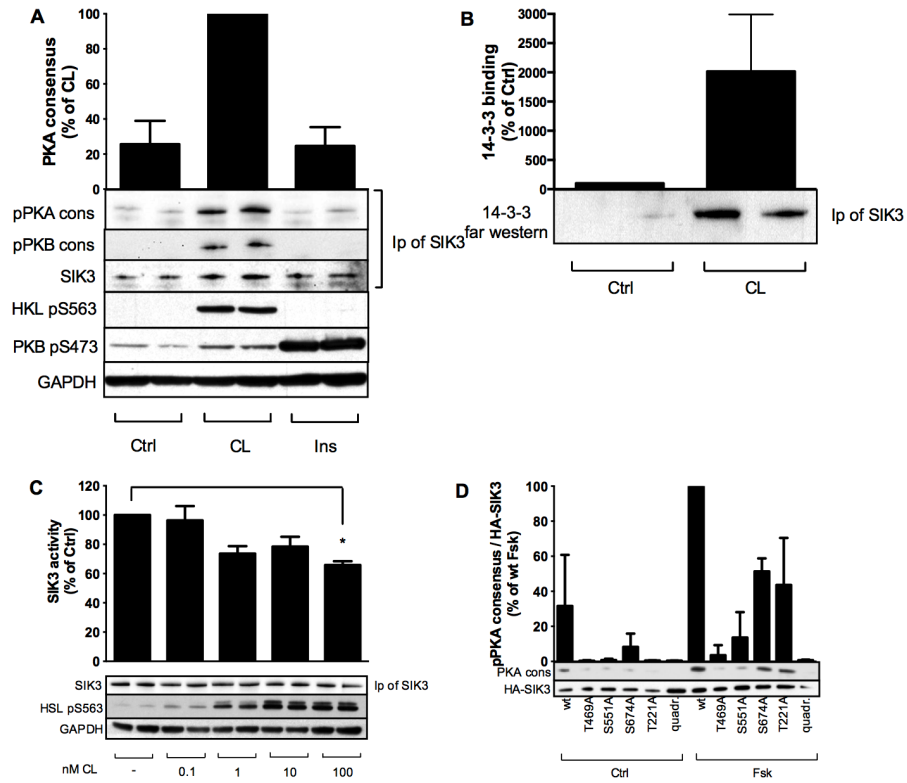


**Figure 8. cAMP-mediated regulation of SIK3 in primary rat adipocytes**
Primary rat adipocytes (a, b and c) and HEK293 cells overexpressing human SIK3 (d) were treated with the cAMP-elevating agent CL and SIK3 was immunoprecipitated. These graphs illustrate the resulting phosphorylation of PKA consensus motifs, 14-3-3 binding and decreased SIK3 kinase activity, as well as the contribution of selected and mutated PKA-sites to total the cAMP-mediated regulation of SIK3.

To summarize, we identify cAMP/PKA as a novel regulatory mechanism for SIK3 regulation, an example of how this kinase can respond to a hormonal stimulus in adipocytes. As we could not replicate the insulin-mediated regulation of an SIK-isoform seen in drosophila [99], this constitutes the first example of SIK3-regulation in mammalian cells. The physiological relevance of this regulatory mechanism remains to be discovered, although it is apparent that the kinase activity of SIK3 is

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The regulation of SIK3 by β-adrenergic stimuli could also involve binding of A-kinase anchoring proteins (AKAP), which are scaffolding proteins with a tissue-specificexpression that anchor proteins together with cAMP-regulated/regulating signaling components, such as PKA, phosphoridesterases, phosphatases or Epac's (molecules that participate in PKA-independent cAMP-mediated regulation) in multiprotein complexes [175]. The slightly punctate visible structure of SIK3 in the cytosol could warrant such speculations, as could the hypothesis that only a part of SIK3-molecules are regulated by cAMP. AKAP's constitute one of several mechanisms by which cAMP-signalling can be tailored to target particular cellular events [175].

# Conclusions

To conclude, we have provided novel findings of how kinases are under hormonal control or modulate hormonal influences in adipocytes. We have established that PKB is an integral part of how insulin exerts its effects on lipid metabolism (lipogenesis and anti-lipolysis) in this tissue, using a new pharmacological approach. We also demonstrate that PKB can regulate AMPK in adipocytes, which in essence means that AMPK activity is possibly under the hormonal influence of insulin. We propose that this regulatory mechanism and the resulting decrease in AMPK activity might contribute to the positive effects of insulin on lipogenesis in adipocytes. We have also revisited previous findings regarding the ability of AMPK to modulate the effects of hormones on lipid- and carbohydrate metabolism in adipocytes. Our data challenge the previous notion (based on studies with AICAR) of AMPK as a negative regulator of glucose uptake and lipolysis in adipocytes. Although A769662 robustly activated AMPK in these cells, its only effect on the metabolic pathways we monitored was an inhibition of de novo fatty acid synthesis. Finally we show that the AMPK-related kinase SIK3 is also under hormonal control in adipocytes. We find that beta-adrenergic stimulation of adipocytes leads to phosphorylation, 14-3-3 binding and reduced activity of SIK3. The physiological relevance of this regulation remains to be uncovered, but as cAMP is such an integral part of adipocyte metabolism, it seems likely that SIK3 participates in the regulation of adipocyte function downstream of this second messenger.

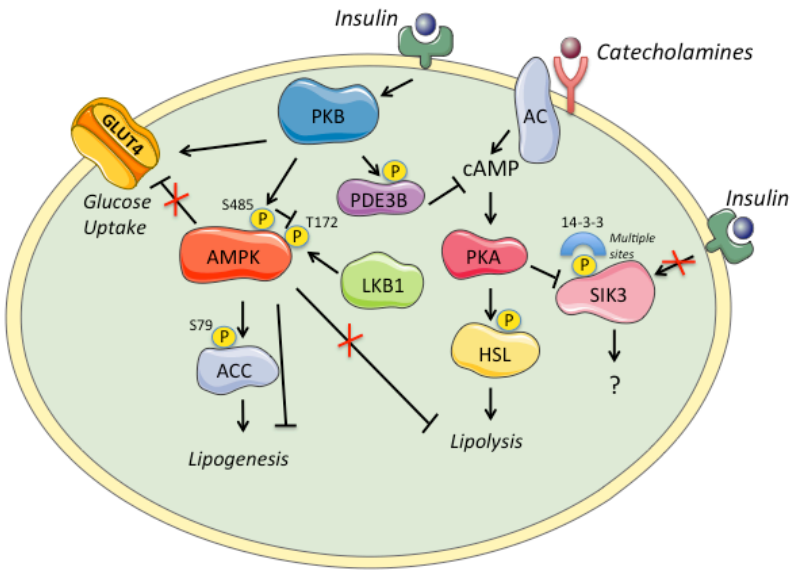


**Figure 9. The role for selected kinases in adipocyte metabolism**
PKB, AMPK and SIK3 all appear to take part in the hormonal regulation of biological responses in adipocytes. PKB is required for the anti-lipolytic action of insulin. Direct activation of AMPK with A759662 modulates the de novo lipogenesis-inducing action of insulin, but not insulin-induced glcuose uptake or isoproterenol-induced lipolysis. AMPK also appears to be controlled by insulin, a mechanism that may explain the lipogenic action of insulin. SIK3 cannot be regulated by insulin in adipocytes, but catecholamines reduce its kinase activity and mediates 14-3-3 binding.

# Future Perspectives

The starting point for continued studies of the findings from paper I (regarding PKB-mediated regulation of AMPK) should be to determine whether S485 phosphorylation is required for the effects of insulin on AMPK activity as well as fatty acid synthesis. In order to accomplish this, we have obtained point-mutated human constructs of the human α1-subunit, that either lack the ability to be phosphorylated on S485 or are mutated in manner that mimicks phosphorylation. These constructs act in a dominant fashion when transfected adenovirally into primary rat adipocytes, in the sense that they replace the wild type AMPK α1-subunit.

As there are difficulties in extrapolating data from studies on rodent metabolism, which differs substantially from human metabolism, confirming insulin-mediated regulation of AMPK in human adipocytes would be of great interest. This work has been initiated and we can reproduce S485-regulation in human adipocytes. The impact of this regulation on lipogenesis in humans remains to be explored.

Another aim could be to determine whether AMPK is differentially expressed or regulatedin adipocytes from obese or insulin reistant individuals.

As for the findings regarding SIK3 (paper II), the presence of this kinase in adipocytes, along with the fact that it can be regulated by β-adrenergic stimuli, one of the main hormones that act upon this tissue, suggests that SIK3 has one or potentially several functions in this tissue. Methods for viral expression and siRNA silencing of SIK3 are established in the laboratory and could be used to address the functional role as well as molecular targets of SIK3 in adipocytes. Another way of identifying (new) substrates or regulators of SIK3 would be to search for interacting proteins. As we believe that the modest decrease in SIK3 activity by β-adrenergic stimuli could reflect that SIK3 is regulated by specific pools of cAMP, in the sense that some SIK3 molecules are in fact highly inactivated by this regulation, and others are not deactivated at all, it would be interesting to explore the possibility of SIK3 binding to AKAP scaffolding proteins. It is likely that SIK3 binds to some form of scaffolding protein as its presence in punctate structures in the cytosol could reflect its participation in some form of protein complex.

It would be interesting to study the adipose-tissue in the existing SIK3 KO mice in order to establish roles for SIK3 in this tissue. Additionally, the regulation, expression and role of SIK3 in humans remains to be discovered.

When it comes to the final and as of yet unpublished paper (paper III), we aim to address a number of points. The assumption that the AMPK β1-subunit is the primary isoform expressed in adipocytes is based on different KO studies in mice. An obvious step before submission of this manuscript is to confirm that the β1-subunit is indeed expressed to a large degree in adipocytes, something that we will do by assessing the amount of AMPK activity associated with either of the two β subunits using isoform-specific antibodies. Furthermore, it would be of interest to study the impact of A769662 on the phosphorylation of proteins that are proposed to mediate the effect of AMPK on lipolysis and glucose uptake, such as HSL and TBC1D4.

From a wider perspective generation of an adipose tissue-specific AMPKα1/α2 KO mouse would benefit the adipose tissue and AMPK fields. This would allow for analysis of the role of AMPK in adipose tissue, as well as testing of the true dependency of AMPK for the effect of different AMPK activators.

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**Shit Academics Say** @AcademicsSay · 24 maj

I pledge to work less this weekend, and spend more time with the family. They seem like nice people.

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# Paper I

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# Protein kinase B activity is required for the effects of insulin on lipid metabolism in adipocytes

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**Berggreen C, Gormand A, Omar B, Degerman E, Göransson O.** Protein kinase B activity is required for the effects of insulin on lipid metabolism in adipocytes. *Am J Physiol Endocrinol Metab* 296: E635–E646, 2009. First published January 21, 2009; doi:10.1152/ajpendo.90596.2008.—Protein kinase B (PKB) is known to mediate a number of biological responses to insulin and growth factors, its role in glucose uptake being one of the most extensively studied. In this work, we have employed a recently described allosteric inhibitor of PKB, Akti, to clarify the role of PKB in lipid metabolism in adipocytes—a subject that has received less attention. Pretreatment of primary rat and 3T3L1 adipocytes with Akti resulted in dose-dependent inhibition of PKB phosphorylation and activation in response to insulin, without affecting upstream insulin signaling [insulin receptor (IR), insulin receptor substrate (IRS)] or the insulin-induced phosphoinositide 3-kinase (PI3K)-dependent activation of the ERK/p90 ribosomal kinase (RSK) pathway. PKB activity was required for the insulin-induced activation of phosphodiesterase 3B (PDE3B) and for the antilipolytic action of insulin. Moreover, inhibition of PKB activity resulted in a reduction in de novo lipid synthesis and in the ability of insulin to stimulate this process. The regulation of the rate-limiting lipogenic enzyme acetyl-CoA carboxylase (ACC) by insulin through dephosphorylation of S79, which is a target for AMP-activated protein kinase (AMPK), was dependent on the presence of active PKB. Finally, AMPK was shown to be phosphorylated by PKB on S485 in response to insulin, and this was associated with a reduction in AMPK activity. In summary, we propose that PKB is required for the positive effects of insulin on lipid storage and that regulation of PDE3B and AMPK by PKB is important for these effects.

Akt; phosphodiesterase 3B; acetyl-coenzyme A carboxylase; adenosine 5′-monophosphate-activated protein kinase; lipogenesis

THE SERINE/THREONINE protein kinase B (PKB/Akt) is a key target for signals that activate phosphoinositide 3-kinase (PI3K), such as insulin and growth factors. There is strong evidence, including results obtained from genetic mouse models, to support the idea that PKB mediates the effects of these stimuli on a multitude of cellular events such as glucose uptake, glycogen synthesis, gluconeogenesis, protein synthesis, and cell survival (12, 17). The importance of PKB in the acute regulation of lipid metabolism by insulin is, however, less well characterized.

Perturbations in the ability of adipose tissue to adequately store triglycerides, and also in its endocrine function such as the production of adipokines, have been predicted to be an underlying cause of insulin resistance and type 2 diabetes. Lipid metabolism in adipocytes is controlled by the concerted actions of cAMP-elevating hormones and insulin. Insulin counteracts catecholamine-induced lipolysis (hydrolysis of triglycerides) mainly by inducing phosphorylation and activation of phosphodiesterase 3B (PDE3B) (13, 18, 19). Activation of PDE3B leads to increased hydrolysis of cAMP, resulting in reduced activity of protein kinase A (PKA), which phosphorylates and activates hormone-sensitive lipase (HSL) as well as other target enzymes that carry out or regulate lipolysis (28, 40). Overexpression and site-directed mutagenesis studies have suggested that PKB is the upstream kinase that phosphorylates PDE3B in response to insulin (2, 33).

In addition to its antilipolytic action, insulin also promotes lipid storage by activating lipogenesis, i.e., de novo generation of lipids. The rate-limiting enzyme in fatty acid synthesis is acetyl-CoA carboxylase (ACC) (25), which catalyzes the conversion of acetyl-CoA to the active fatty acid precursor malonyl-CoA. Malonyl-CoA at the same time limits fatty acid oxidation by inhibiting carnitine palmitoyltransferase I, which transports fatty acids into the mitochondrion (1). One way in which insulin activates ACC is by inducing a net dephosphorylation of ACC at S79 (21, 56), the site phosphorylated by AMP-activated protein kinase (AMPK) (15). The mechanisms underlying insulin-induced dephosphorylation of this residue in ACC are not known, but overexpression of active PKB in adipocytes has been shown to increase lipogenesis (34, 56). Moreover, AMPK in cardiac muscle has been shown to be phosphorylated by PKB in response to insulin, leading to an inhibition of phosphorylation of AMPK at T172 and therefore inhibition of its activity (29, 36).

PKB is a member of the AGC (denoting PKA, PKG, PKC) family of kinases, and it is expressed as three isoforms in mammals: PKBα, PKBβ, and PKBγ (14). Like many of the AGC kinases, PKB is activated through phosphorylation of a residue in the activation loop (T308 in PKBα) by 3-phosphoinositide-dependent kinase (PDK)1 (4, 5) and by phosphorylation of a hydrophobic motif residue close to the COOH terminus (S473 in PKBα) (4). In the case of PKB, the kinase that brings about phosphorylation of the hydrophobic motif (PDK2) is the complex of mammalian target of rapamycin (mTOR) and rapamycin-insensitive companion of mTOR (RICTOR) (30).

Previous studies addressing the role of PKB in lipid metabolism have mainly been based on the overexpression of constitutively active or dominant-negative versions of the kinase (2, 33, 34, 56). Although useful in many regards, overexpression strategies are associated with certain pitfalls, which are discussed below. Pharmacological inhibition is a powerful method for the study of endogenous proteins and their role in

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acute hormone action. Moreover, pharmacological inhibition (or activation) is still by far the most common mechanism of action of drugs in clinical use, which demonstrates the importance of developing and characterizing such tools. Akti-1/2, hereafter referred to as Akti, is a recently described pharmacological inhibitor of PKBα and PKBβ that inactivates PKB in vitro as well as in cells (8). Akti has been found to have a 10- to 100-fold selective preference for PKBα and PKBβ over 70 kinases in vitro, including PI3K and PDK1 (39). Akti has also been shown to be capable of inhibiting insulin-induced phosphorylation and activation of PKB in cells, most likely by making it a worse substrate for its upstream kinases, without affecting other tested insulin-activated signaling pathways (24, 39). Akti has been used to study the potential of PKB as a therapeutic target for anticancer drugs (8) and also to investigate the role of PKB in glucose uptake in L6 myotubes and 3T3L1 adipocytes (24, 23) and in the regulation of gluconeogenic gene expression (39). In this study, we have used Akti in order to evaluate the role of PKB in the regulation of lipid metabolism in adipocytes.

## MATERIALS AND METHODS

*Materials.* Akti was a generous gift from Dario Alessi and Michael Keane (Univ. of Dundee, Dundee, UK), who synthesized it in-house (39). Akti is also available commercially through Calbiochem, where it is termed Akt inhibitor VIII. 3T3L1 cells were obtained from the American Type Culture Collection; rats from Charles River Laboratories; DMEM, dexamethasone, wortmannin, and IBMX from Sigma; and insulin from Novo Nordisk (Copenhagen, Denmark). D-[6-$^3$H]-glucose (catalog no. TRK85) and protein G-Sepharose were purchased from GE Healthcare and [γ-$^{32}$P]ATP from PerkinElmer. Glycerokinase, glycerol-3-phosphate dehydrogenase, and NAD used for the glycerol determinations were from Roche, as were Complete protease inhibitor cocktail tablets. Precast Novex SDS polyacrylamide Bis-Tris gels, lauryl dodecyl sulfate (LDS) sample buffer, and fetal calf serum (FCS) were obtained from Invitrogen and phosphocellulose P81 paper from Whatman. The PDE3 inhibitor OPC3911 was a kind gift from Otsuka Pharmaceuticals. All peptides were synthesized by Dr. Graham Bloomberg (Univ. of Bristol, Bristol, UK). Enhanced chemiluminescence (ECL) Super Signal Reagent was purchased from Pierce.

The following antibodies were used for Western blotting: anti-PKB(pT308), anti-glycogen synthetase kinase (GSK)3(pS21/9), anti-HSL(pS563), anti-ACC, anti-ACC(pS79), anti-AMPK, anti-AMPK-(pT172), anti-AMPK(pS485), anti-ERK, anti-ERK(pT202/Y204) and anti-insulin receptor substrate (IRS)1 were all from Cell Signaling Technology (Beverly, MA), and anti-PKB(pS473), anti-GSK3, anti-insulin receptor (IR)(pY1162/1163) and anti-IRS1(pY612) were purchased from Biosource. Antibody to total PKB was a gift from Dario Alessi (Univ. of Dundee) and was raised against a peptide corresponding to residues 466-480 of rat PKBα. Anti-HSL antibody was a gift from Cecilia Holm (Lund Univ.) and was raised against rat HSL. Anti-PDE3B antibodies were generated as described previously (49). The following antibodies used for immunoprecipitation were all kindly provided by Dario Alessi and Kei Sakamoto (Univ. of Dundee): anti-PKBα, raised against a fragment of human PKBα corresponding to residues 1-149; anti-mPKBβ (used for immunoprecipitation of PKBβ in 3T3L1 adipocytes), raised against a peptide corresponding to residues 455-469 of mouse PKBβ; anti-PKBγ, raised against a peptide corresponding to residues 116-128 of human PKBγ; and anti-p90 ribosomal kinase (RSK), raised against a peptide corresponding to residues 712-734 of human RSK2. The antibody used for immunoprecipitation of rat PKBβ was generated in-house against a peptide corresponding to residues 455-469 of rat PKBβ. AMPKα1 antibodies were generously provided by Grahame Hardie (Univ. of Dundee) and were raised against a peptide corresponding to residues 344-358 of rat AMPKα1. The isoform specificity of the anti-PKB antibodies used for immunoprecipitation was validated in adipocyte lysates and with purified proteins, both by our group (data not shown) and by others (50). Horseradish peroxidase-conjugated secondary antibodies were obtained from Biosource (anti-rabbit), Pierce (anti-sheep), and GE Healthcare (anti-mouse).

*Culture and stimulation of 3T3L1 adipocytes.* 3T3L1 fibroblasts were cultured at subconfluence in DMEM containing 10% FCS and 1% penicillin-streptomycin at 37°C and 95% air-5% $CO_2$. Two-day postconfluent cells were incubated for 72 h in DMEM supplemented with 0.5 mM IBMX, 10 μg/ml insulin, and 1 μM dexamethasone, and the cells were thereafter cultured in normal growth medium. Experiments were performed on *days 8–14* after the initiation of differentiation, at which time the differentiation rate was typically 80–95%. Cells were stimulated as indicated in Figs. 1–4 and 6, rinsed briefly with PBS, and scraped in lysis buffer containing (in mM) 50 Tris·HCl, pH 7.5, 1 EGTA, 1 EDTA, 1 sodium orthovanadate, 10 sodium-β-glycerophosphate, 50 sodium fluoride, 5 sodium pyrophosphate, and 1 dithiothreitol (DTT), with 1% (wt/vol) NP-40, 0.27 M sucrose, and Complete protease inhibitor cocktail (1 tablet/0.50 ml). Lysates were centrifuged at 13,000 *g* for 15 min at 4°C, and the infranatants were collected by punching a hole, with a needle, at the bottom of the tube. Total protein content of the lysates was determined by the method of Bradford.

For PDE3B measurements, cells were scraped and homogenized in lysis buffer without NP-40. PDE activity was measured either in a crude homogenate generated by centrifugation at 13,000 *g* for 15 min at 4°C or in membrane fractions generated by centrifugation of the homogenates at 35,000 *g* for 45 min at 4°C. Pellets were rehomogenized in the same buffer, and protein content was determined by the method of Bradford (11).

*Isolation and stimulation of primary rat adipocytes.* Adipocytes were prepared from epididymal adipose tissue of 36- to 38-day-old male Sprague-Dawley rats (47) under a protocol approved by the ethical review committee at Lund University (approval no. M158-06). Adipocytes were suspended in Krebs-Ringer medium, 25 mM HEPES pH 7.4, 200 nM adenosine, 2 mM glucose, and 1% bovine serum albumin (BSA) (typically 2 ml of a 10% suspension), and stimulated as indicated in Figs. 1–6. To stop incubations, cells were washed in Krebs-Ringer medium without BSA and subsequently homogenized in 0.5–1 ml of homogenization buffer containing (in mM) 50 Tris·HCl pH 7.5, 1 EGTA, 1 EDTA, 1 sodium orthovanadate, 10 sodium-β-glycerophosphate, 50 sodium fluoride, 5 sodium pyrophosphate, and 1 DTT, with 0.27 M sucrose and Complete protease inhibitor cocktail (1 tablet/0.50 ml). Homogenates were centrifuged for 5 min at 5,000 *g*, and the fat cake was removed. The remaining supernatant was supplemented with 1% (vol/vol) NP-40, left on ice to solubilize for 30 min, and centrifuged at 13,000 *g* for 10 min. Protein concentrations were determined by the method of Bradford.

PDE3B measurements were carried out either in detergent-free homogenates, prepared as above, or in membrane fractions generated by centrifugation of the homogenates at 35,000 *g* for 45 min at 4°C. Pellets were rehomogenized in the same buffer, and protein content was determined by the method of Bradford.

*Western blot analysis.* Total cell lysates or homogenates (5–30 μg) were heated at 95°C for 2 min in LDS sample buffer, subjected to polyacrylamide gel electrophoresis on precast Novex gradient gels, and electrotransferred to nitrocellulose membranes. The membranes were blocked for 30 min in 50 mM Tris·HCl pH 7.6, 137 mM NaCl, and 0.1% (wt/vol) Tween 20 (TBS-T) containing 10% (wt/vol) skimmed milk. The membranes were then probed with 0.5–1 μg/ml of the indicated antibodies in TBS-T containing 5% (wt/vol) skimmed milk or in 5% (wt/vol) BSA for 16 h at 4°C. Detection was performed with horseradish peroxidase-conjugated secondary antibodies and the ECL reagent (Pierce). Quantification of the bands was performed by

digitizing the ECL films with a Fuji LAS 1000 charge-coupled device camera and analysis of the intensities with Image Gauge software (Fuji).

*Kinase assays.* Five to three hundred micrograms of cell lysate was incubated at 4°C for 1 h on a shaking platform with 1–5 μg of anti-PKB, anti-RSK, or anti-AMPK antibodies (as described under *Materials*) conjugated to 5 μl of protein G-Sepharose. The immunoprecipitates were washed twice with 0.5 ml of lysis buffer containing 0.5 M NaCl and 1 mM DTT and twice with 0.5 ml of 50 mM Tris·HCl pH 7.5, 0.1 mM EGTA, and 1 mM DTT (*buffer A*). Phosphotransferase activity toward peptide substrates was then measured in a total assay volume of 50 μl containing 50 mM Tris·HCl pH 7.5, 0.1% (vol/vol) 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM [$\gamma$-$^{32}$P]ATP (300 cpm/pmol), and 200 μM RPRAATF (PKB), 30 μM Crosstide (RSK), or 200 μM AMARA (AMPK) peptide for 20 min at 30°C. Incorporation of [$^{32}$P]phosphate into the peptide substrate was determined by applying 40 μl of the reaction mixture onto P81 phosphocellulose paper, followed by washing of the paper in 50 mM phosphoric acid and scintillation counting. One unit of activity was defined as that which catalyzed the incorporation of 1 nmol of $^{32}$P into the substrate per minute.

*Assay of PDE3B.* PDE3 activity was measured in 20–30 μg of adipocyte membrane fractions or total homogenates, as described previously (42). The assay was performed at 30°C in a total volume of 300 μl containing (in mM) 50 TES pH 7.4, 250 sucrose, 1 EDTA, 0.1 EGTA, 8.3 $MgCl_2$, and 0.5 cAMP, with 1 μCi/ml [$^3$H]cAMP, and 0.6 μg/ml ovalbumin, in the presence or absence of the PDE3 inhibitor OPC3911 at 3 μM.

*Measurement of lipolysis and lipogenesis in rat adipocytes.* To measure lipolysis, adipocytes [400 μl of a 5% (vol/vol) suspension] were suspended in Krebs-Ringer medium, 25 mM HEPES pH 7.4, 200 nM adenosine, 2 mM glucose, and 1% BSA, and treated with inhibitors and hormones as indicated in Fig. 4 (at 37°C, with shaking at 150 cycles/min). After 30 min the cells were placed on ice for 20 min, and 200 μl of the cell medium was subsequently removed for enzymatic determination of the glycerol content as described previously (53, 57).

Lipogenesis was assayed in 700 μl of a 2% (vol/vol) suspension of adipocytes in Krebs-Ringer medium, 25 mM HEPES pH 7.4, 200 nM adenosine, 0.55 mM glucose, and 3.5% BSA, as the incorporation of D-[6-$^3$H]glucose into adipocyte triglycerides as previously described (44). Briefly, reactions were stopped with 3.5 ml of a toluol-based scintillation liquid containing 0.3 g/l 1,4-bis[5-phenyl-2-oxazolyl]benzene, 2,2′-*p*-phenylene-bis[5-phenyloxazole] (POPOP) and 5 g/l 2,5-diphenyl oxazole (PPO). Incorporation of [$^3$H]glucose into cellular lipids was measured by scintillation counting.

*Measurement of lipolysis and lipogenesis in 3T3L1 adipocytes.* 3T3L1 adipocytes cultured in six-well plates were rinsed with PBS, preequilibrated in 800 μl of Krebs-Ringer medium, 25 mM HEPES pH 7.4, 5 mM glucose, and 3% (wt/vol) BSA, and then treated with inhibitors and hormones as indicated in Fig. 4. After 1 h of glycerol accumulation, the medium was removed and centrifuged, and 200 μl was used for the enzymatic determination of glycerol content as described previously (53).

For lipogenesis measurements, 3T3L1 adipocytes cultured in six-well plates were rinsed with PBS, preequilibrated in 1 ml of Krebs-Ringer medium pH 7.4, 25 mM HEPES pH 7.4, 200 nM adenosine, 0.55 mM glucose, and 3.5% BSA, for 1 h and then treated with inhibitors and hormones, in the presence of D-[6-$^3$H]glucose, as indicated in Fig. 6. The incorporation of D-[6-$^3$H]glucose into adipocyte triglycerides was determined by scintillation counting of the cells as described for rat adipocytes.

*Statistical methods.* Differences were analyzed with Student's *t*-test (2-tailed, paired) and were considered statistically significant when $P < 0.05$. Results are presented as means + SE.

## RESULTS

*Akti is an efficient inhibitor of PKB in adipocytes.* To evaluate the efficiency of Akti in inhibiting PKB in different adipocyte cell types, primary rat and 3T3L1 adipocytes (of mouse origin) were pretreated with different doses of Akti or the PI3K inhibitor wortmannin and then stimulated with insulin. Increasing doses of either Akti or wortmannin prevented the ability of insulin to induce phosphorylation of PKB at the regulatory T308 and S473 sites in both cell types (Fig. 1, *A* and *B*). In rat adipocytes, 7.5 μM Akti was sufficient to inhibit 95% and 80% of the insulin-induced phosphorylation of T308 and S473, respectively. In 3T3L1 adipocytes, 3 μM Akti abolished insulin-stimulated phosphorylation of PKB altogether. Akti-mediated inhibition of PKB correlated with a similar prevention of insulin-induced phosphorylation of a well-established cellular PKB substrate, GSK3 (Fig. 1, *A* and *B*), demonstrating that the inhibition of PKB phosphorylation does indeed translate into an inactivation of PKB in cells.

In vitro, Akti was reported to efficiently inhibit the α- and β-isoforms, with a slight preference for PKBα (with an $IC_{50}$ of 58 nM as opposed to 210 nM), whereas PKBγ was poorly inhibited ($IC_{50}$ = 2.1 μM). It was also reported that Akti inhibited PKBα and PKBβ activity, but not PKBγ, in a prostate tumor cell line (16). Since pretreatment with Akti resulted in a near-complete ablation of the insulin-induced phosphorylation of PKB and since at least 3T3L1 adipocytes have been reported to express appreciable levels of PKBγ (9), we were interested in analyzing the effect of Akti on the activities of individual PKB isoforms in adipocytes. To do this, we employed isoform-specific immunoprecipitation (see MATERIALS AND METHODS) of PKBα, -β, and -γ in lysates from adipocytes treated with Akti and insulin and measured their activities toward a peptide substrate. As demonstrated in Fig. 1, *C* and *D*, all three isoforms were detectable and activated by insulin in rat and 3T3L1 adipocytes. The activities of PKBα and PKBβ were similarly inhibited in the two cell types, with a slightly higher efficiency toward the α-isoform. Submicromolar (0.1 μM) Akti concentrations were sufficient to significantly reduce the insulin-induced activity of PKBα in 3T3L1 cells (Fig. 1*D*) as well as in rat adipocytes (data not shown). Although significantly less sensitive to Akti, PKBγ activation was also observed to be reduced in a dose-dependent manner. The PKBγ activity remaining at Akti concentrations of 10 μM and 3 μM, in rat and 3T3L1 adipocytes, respectively, did not significantly contribute to total PKB activity/phosphorylation, as demonstrated by the near to complete abrogation of insulin-stimulated phosphorylation of T308 and S473 (Fig. 1, *A* and *B*). We also analyzed the sensitivity of glutathione *S*-transferase (GST)-PKBγ expressed in HEK293 cells to Akti, and in this setting an Akti concentration of 10 μM was sufficient to completely prevent phosphorylation and activation of PKBγ in response to IGF-I (data not shown). In summary, Fig. 1 shows that Akti prevents total PKB phosphorylation/activity at 10 μM and 3 μM for rat and 3T3L1 adipocytes, respectively, and that concentrations of 1–3 μM Akti are sufficient to inhibit PKBα and PKBβ activities in both cell types.

*Akti does not affect upstream insulin signaling or PI3K-dependent insulin-induced activation of ERK/RSK pathway.* To rule out the possibility that Akti prevents the phosphorylation

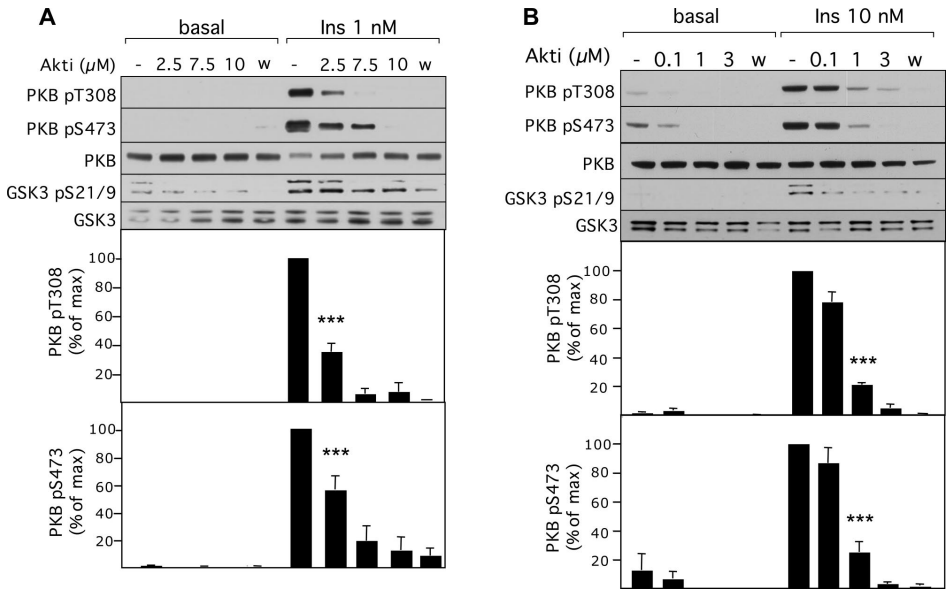


Fig. 1. Effect of Akti on insulin-induced protein kinase B (PKB) phosphorylation (*A* and *B*) and activation (*C* and *D*) in rat and 3T3L1 adipocytes. Rat (*A* and *C*) and 3T3L1 (*B* and *D*) adipocytes were preincubated with or without Akti (1 h) or wortmannin (w; 100 nM, 20 min) as indicated, and then stimulated with insulin (Ins) for 10 min or left untreated (basal). *A* and *B*: cell lysates were analyzed by Western blot using phospho-specific antibodies as indicated. PKB immunoblots from 3–7 separate experiments were quantified and expressed as % of the insulin-stimulated sample and are presented as means + SE. Representative blots are shown. GSK, glycogen synthase kinase. *C* and *D*: lysates were analyzed with regard to PKB activity by immunoprecipitation with isoform-specific antibodies and a peptide substrate. Activities were expressed as % of the insulin-stimulated sample and are presented as means + SE of 2–5 separate experiments. Insulin-stimulated PKB activities in rat adipocytes ranged from 1.8 to 4.82 mU/mg (mean 2.47) for PKBα, 1.0 to 4.61 mU/mg (mean 3.25) for PKBβ, and 0.14 to 0.42 mU/mg (mean 0.32) for PKBγ. Insulin-stimulated PKB activities in 3T3L1 cells ranged from 3.91 to 8.65 mU/mg (mean 5.71) for PKBα, 0.92 to 1.51 mU/mg (mean 1.27) for PKBβ, and 2.17 to 5.73 mU/mg (mean 3.47) for PKBγ. Significant decrease from the sample stimulated with insulin in the absence of Akti: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

of PKB in cells by affecting upstream insulin signaling, we analyzed the phosphorylation of the IR at the activity-controlling sites Y1162 and Y1163 and IRS1 at Y612—one of the sites that mediate PI3K docking (20). Increasing doses of Akti did not affect these tyrosine phosphorylations in rat (Fig. 2*A*) or 3T3L1 (Fig. 2*B*) adipocytes. Surprisingly, wortmannin appeared to inhibit insulin-induced tyrosine phosphorylation of the IR in 3T3L1 adipocytes, although this was not the case in rat adipocytes and, furthermore, did not translate into reduced IRS phosphorylation in either cell type (Fig. 2*B*). Moreover, Akti did not affect insulin-induced phosphorylation/activation of ERK1/2 and RSK in 3T3L1 adipocytes (Fig. 2*C*), although this activation was sensitive to the PI3K inhibitor wortmannin, indicating that PI3K is not a target for Akti. Insulin-induced phosphorylation of ERK and activation of RSK2 appeared to be intact in the presence of as much as 30 μM Akti ($n = 2$ and 1, respectively; data not shown).

Collectively, the results presented in Figs. 1 and 2 demonstrate that Akti efficiently and specifically prevents the insulin-induced phosphorylation and activation of PKB in rat and 3T3L1 adipocytes and is a useful tool in studying the role of PKB in lipid metabolism in these cells.

*PKB is required for the antilipolytic action of insulin, through regulation of PDE3B.* That PKB has a role as an upstream kinase for PDE3B, the rate-limiting enzyme in insulin-induced antilipolysis, is widely accepted, but this has never been supported by pharmacological inhibition or genetic deletion of PKB (52). As shown in Fig. 3, acute inhibition of PKB by Akti resulted in significantly reduced activation of PDE3B in response to insulin in both adipocyte systems. Maximal inhibition occurred at 7.5 μM and 3 μM Akti in rat and 3T3L1 adipocytes, respectively, and these concentrations were as efficient as wortmannin in preventing PDE3B activation in response to insulin.

From studies employing PDE3B inhibitors or PDE3B-deficient mice (13, 19), it is known that a major role of PDE3B in adipocytes is to mediate the antilipolytic effect of insulin. We therefore investigated the effect of Akti pretreatment on this biological response to insulin and monitored the phosphorylation status of HSL, which carries out catecholamine-induced lipolysis in adipocytes (51). Stimulation of rat adipocytes with the β-adrenergic agonist isoproterenol resulted in increased phosphorylation of HSL at S563 (Fig. 4*A*), which is a target for PKA and controls the

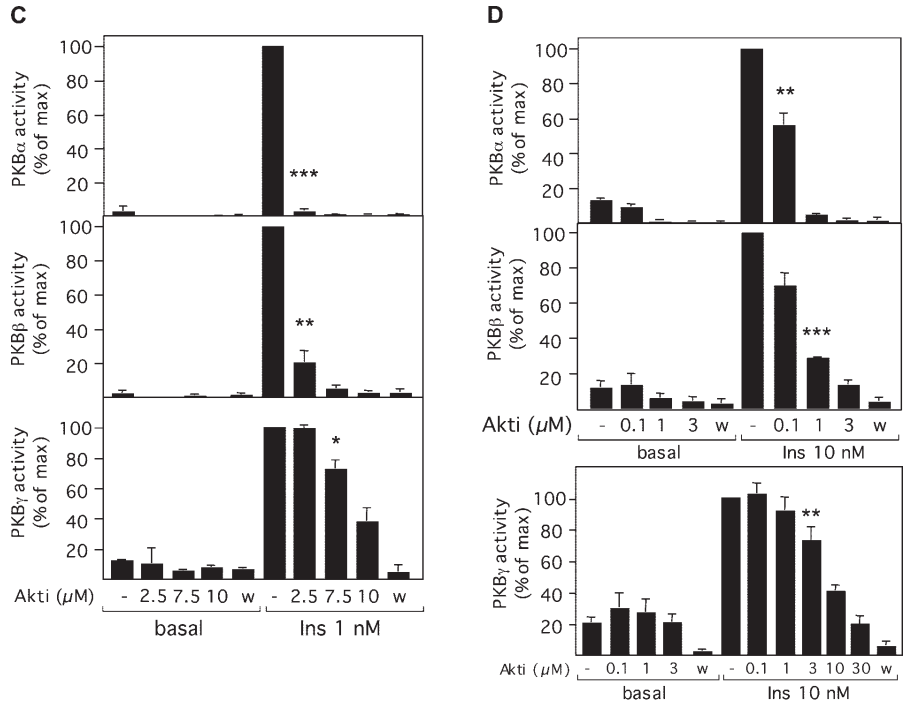


Fig. 1.—*Continued*

lipolytic activity of HSL in vivo. The phosphorylation/activation of HSL was reflected by an increased lipolytic rate (Fig. 4*B*). In the absence of inhibitors, insulin treatment resulted in a complete reversal of HSL phosphorylation and a reduction of lipolysis back to the basal level. PKB inhibition, by increasing doses of Akti, did, however, potentiate the effects of isoproterenol on HSL phosphorylation and, most importantly, prevented the ability of insulin to counteract isoproterenol-induced HSL phosphorylation (Fig. 4*A*) and lipolysis (Fig. 4, *B* and *C*). The PDE3 inhibitor OPC3911, in addition to wortmannin, also inhibited the antilipolytic effect of insulin. In 3T3L1 adipocytes, Akti similarly inhibited the antilipolytic action of insulin, although higher concentrations of Akti were required (Fig. 4, *D–F*).

*PKB is required for the ability of insulin to regulate ACC and for lipogenesis in adipocytes.* Another important manner in which insulin promotes the storage of lipids, at least in rodents, is by stimulating de novo lipid formation. This is achieved mainly by activating ACC, the rate-limiting enzyme in fatty acid synthesis (25). Insulin activates ACC, at least in part, by causing a net dephosphorylation of ACC at S79 (21, 56). This site is known to be phosphorylated by AMPK, leading to inhibition of enzyme activity. As shown in Fig. 5*A*, insulin induced a significant (40%) decrease in the phosphorylation of ACC at S79. The dephosphorylation was dependent on the presence of active PKB, as demonstrated by the reversal of ACC S79 phosphorylation as a result of Akti pretreatment (Fig. 5*A*). This reversal was observed at Akti concentrations down to 1 μM (data not shown). These results would be consistent with AMPK being negatively regulated by insulin, through PKB phosphorylation, as has been demonstrated for AMPK in the heart (29, 36). As shown in Fig. 5*B*, AMPK was indeed phosphorylated on S485, the suggested PKB site, in response to insulin. This was associated with a reduced phosphorylation of T172, and a significant (25%) decrease in activity of AMPKα1, which is the major AMPK isoform in adipocytes (Fig. 5*C*). PKB inhibition reversed the effects of insulin on AMPK phosphorylation (S485, T172) and activity.

Our results (Fig. 5) clearly show that active PKB is required for the ability of insulin to regulate the key lipogenic enzyme ACC, and that one mechanism involved in this regulation may be PKB-mediated inhibition of AMPK. We therefore next investigated the effect of PKB inhibition on lipogenesis, measured as the incorporation of [$^3$H]glucose into neutral lipids. In rat and 3T3L1 adipocytes, insulin induced two- and fourfold increase in the lipogenic rate, respectively (Fig. 6). In rat adipocytes PKB inhibition resulted in a dose-dependent reduction of basal and insulin-induced lipogenesis, with a complete abolishment of the

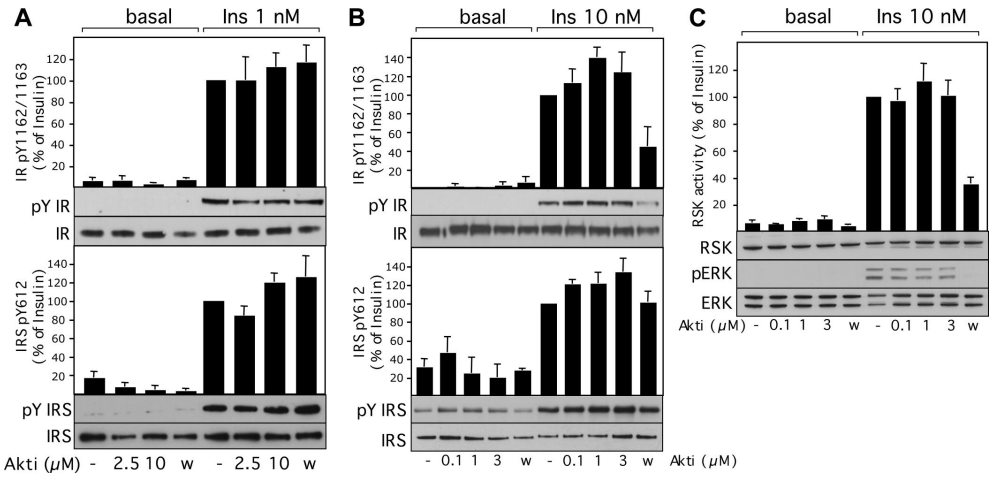


Fig. 2. No effect of Akti on insulin receptor (IR) or insulin receptor substrate (IRS) (*A* and *B*) or on ERK/p90 ribosomal kinase (RSK) (*C*) activation. Rat (*A*) and 3T3L1 (*B* and *C*) adipocytes were preincubated with or without Akti (1 h) or wortmannin (w; 100 nM, 20 min) as indicated and then stimulated with insulin (Ins) for 10 min or left untreated (basal). Cell lysates were analyzed by Western blot using phospho-specific antibodies to IR, IRS1 (*A* and *B*), or ERK (*C*). Immunoblots from 3–7 separate experiments were quantified and expressed as % of the insulin-stimulated sample and are presented as means + SE. Representative blots are shown. RSK2 activity was determined by immunoprecipitation kinase assay employing a peptide substrate (*C*). Results are presented as means + SE of 4 experiments. Insulin-stimulated RSK activity ranged from 3.3 to 19.4 mU/mg (mean 10.7).

ability of insulin to increase lipogenesis at an Akti concentration of 7.5 μM (Fig. 6*A*). In 3T3L1 adipocytes, inhibition of PKB led to greatly reduced insulin-induced lipogenesis, as well as a 50% reduction in the basal lipogenic rate (Fig. 6*B*).

## DISCUSSION

In this work, we show that Akti is a useful experimental tool to study the consequences of PKB inhibition in two adipocyte cell types, including primary rat adipocytes, which are physiological target cells for insulin (Figs. 1 and 2). Moreover, we provide definitive evidence that PKB is critical for the ability of insulin to activate PDE3B, and thereby antilipolysis (Figs. 3 and 4). We also propose a novel mechanism for the regulation of ACC and lipogenesis, in which PKB carries out inhibitory phosphorylation of AMPK, leading to a net dephosphorylation, and hence activation, of ACC (Figs. 5 and 6). Our current view

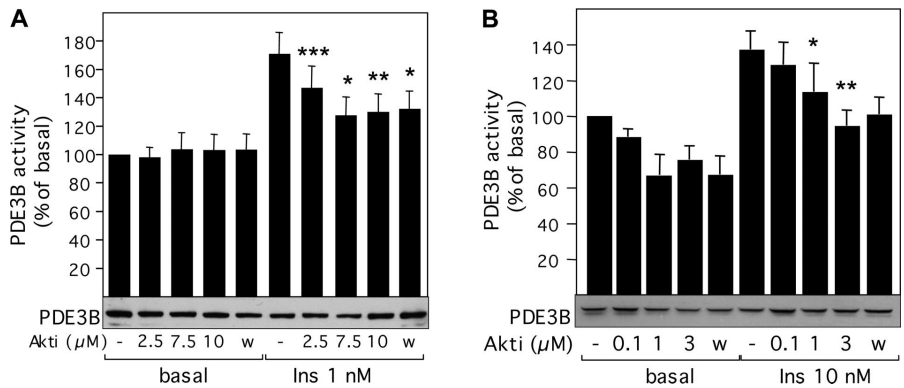


Fig. 3. Akti inhibits insulin-induced activation of phosphodiesterase 3B (PDE3B) in rat (*A*) and 3T3L1 (*B*) adipocytes. Rat and 3T3L1 adipocytes were preincubated with or without Akti (1 h) or wortmannin (w; 100 nM, 20 min) as indicated and then simulated with insulin (Ins) for 10 min or left untreated (basal). PDE3B activity was measured in detergent-free homogenates or in crude membrane fractions, and the results were expressed as % of the unstimulated sample. The results presented are means + SE from 6–8 independent experiments. Insulin-induced PDE3B activity in rat adipocytes ranged from 41.0 to 129.7 pmol·min$^{-1}$·mg$^{-1}$ (membrane fractions) and from 6.2 to 20.9 pmol·min$^{-1}$·mg$^{-1}$ (homogenates) and in 3T3L1 adipocytes from 34.5 to 39.1 pmol·min$^{-1}$·mg$^{-1}$ (membrane fractions) and from 4.9 to 20.9 pmol·min$^{-1}$·mg$^{-1}$ (homogenates). Significant decrease from the sample stimulated with insulin in the absence of Akti: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

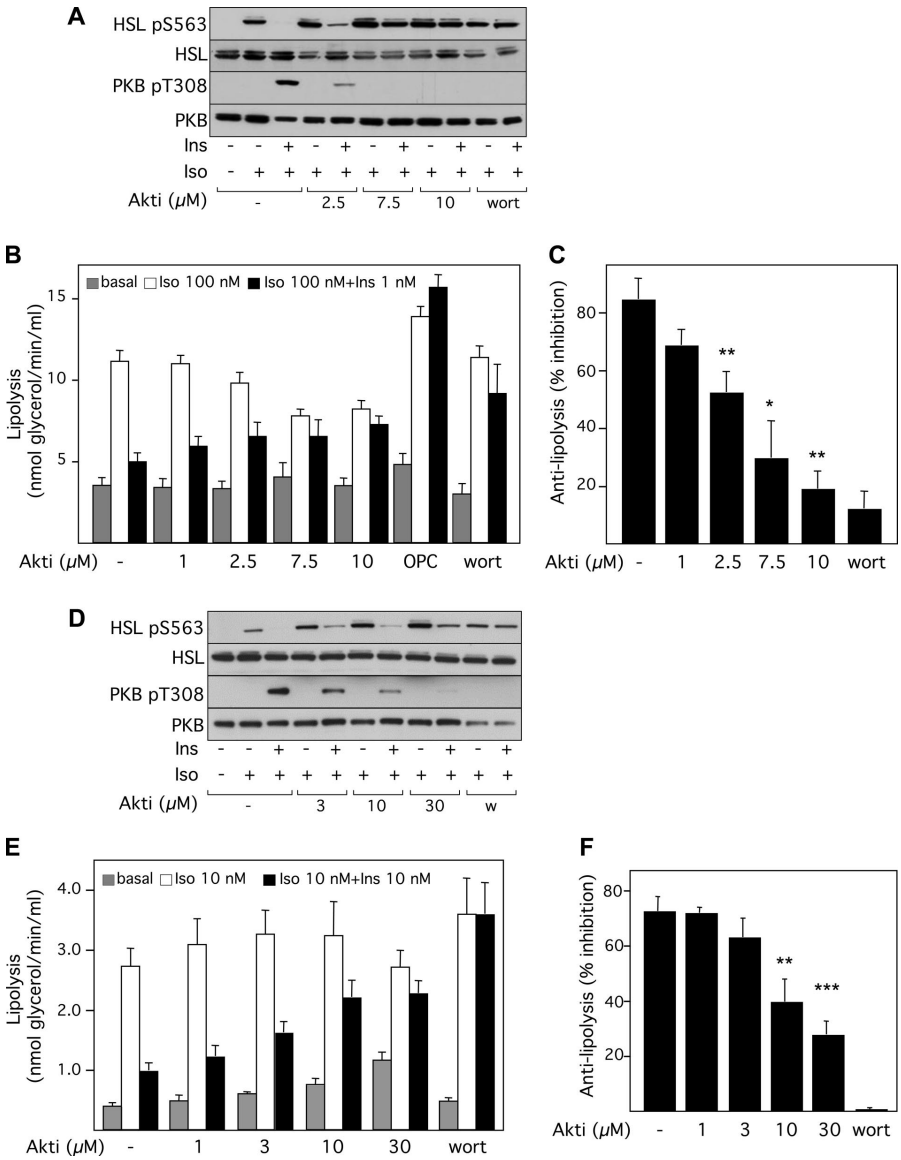


Fig. 4. Effect of Akti on hormone-sensitive lipase (HSL) phosphorylation and lipolysis in rat (*A–C*) and 3T3L1 (*D–F*) adipocytes. Rat and 3T3L1 adipocytes were preincubated with or without Akti (1 h), wortmannin (wort; 100 nM, 20 min), or the PDE3 inhibitor OPC3911 (OPC, 10 μM, 30 min) as indicated and then simulated with insulin (Ins) and isoproterenol (Iso) for 30 min (rat adipocytes) or 60 min (3T3L1 adipocytes) or left untreated (basal). Cell lysates were analyzed by Western blot using phospho-specific antibodies as indicated (*A* and *D*). Lipolysis was measured as accumulated glycerol release into the medium. Results are presented as means of absolute glycerol release values (*B* and *E*) or as % inhibition of isoproterenol-induced lipolysis in the presence of insulin (*C* and *F*) + SE from 5 or 6 independent experiments. *C* and *F*: significant decrease from the sample stimulated with isoproterenol and insulin in the absence of Akti: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

of how insulin regulates lipid metabolism, and the role of PKB therein, is summarized in Fig. 7.

Before the present study, because of the lack of specific PKB inhibitors, the role of PKB in lipid metabolism had only been investigated with overexpression (34, 56). There are notable limitations to the use of these strategies: for example, overexpression of active PKB may lead to triggering of signaling pathways that are perhaps normally activated by other members of the AGC family of kinases; also, such overexpression does not determine the absolute requirement for PKB in a given process. Dominant-negative constructs may affect other signaling molecules besides PKB, such as AGC kinases that are also activated by PDK1 phosphorylation. Although the use of PKB-null mouse models is complicated by isoform redundancy, as well as the fact that compensatory mechanisms may arise as a consequence of permanent gene loss, these models still represent interesting systems for further study of the role of PKB in lipid metabolism. Our results show that Akti can potently inhibit PKB in adipocytes without affecting upstream signaling (IR, IRS) or other insulin-stimulated, PI3K-dependent signaling pathways (ERK/RSK). This is in line with recent data from Green et al. (24), who reported similar findings in L6 myotubes. In that study, it was also demonstrated that Akti does not affect in vitro PI3K activity, the association between IRS1 and PI3K p85, or the insulin-induced generation of 3-phosphoinositides.

The specificity of Akti for different isoforms of PKB has mainly been studied in vitro, and it was shown to preferentially act on PKBα and PKBβ. Our immunoprecipitation kinase assays show that in adipocytes (Fig. 1, *C* and *D*) and in HEK293 cells expressing GST-PKBγ (data not shown), Akti also prevents the activation of PKBγ, albeit at higher concentrations. The PKBγ activity remaining at Akti concentrations of 10 μM and 3 μM in rat and 3T3L1 adipocytes, respectively, was not reflected in the analysis of total T308 and S473 phosphorylation (Fig. 1, *A* and *B*), most likely suggesting that PKBγ constitutes a relatively small portion of the total PKB protein in adipocytes. Although there have been reports of the existence of short forms of PKBγ lacking the COOH-terminal regulatory S473 site (35), the information now available in the database suggests that the two regulatory sites in PKBγ, and the sequences surrounding them, are completely conserved when comparing the rat, mouse (3T3L1), and human sequences. Phosphorylation of human GST-PKBγ overexpressed in HEK293 cells was readily detected (data not shown), suggesting that the T308 and S473 phospho-specific antibodies do indeed recognize the phosphorylated PKBγ epitopes. The relative abundance of the three isoforms is difficult to determine in detail because of the different efficiencies of the antibodies, as well as different specific activities of PKB isoforms toward the peptide substrate. We nevertheless addressed this issue by measuring the activity of PKBα, PKBβ, and PKBγ, simultaneously, in lysates from insulin-stimulated adipocytes. PKBγ only accounted for 5% of the total insulin-stimulated PKB activity in rat adipocytes and 16% in 3T3L1 adipocytes (data

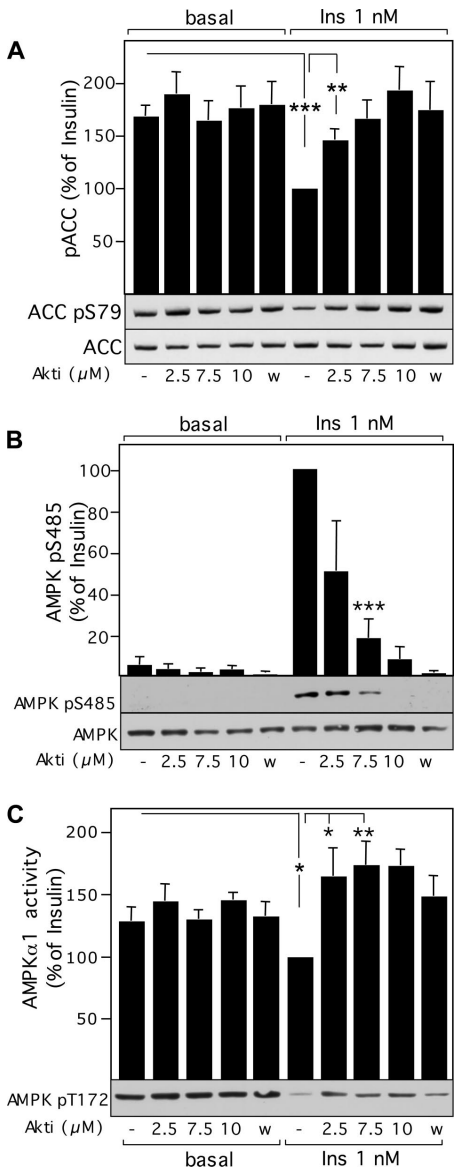


Fig. 5. Effect of Akti on the regulation of acetyl-CoA carboxylase (ACC; *A*) and AMP-activated protein kinase (AMPK; *B* and *C*). Rat adipocytes were preincubated with or without Akti (1 h) or wortmannin (w; 100 nM, 20 min) as indicated and then simulated with insulin (Ins) for 10 min or left untreated (basal). Cell lysates were analyzed by Western blot using phospho-specific antibodies to ACC (*A*) and AMPK (*B* and *C*). ACC pS79 and AMPK pS485 immunoblots were quantified and expressed as % of the insulin-stimulated sample and are presented as means + SE of 3–7 separate experiments. AMPK activity (*C*) was determined in AMPKα1 immunoprecipitates with a peptide substrate. Significant difference between samples: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

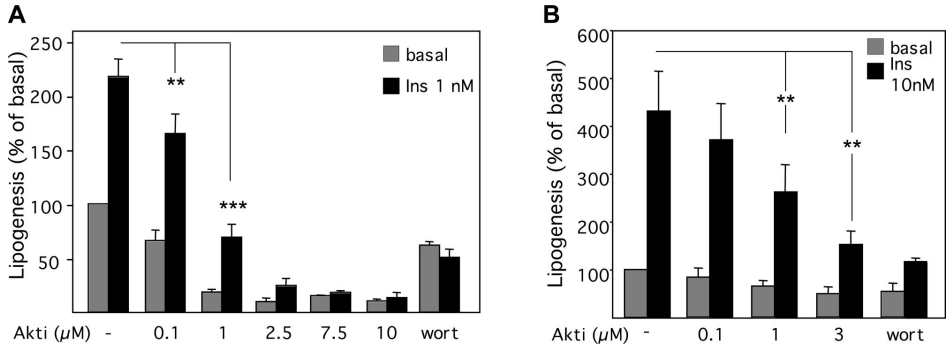


Fig. 6. Effect of Akti on lipogenesis in rat (*A*) and 3T3L1 (*B*) adipocytes. Rat and 3T3L1 adipocytes were preincubated with or without Akti (1 h) or wortmannin (wort; 100 nM, 20 min) as indicated and then stimulated with insulin (Ins) for 30 min or left untreated (basal). Lipogenesis was measured as the incorporation of [$^3$H]glucose into adipocyte neutral lipids. Results are presented as % of the basal value and are means + SE from 4–6 independent experiments. Significant decrease from the sample stimulated with insulin in the absence of Akti: $^{**}P < 0.01$, $^{***}P < 0.001$.

not shown). These results suggest that the contribution of PKBγ to total PKB activity/phosphorylation is relatively small in both cell types.

To understand the differences in the efficiency by which Akti acts on PKB isoforms in vitro and in vivo, it will be critical to fully characterize its molecular mechanism of action. Akti belongs to a series of compounds developed in 2005 by Lindsley et al. (38), who showed that these inhibit PKB in a non-ATP- or substrate-competitive manner. This is one explanation as to why Akti displays a high degree of specificity for PKB over other kinases, most importantly members of the AGC family, which are highly homologous in their kinase domains (8, 39). Instead, it was proposed that Akti acts allosterically by binding to a site that requires the pleckstrin homology (PH) domain of PKB, since antibodies to the PH domain or the hinge region were found to prevent the action of Akti, as did deletion of the PH domain (8). Binding of the inhibitor has been postulated to promote a conformational change that renders the enzyme inactive but also prevents phosphorylation by upstream kinases. A recent study in fact identified a tryptophan residue (W80) in the PH domain of PKB, the mutation of which rendered the kinase insensitive to inhibition by Akti (24). In L6 myotubes expressing the W80A mutant, but not the wild-type PKB, insulin-induced glucose uptake was maintained even in the presence of Akti. This demonstrates that Akti does indeed inhibit glucose uptake by specifically affecting PKB, and that W80 is critical for the action of Akti.

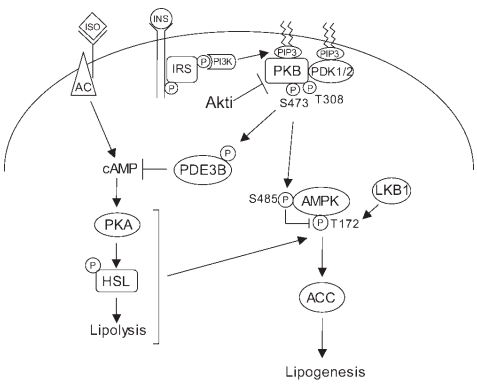


Fig. 7. Role of PKB in lipid metabolism. This schematic overview summarizes our current understanding of the role of PKB in the regulation of lipogenesis and antilipolysis by insulin. Our data are consistent with PKB being required for the antilipolytic action of insulin, and with PKB being upstream of the antilipolytic enzyme PDE3B. Lipolysis is induced by the action of catecholamines [for example, isoprenaline (Iso)] on G protein-coupled receptors that activate adenylate cyclase (AC), resulting in elevated levels of cAMP and increased protein kinase A (PKA) and HSL activities. Insulin counteracts lipolysis by inducing the phosphorylation and activation of PDE3B by PKB, leading to hydrolysis of cAMP and attenuation of the lipolytic pathway. Moreover, we have shown that PKB is critical for lipogenesis and for the insulin-induced dephosphorylation/activation of the lipogenic enzyme ACC. One way in which PKB may regulate ACC is by direct phosphorylation of AMPK at S485, resulting in inhibition of phosphorylation of AMPK at T172 and of AMPK activity. This in turn leads to a net dephosphorylation and activation of ACC. Alternatively, since increased levels of cAMP and lipolysis have been shown to activate AMPK, PKB may inhibit AMPK indirectly by activating PDE3B, resulting in reduced cAMP levels. LKB1 is the main AMPK T172 kinase, at least in liver and muscle.

By employing pharmacological, selective inhibition of PKB, we have provided strong evidence that PKB is indeed an important upstream kinase for PDE3B (Fig. 3), both in primary rat and 3T3L1 adipocytes. Our data are in line with previous reports demonstrating that PKB phosphorylates and thereby activates PDE3B in vitro (2, 33). Moreover, several groups have also employed constitutively active and dominant negative versions of PKB to suggest that PKB acts upstream of PDE3B in 3T3L1 adipocytes, the promyeloid cell line FDCP2, and *Xenopus* oocytes (2, 7, 33). It has also been shown that PKB and PDE3B coimmunoprecipitate and colocalize in macromolecular complexes, and that these complexes are important for the regulation of PDE3B by hormones (3). In contrast to our data, Smith et al. (48) suggested that PKB activity was not critical for activation of PDE3B and for antilipolysis in adipocytes. These conclusions were made from experiments

using a myosin light chain kinase inhibitor, ML9, that also appeared to act on PKB. However, ML9 also efficiently inhibited other AGC kinases such as PKA and RSK. Especially in the case of lipolysis, results achieved with this tool are difficult to interpret, given the critical role of PKA in the regulation of lipolysis.

In Fig. 4 we demonstrate that the inability of insulin to normally activate PDE3B in the presence of Akti (see Fig. 3) is associated with an inhibition of the antilipolytic action of insulin in both cell types. However, the concentration of Akti required to prevent antilipolysis was greater in 3T3L1 adipocytes than in rat adipocytes, although 3T3L1 cells displayed a higher sensitivity to the inhibitor at the level of PKB, as judged by the results presented in Fig. 1. It should be noted, however, that in the context of the glycerol accumulation experiment in 3T3L1 adipocytes, the sensitivity of PKB T308 phosphorylation to Akti was significantly lower (compare Fig. 4*D* with Fig. 1*B*) and did in fact correlate with the inhibition of antilipolysis. The reasons for this apparent discrepancy in sensitivity are not clear; however, differences in the experimental conditions under which these two types of experiments were carried out (see MATERIALS AND METHODS) may have affected the efficiency of Akti.

Our data support the idea that PKB has an important role in the regulation of ACC and lipogenesis (Figs. 5 and 6). It is well established that insulin promotes the activation of ACC in several tissues and cell types, including liver and adipocytes; however, the mechanism underlying this effect of insulin has been less clear (26, 41, 54). ACC is regulated by changes in its phosphorylation status, as well as allosterically by citrate (32). In the 1980s, several papers reported the ability of insulin to increase phosphorylation of specific sites on ACC in adipocytes, as well as the identification of kinases capable of phosphorylating these in vitro (27, 32). However, neither of the sites appeared to control ACC activity or its regulation by citrate (27). Subsequently, Hardie and coworkers (15, 46) reported that phosphorylation of S79 in ACC by AMPK leads to its inactivation and also to a reduced sensitivity to citrate, and this is now believed to be the major regulatory mechanism for ACC. Our results showing that S79 phosphorylation is reduced in response to insulin (Fig. 5*A*) are consistent with previous reports (21, 56), and this most likely explains the activation of ACC by insulin. Moreover, our data with Akti support the notion that PKB has a critical role in this regulation (Fig. 5*A*). Interestingly, recent work on the regulation of AMPK signaling in heart has identified a pathway by which insulin negatively regulates AMPK through phosphorylation by PKB (29, 36). Our work is consistent with AMPK (S485) being a substrate for PKB in adipocytes (Fig. 5*B*), and Akti inhibition of the PKB-mediated phosphorylation of AMPK was associated with increased AMPK activity (Fig. 5*B*). Phosphopeptide mapping would have to be carried out in order to determine whether S485 is phosphorylated to a physiologically relevant level. Moreover, a nonphosphorylatable mutant of AMPK would be useful to determine the requirement for S485 in the negative regulation of AMPK by insulin and PKB in cells. Elevated cAMP levels in adipocytes are well known to stimulate AMPK activity (55), and this was recently shown to be mediated by changes in AMP/ATP levels, as a result of reesterification of fatty acids released during lipolysis (22). An alternative way in which PKB could inhibit AMPK in adipocytes is therefore via the activation of PDE3B, increased cAMP hydrolysis, and thus reduced lipolysis (Fig. 7). This is, however, unlikely to account for the negative effects of insulin on AMPK in heart muscle, in which insulin-regulated isoforms of PDE do not appear to be expressed (31, 43) and lipolysis is not a significant metabolic route.

In our hands, activation of PKB in response to insulin resulted in a 25% reduction in AMPK activity. This is consistent with the results of previous studies showing that insulin stimulation reduced AMPK activity in a hepatoma cell line by 20–30% (54) as well as inhibiting T172 phosphorylation of AMPK in 3T3L1 adipocytes (55) and in heart (36). In contrast, another study, performed in rat adipocytes, reported that the activity of AMPK remained unchanged in response to insulin (45). The reduction in AMPK activity that we observed could at least in part account for the effect of insulin on phosphorylation of S79 in ACC. In addition, it is possible that PKB is involved in the regulation of other sites in ACC, the phosphorylation of which may in turn prevent phosphorylation of S79, or that PKB regulates a phosphatase that can act on ACC S79. Insulin has indeed been shown to promote the association between ACC and an ACC phosphatase (37).

Inhibition of PKB had profound effects on the lipogenic rate, leading to a reduction in basal and insulin-stimulated lipogenesis in both cell types (Fig. 6). Although pretreatment with Akti did slightly reduce basal PKB phosphorylation and activity (Fig. 1), basal phosphorylation of ACC remained unchanged in the presence of Akti (Fig. 5*A*). It is possible that PKB-mediated regulation of other enzymes involved in lipogenesis could account for the reduction in basal lipogenesis, as well as in the insulin-induced lipogenesis observed in the presence of Akti. For example, ATP-citrate lyase, which catalyzes the formation of acetyl-CoA from citrate, has been reported to be a substrate for PKB; however, it is not clear whether PKB phosphorylation affects ATP-citrate lyase activity (10). In rat adipocytes, the insulin-induced increase in lipogenesis was completely abolished in the presence of Akti. In Akti-treated 3T3L1 cells, insulin was still able to stimulate lipogenesis threefold, although the maximal lipogenic rate under these conditions was greatly reduced. This preserved stimulation could be accounted for by the remaining PKBγ activity in these cells; alternatively, insulin may in a PKB-independent manner activate lipogenic enzymes other than ACC in 3T3L1 adipocytes.

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# Paper II

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# cAMP-elevation mediated by β-adrenergic stimulation inhibits salt-inducible kinase (SIK) 3 activity in adipocytes

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ABSTRACT

Salt-inducible kinase (SIK) 3 is a virtually unstudied, ubiquitously expressed serine/threonine kinase, belonging to the AMP-activated protein kinase (AMPK)-related family of kinases, all of which are regulated by LKB1 phosphorylation of a threonine residue in their activation (T)-loops. Findings in adrenal cells have revealed a role for cAMP in the regulation of SIK1, and recent findings suggest that insulin can regulate an SIK isoform in Drosophila. As cAMP has important functions in adipocytes, mainly in the regulation of lipolysis, we have evaluated a potential role for cAMP, as well as for insulin, in the regulation of SIK3 in these cells. We establish that raised cAMP levels in response to forskolin and the β-adrenergic receptor agonist CL 316,243 induce a phosphorylation of SIK3 in HEK293 cells and primary adipocytes. This phosphorylation coincides with increased 14-3-3 binding to SIK3 in these cell types. Our findings also show that cAMP-elevation results in reduced SIK3 activity in adipocytes. Phosphopeptide mapping and site-directed mutagenesis reveal that the cAMP-mediated regulation of SIK3 appears to depend on three residues, T469, S551 and S674, that all contribute to some extent to the cAMP-induced phosphorylation and 14-3-3-binding. As the cAMP-induced regulation can be reversed with the protein kinase A (PKA) inhibitor H89, and a role for other candidate kinases, including PKB and RSK, could be excluded, we believe that PKA is the kinase responsible for SIK3 regulation in response to elevated cAMP levels. Our findings of cAMP-mediated regulation of SIK3 suggest that SIK3 may mediate some of the effects of this important second messenger in adipocytes.



## 1. Introduction

Salt-inducible kinase (SIK) 3, one of three SIK isoforms, is a member of the AMP-activated protein kinase (AMPK) subfamily of serine/threonine kinases, all of which share homology with AMPK in their kinase domains. The activity of AMPK and most of its related kinases, is regulated by phosphorylation of a specific threonine residue in their activation (T)-loop, a phosphorylation mediated by the upstream kinase LKB1 [1–4].

SIK1 was discovered in the adrenal glands of rats fed a high-salt diet [5] and subsequent homology searches revealed two additional isoforms, SIK2 and SIK3 [6]. While SIK1 appears to participate in steroidogenesis [7] and SIK2 has mainly been implicated in inhibition of gluconeogenesis in the liver [8], SIK3 is the least investigated of the SIK isoforms, its function and regulation largely unstudied. Recent findings in Drosophila, suggest that an SIK isoform is activated by protein kinase B (PKB) in response to insulin, resulting in regulation of energy balance in the fly [9]. Previous findings in Drosophila suggest a role for SIK3 in spindle pole formation [10], a result corroborated in studies of ovarian cancer cell lines, where overexpression of SIK3 resulted in promotion of cell cycle progression [11]. The recent generation of an SIK3 knock-out mouse model revealed that SIK3 is essential for chondrocyte hypertrophy during skeletal development in mice [12]. Sasagawa et al. also report that SIK3-deficient mice display impaired cholesterol metabolism (unpublished data).

It has been discovered that some 14-3-3 isoforms can bind directly to the T-loop threonine residue (T221 in human SIK3) of SIK1 and SIK3 upon LKB1-phosphorylation, resulting in a change in the subcellular distribution of these kinases [13]. 14-3-3s are scaffolding proteins that homo- and heterodimerize and bind to specific phosphorylated motifs on more than 300 targets, mainly affecting their subcellular localization or interactions with other proteins [14]. In adrenal cells, SIK1 undergoes nucleocytoplasmic shuttling in response to stimuli that raise cAMP levels, a translocation that depends on the phosphorylation of a specific serine residue, S577, which is conserved in SIK3 (S551) [15]. Moreover, we

*Abbreviations:* AMPK, AMP-activated protein kinase; ATP, adenosine tri-phosphate; cAMP, cyclic adenosine mono-phosphate; CRTC2, CREB regulated transcription coactivator 2; GST, Glutathione-S-Transferase; HEK293, human embryonic kidney 293 cells; HSL, hormone sensitive lipase; IPTG, isopropyl-β-D-thiogalactopyranoside; PKA, protein kinase A; PKB, protein kinase B; RSK, ribosomal S6 kinase; SIK, salt-inducible kinase.

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recently demonstrated that SIK2 is phosphorylated on S358 in response to cAMP in adipocytes, and that this induces its binding to 14-3-3 and relocalization [16]. However, the effect of cAMP-increasing agents, or any other extra-cellular signals, such as insulin/IGF1, on SIK3 in mammalian cells, remains unclear.

In this paper, we investigated potential effects of cAMP as well as insulin on SIK3 phosphorylation, 14-3-3 binding, kinase activity and sub-cellular localization. In order to study the regulation of endogenous SIK3 in a physiologically relevant mammalian cell model, we employed primary rat adipocytes, the function of which is under critical control by both cAMP and insulin [17].

We demonstrate that increased cAMP levels induce a PKA-dependent phosphorylation of SIK3, which correlates with an increase in 14-3-3 binding to sites other than the T-loop T221. Moreover, we demonstrate that this phosphorylation correlates with an inhibition of SIK3 kinase activity. Insulin/IGF1 did however not affect SIK3 phosphorylation or its binding to 14-3-3.

## 2. Materials and methods

### 2.1. Materials

Human embryonic kidney (HEK) 293 cells were kindly provided by Dr Kei Sakamoto (University of Dundee, UK) and Sprague–Dawley rats were obtained from Charles River laboratories. Dulbecco's Modified Eagle Medium (DMEM), trypsin/EDTA, forskolin, CL 316,243, pre-coupled HA-agarose, isopropyl-β-D-thiogalactopyranoside (IPTG), glutathione, fish skin gelatine and ampicillin were all obtained from Sigma Aldrich. Fetal Calf serum (FCS), human recombinant epidermal growth factor (EGF), pre-cast Novex SDS Polyacrylamide Bis-Tris gels, dithiothreitol (DTT), lauryl dodecyl sulfate (LDS) sample buffer and Hoechst nuclear stain were all purchased from Invitrogen (Carlsbad, USA). BL21 and DH5α-strains of *Escherichia coli* were kindly provided by Maria Deak at the Division of Signal Transduction Therapy (DSTT) (University of Dundee, UK). Glutathione-S-Transferase (GST)-Sepharose and Protein-G-Sepharose were purchased from GE Healthcare Biosciences (Uppsala, Sweden), complete protease-inhibitor tablets from Roche (Mannheim, Germany), polyethylenimine (PEI) was obtained from Polysciences (Eppelheim, Germany), Hydromount from National Diagnostics (Georgia, USA) and HiSpeed Maxi Kit from Qiagen. P81 phosphocellulose cation-exchange paper was purchased from Whatman (Dassel, Germany), $^{32}$P-γ-ATP from Perkin Elmer (Boston, USA), Sakamototide peptide was synthesized by Pepceuticals (Enderby, UK) and AMARA peptide was synthesized by Dr. Graham Bloomberg (University of Bristol, UK). Enhanced Chemiluminescence SuperSignal Reagent, cover slips and slides were purchased from Thermo Scientific (Schwerte, Germany). H89 was purchased from Biomol and IGF-1 from Tocris Bioscience (Bristol, UK). MK2206, PD 0325901 and full-length CREB regulated transcription coactivator 2 (CTRTC2) were kindly provided by the Division of Signal Transduction Therapy (University of Dundee, UK). pCMV5 and pEGFP-C1 cDNA constructs encoding wild type, T221A and kinase inactive (D206A) versions of an HA-tagged human SIK3 sequence obtained from www.kinase.com, were kindly provided by Dario Alessi, University of Dundee, and all other SIK3 mutants were generated by DNA-Cloning-Service (Hamburg, Germany). pGEX vectors encoding GST-tagged human 14-3-3ζ were kindly provided by Professor Carol Mackintosh (University of Dundee, UK). Insulin was supplied by Novo Nordisk (Copenhagen, Denmark).

### 2.2. Antibodies

The anti-SIK3 and anti-SIK2 C-terminal antibodies used for immunoprecipitation and western blotting of SIK3 and SIK2 respectively, were raised in rabbit against peptides corresponding to residues 1349–1369 (TDILLSYKHPEVSFSMEQAGV) of human SIK3 and residues 906–926 (LFDCEMLDAVDPQHNGYVLVN) of human SIK2 and affinity-purified by Innovagen (Lund, Sweden). The antibody used for immunoprecipitation and kinase assay of AMPKα1 was a kind gift from Professor D. Grahame Hardie (University of Dundee). The following total protein antibodies were used for Western Blotting: anti-HA and anti-GAPDH antibodies were obtained from Sigma Aldrich, anti-AMPK antibody was from Cell Signaling Technology and anti-14-3-3 antibody from Santa Cruz Biotechnology. The following phosphospecific antibodies were used for Western Blotting: anti-phospho HSL S563, anti-phospho PKA consensus, anti-phospho PKB consensus, anti-phospho AMPK T172 and anti-phospho ERK T42/44 were all purchased from Cell Signaling Technologies. Anti-phospho PKB S473 antibody, Alexa Fluor 594 anti-mouse antibody and horseradish-peroxidase (HRP)-conjugated secondary anti-rabbit antibody were all purchased from Invitrogen (Carlsbad, USA). HRP-conjugated secondary anti-mouse antibody was purchased from GE Healthcare.

### 2.3. Isolation and stimulation of primary rat adipocytes

Primary rat adipocytes were isolated from epididymal adipose tissue of male 36–38 day old Sprague–Dawley rats [18], under a protocol approved by the ethical review committee at Lund University (approval no. M212-09). Adipocytes were suspended in Krebs-Ringer medium containing 25 mM HEPES pH 7.4, 200 nM adenosine, 2 mM glucose and 1% BSA (Krebs-Ringer medium) and stimulated at 37 °C in a shaking water bath (120 rpm). The cells were subsequently washed twice in Krebs-Ringer medium without BSA and homogenized in 0.5–1 ml of homogenization buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT) and complete protease inhibitor (1 tablet/50 ml). The cell homogenates were centrifuged for 5 min at 5000 *g* and the fat layer was removed. 1% NP40 was added to the remaining supernatant, the samples were left on ice for 30 min to solubilize and finally centrifuged for 15 min at 4 °C and 13000 *g*. Protein concentrations were determined according to the Bradford protocol.

### 2.4. Culture, transfection and stimulation of HEK293 cells

HEK293 cells were cultured in DMEM to 100% confluence in 10 cm dishes and split 1:5 into new plates. Approximately six hours post-seeding, each 10 cm plate was transfected with 5 μg DNA of pCMV5 constructs (amplified in *E. coli* DH5α bacteria according to HiSpeed Qiagen maxi preparation protocol) encoding wild type, kinase inactive D206A, T-loop mutant T221A, S469A, S551A, S674A and T221A/S469A/S551A/S674A HA-SIK3 using the PEI method [19]. Cells were grown in serum-free medium for 16 h before stimulation and harvesting. 36 h post-transfection, cells were stimulated, washed once with PBS and lysed in 500 μl lysis buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT), 1% NP40 and complete protease inhibitor (1 tablet/50 ml) (lysis buffer).

### 2.5. Generation of recombinant 14-3-3 for far western

pGEX vectors encoding human GST-tagged wild type 14-3-3ζ were transformed into *E. coli* BL21 bacteria. One liter cultures were grown at 37 °C and 220 rpm in Luria Broth medium containing 100 μg/ml of ampicillin, until the optic density was 0.8. IPTG was added to a concentration of 100 μM in order to induce protein expression and the bacteria were cultured for an additional 16 h at 26 °C at 220 rpm. The cultures were centrifuged for 20 min at 4 °C and 4000 rpm and the bacterial pellet was resuspended in 10 ml ice-cold lysis buffer containing 1 mM DTT and complete protease inhibitors. After one cycle of freeze thawing, the extracts were sonicated for complete bacterial lysis and then

centrifuged for 20 min at 4 °C and 20000 g. The supernatant was collected and the recombinant protein affinity-purified on 200 µl glutathione-sepharose beads during 1 h of incubation at 4 °C on a rolling platform. The beads were washed twice with 10 ml lysis buffer containing 1 mM DTT and 0.5 M NaCl, and twice with 10 ml 50 mM Tris–HCl, pH 7.5 and 0.1 mM EGTA (buffer A) containing 1 mM DTT and 0.27 M sucrose. A portion of the recombinant material was kept coupled to the sepharose-beads for SIK3-pulldown experiments while a portion was eluted from the beads in Buffer A with 30 mM glutathione and 0.27 M sucrose.

### 2.6. Immunoblotting

Lysates and immunoprecipitates of SIK3 were heated at 95 °C for 2–5 min in LDS sample buffer and subjected to electrophoresis on Novex pre-cast SDS-PAGE gels and subsequent electrotransfer to nitrocellulose membrane. Membranes from lysate samples were blocked for 30 min in 10% (w/v) dried skimmed milk. Membranes were probed with 1 µg/ml or 1:1000–5000 dilutions of primary antibodies for 16 h at 4 °C in 5% (w/v) dried skimmed milk or 5% (w/v) BSA. Far western assays were performed using a previously described method [20]. Protein detection was performed with horseradish-peroxidase-conjugated secondary antibodies followed by treatment with chemiluminescence reagent. Films were developed using an AGFA CURIX 60. Quantification of the bands was performed by digitalizing the ECL-films in a Fuji LAS 1000 CCD camera followed by analysis of the intensities using the Image Gauge software or development in a ChemiDoc XRS+ camera followed by analysis of band intensities in the Image Lab software (both from BioRad).

### 2.7. Immunoprecipitation and in vitro assay of SIK2, SIK3 and AMPK kinase activity in adipocytes

Lysates containing 25–50 µg of protein were incubated at 4 °C for 1–2 h on a shaking platform with 1–3 µg of the relevant antibody conjugated to 5 µl protein G-Sepharose. The immunoprecipitates were washed twice with 500 µl lysis buffer supplemented with 0.5 M NaCl and 1 mM DTT, and twice with 500 µl buffer A. The kinase activity was measured in a volume of 50 µl containing 50 mM Tris–HCl pH 7.5, 0.1% 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM $^{32}$Pγ-ATP (300 cpm/pmol) and a relevant peptide substrate for 20 min at 30 °C. For SIK2 and SIK3, 200 µM Sakamototide peptide (ALNRTSSDSALHRRR), which encompasses S171 of CRTC2 shown to be phosphorylated by SIKs in vitro [21], was used as a substrate. For AMPK, the AMARA (AMARAASAAALARRR) peptide was used, which is based on the AMPK consensus peptide substrate sequence. The assay was terminated by applying 40 µl of the reaction mixture on p81 cation-exchange phosphocellulose paper, followed by immersion in 50 mM phosphoric acid and 5–6 subsequent washes in 50 mM phosphoric acid before liquid scintillation counting. Incorporation of $^{32}$P-phosphate was expressed as pmol ATP incorporated/mg protein/min (mU/mg). Phosphotransferase activity of SIK3 towards the full-length protein substrate CRTC2 was measured in a total assay volume of 25 µl containing 50 mM Tris/HCl pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 10 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM $^{32}$P-ATP-ATP (300 cpm/pmol) and 1 µg CRTC2 protein for 20 min at 30 °C. Following polyacrylamide gel electrophoresis and colloidal Coomassie staining, the gels were fixed, dried and analyzed in a FLA 3000 FUJIFILM reader using FUJI imaging plates type BAS-III. One Unit (U) of activity was defined as that which catalyzed the incorporation of 1 nmol of $^{32}$P/min into the substrate.

### 2.8. Immunocytochemistry of SIK1 and SIK3 in HEK293 cells

HEK293 cells were cultured on glass cover slips in 12 well plates with DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Approximately 24 h post-seeding, the cells were transfected with 0.5 µg DNA of pCMV5 constructs (amplified in *E. coli* DH5α bacteria according to HiSpeed Qiagen maxi preparation protocol) encoding wild type and S575A HA-SIK1 (mouse S577) as well as wild type and T221A/S469A/S551A/S674A (quadr.) HA-SIK3 using the PEI method [19]. After 22 h in regular growth medium, cells were kept in serum-free medium for 2 h before stimulation.

Cells were fixed in PBS containing 4% formaldehyde for 10 min and permeabilized in PBS containing 0.25% Triton-X-100 for 5 min. The cells were incubated for one hour with anti-HA antibody (1:1000) at room temperature, followed by incubation for one hour with 1:2000 anti-mouse Alexa 594 antibody interspersed with washing. Hoechst Nuclear stain was included (1:10000) in one of the final washing steps before mounting on glass slides.

The cells were imaged using a Zeiss LSM 510 META microscope with excitation wavelengths 405 (Hoechst nuclear stain) or 561 (Alexa 594) nm. A Plan-Apochromat 63×/1.4 oil DIC objective was used and a frame size of 024×1024×1.

### 2.9. Phosphopeptide mapping of human SIK3 expressed in HEK293 cells and identification of 14-3-3 isoforms

Wild type and D206A (kinase inactive) human HA-SIK3 were expressed in HEK293 cells as described above. SIK3 was immunoprecipitated from 5 mg of total protein and loaded on a polyacrylamide gel. The bands representing HA-SIK3 and 14-3-3 were excised, incubated with 50 mM iodoacetamide to alkylate Cys residues, washed and digested with trypsin for 16 h. Tryptic digests were analyzed by LC–MS on a LTQ-Orbitrap Velos mass spectrometer system (ThermoElectron, Bremen, Germany) coupled to a Proxeon Easy-LC HPLC system. The peptide mixture was separated on an LC-Packings PepMap C18 column (0.075×150 mm) equilibrated in 0.1% formic acid/water and eluted with a 60 min discontinuous gradient of acetonitrile/0.1% formic acid at a flow rate of 300 nl/min. The orbitrap was set to analyze the survey scans at 60000 resolution and top 10 ions (>50000 cps) in each duty cycle, were selected for MSMS in the high energy collision cell (HCD) at a resolution of 7500. The maximum fill time for the HCD cell was set to 150 ms (30000 ions).

The MSMS spectra were searched against SwissProt database using the Mascot search engine (Matrix Science) run on an in-house server using the following criteria; peptide tolerance = 10 ppm, daughter ion tolerance of 0.1 Da, trypsin as the enzyme, carboxyamidomethylation of cysteine as a fixed modification with oxidation of methionine and phosphorylation of serine, threonine and tyrosine as a variable modification. Any MSMS spectra that could be assigned to a phosphopeptide were inspected manually using QualBrowser software (ThermoElectron, San Jose, CA).

### 2.10. Co-immunoprecipitation of HA-SIK3 and 14-3-3 from HEK293 cells

Lysates containing 150 µg of total protein were incubated at 4 °C for 1–2 h on a shaking platform with 3–4 µl packed anti-HA-agarose. The immunoprecipitates were washed twice with 500 µl lysis buffer, containing 0.15 M NaCl and 1 mM DTT and twice with 500 µl buffer A. 1.5× Novex LDS Sample Buffer was added to the samples, which were subsequently vortexed, heated at 95 °C for five minutes, centrifuged at 7000 *g* after which the supernatant was collected for loading on Novex pre-cast SDS-PAGE gels.

## 3. Results

### 3.1. Stimulation of adipocytes with the β-adrenergic receptor agonist CL 316, 243 results in enhanced phosphorylation and 14-3-3 binding of endogenous SIK3

In order to explore the regulation of SIK3 by cAMP and address recent findings of SIK isoforms being regulated by insulin [9], primary

rat adipocytes were stimulated with the β-adrenergic receptor agonist CL 316,243 and insulin. SIK3 was immunoprecipitated and analyzed by western blot with antibodies directed against phosphorylation of PKA- and PKB consensus motifs (pPKA- and pPKB cons). Phosphorylation of HSL (S563) and PKB (S473) was monitored as positive controls for the activation of the cAMP and insulin pathways respectively. As seen in Fig. 1A, there was a pronounced increase in the phosphorylation of SIK3 on PKA consensus sites, in response to CL 316,243, suggesting a role for cAMP in the regulation of SIK3. The PKB consensus site antibody could also detect this cAMP-induced increase in SIK3 phosphorylation. The fact that PKB was not activated in response to CL 316,243, and that neither of the two phospho-antibodies detected any phosphorylation of SIK3 following insulin stimulation, suggests that the signal observed with the PKB consensus antibody, was due to the overlap between PKA- and PKB consensus sequences, and not to phosphorylation by PKB.

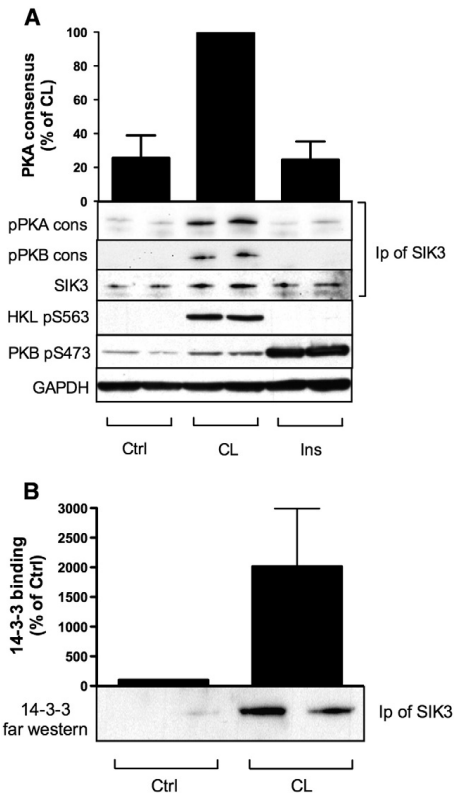



**Fig. 1.** Phosphorylation and 14-3-3 binding of SIK3 in adipocytes treated with the β-adrenergic receptor agonist CL 316,243. Primary rat adipocytes were isolated and stimulated with the β-adrenergic receptor agonist CL 316,243 (CL, 100 nM, 30 min), insulin (ins, 10 nM, 15 min) or left untreated (Ctrl). Endogenous SIK3 was immunoprecipitated and analyzed by western blot with antibodies targeting PKA- and PKB consensus motifs (pPKA cons, pPKB cons, A) as well as for 14-3-3 binding with purified GST-tagged 14-3-3ζ protein by means of far western blotting (B). Phosphoblots of pHSL S563 and pPKB S473 served as controls of successful CL and insulin stimulation and GAPDH served as a loading control (A). The graphs illustrate mean values + SD of quantified blots from three (A) and two experiments (B) respectively, shown together with representative blots.

Western blots of immunoprecipitated SIK3 were also analyzed with purified GST-tagged 14-3-3ζ by means of far western. We found that the extent to which 14-3-3 could interact with SIK3 was enhanced in adipocytes that were stimulated with CL 316,243 (Fig. 1B), suggesting that the cAMP-induced phosphorylation creates new binding sites for 14-3-3 on SIK3.

### *3.2. Stimulation of adipocytes with the β-adrenergic agonist CL 316, 243 inhibits the kinase activity of endogenous SIK3*

We addressed a possible role for cAMP-elevation in the regulation of SIK3 kinase activity and found that subjecting primary adipocytes to increasing concentrations of the β-adrenergic receptor agonist CL 316,243, resulted in a significant, dose-dependent reduction in the activity of endogenous SIK3, as measured in vitro towards a peptide substrate (Fig. 2A) and by autoradiography towards full-length CRTC2 (also known as TORC2) (Fig. 2B) — a well-established in vitro substrate for SIK isoforms [22].

AMPK, as well as the SIK2 isoform of the salt-inducible kinases, are also able to phosphorylate CRTC2, from which the peptide sequence used in the in vitro kinase assay of SIK3 derives. In addition, both AMPK and SIK2 are expressed at high levels in adipocytes [8]. To confirm that the (inhibition of) activity we detected was indeed that of SIK3, and not due to cross-reactivity of the antibodies with either of these two kinases, we also monitored the activity of SIK2 and AMPK. As demonstrated in Fig. 2C, SIK2 activity did not change significantly, and if anything showed a slight increase, in response to increases in cAMP levels. As seen in Fig. 2D, in response to β-adrenergic stimulation, we observed a dose-dependent increase in AMPK activity and phosphorylation of T172, the activity-controlling site of AMPK. This is consistent with previous findings both in cultured adipocytes [23,24] and in primary adipocytes [25]. Collectively, this validates our SIK3 activity data.

### *3.3. Phosphopeptide mapping reveals several residues involved in cAMP-mediated regulation of SIK3*

In order to identify the individual amino acid residues mediating the regulation of SIK3 by cAMP, we performed phosphopeptide mapping of wild type and kinase inactive (D206A) SIK3 expressed in HEK293 cells that had been stimulated with the cAMP-elevating agent forskolin or left untreated. As in adipocytes, SIK3 expressed in HEK293 cells was phosphorylated on PKA consensus sites, and its binding to endogenous 14-3-3 increased in response to cAMP-elevation (Fig. 3A). There was no change in the T-loop phosphorylation of SIK3 following forskolin stimulation (Fig. 3A) and furthermore, cAMP-induced regulation of the kinase inactive D206A version of SIK3 was the same as the wild type (data not shown). The colloidal staining of the indicated bands revealed that a substantially larger number of 14-3-3 molecules bound to SIK3 in stimulated cells when compared to SIK3 in untreated cells (Fig. 3B), and when the 14-3-3 proteins were identified by LC–MS using the same system as used for the phosphopeptide analysis, we found that SIK3 bound all seven isoforms of 14-3-3 (data not shown). Identification of individual phospho-peptides revealed that SIK3 is heavily phosphorylated in non-stimulated cells (Fig. 3C, D). The phosphorylation profile of kinase inactive SIK3 closely resembled that of wild type SIK3, revealing only one phosphorylated peptide less than in the wild type, which suggests that the abundant basal phosphorylation is not a result of auto-phosphorylation. Four peptides generated particularly high peaks, three of which exhibited substantially increased phosphorylation in response to forskolin (Fig. 3C). The sites phosphorylated in these peptides; T469, S551 and S674, all qualified as 14-3-3 binding motifs according to the 14-3-3 consensus binding sequences suggested by Scansite (http://scansite.mit.edu). In addition, we detected two additional peptides that were induced by forskolin, representing phosphorylation on S613 and S1022, albeit at much lower intensity (Fig. 3D).

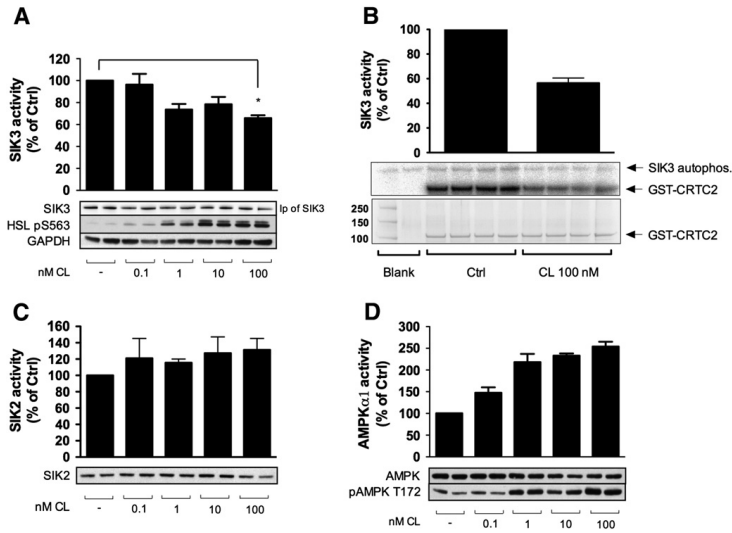


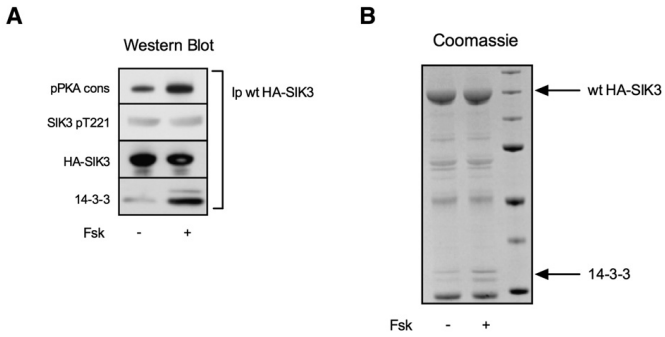

**Fig. 2.** Effect of cAMP-elevation on endogenous SIK3 kinase activity in adipocytes. Primary rat adipocytes were isolated and stimulated with indicated concentrations of the β-adrenergic receptor agonist CL 316,243 (CL, 30 min) or left untreated (Ctrl). The activity of immunoprecipitated endogenous SIK3 (A–B), SIK2 (C) and AMPKα1 (D) was analyzed in an in vitro kinase assay towards the Sakamototide peptide, which encompasses S171 in CRTC2 (A, C), the AMARA peptide (D) or by autoradiography towards full-length CRTC2 (B) as a substrate. The graphs illustrate mean values + SD of five (A) and two (B, C, D) separate experiments. Statistical analysis was performed using one-way ANOVA, followed by Tukey's multiple comparison test and differences were considered statistically significant when $P<0.05$ (*), $P<0.01$, and $P<0.001$ (NS, non-significant) (A). Cell lysates or immunoprecipitates were analyzed by western blotting as indicated in the figure.

In order to determine which of the cAMP-induced phosphorylation sites that are responsible for the increase in 14-3-3 binding observed upon cAMP-induction, we constructed single phosphorylation site mutants of SIK3 in which T469, S551, S674 as well as T221, the SIK3 activity-controlling site known to bind 14-3-3 in non-stimulated cells, were converted to an alanine. We also employed a mutant SIK3 version, in which all four aforementioned residues were mutated simultaneously (quadr.). The mutants were expressed in HEK293 cells and analyzed for phosphorylation by PKA (pPKA consensus blotting) and for their ability to interact with endogenous 14-3-3 in a co-immunoprecipitation assay. cAMP-induction resulted in a two-fold increase in PKA phosphorylation of SIK3 in these cells (Fig. 4A). In parallel, a three-fold increase in 14-3-3 binding to wild type SIK3 could be observed when the cells were stimulated with forskolin (Fig. 4B). Mutating the T-loop residue on SIK3 resulted in a partial decrease in basal 14-3-3 binding, which is consistent with T221 being a known 14-3-3 binding site in the basal state [13]. Mutation of the T-loop residue on SIK3 also resulted in a partial decrease in cAMP-induced phosphorylation of SIK3 (Fig. 4A). This was not expected, as this site was unresponsive to cAMP-elevation in the phosphopeptide mapping of SIK3 and western blotting of SIK3 with a T-loop antibody confirmed that elevated cAMP levels did not exert any effect on the activity-controlling T-loop phosphorylation of SIK3 (Fig. 3A). The T221A mutant maintained a cAMP-induced 14-3-3 binding, further strengthening the findings obtained in primary adipocytes of an increase in 14-3-3 binding sites upon cAMP-elevation. When employing single-site mutants of the most prominent forskolin-induced sites detected in the phosphopeptide mapping, we found that T469, S551 and S674 all participate in the cAMP-mediated increase in phosphorylation of SIK3 as well as in the increased 14-3-3 binding (Fig. 4A–B). Of these three sites, mutation of T469 appeared to have the most dramatic, S551 an intermediate, and S674 only a modest, effect on SIK3 phosphorylation and 14-3-3 binding. The T221A/S469A/S551A/S674A quadruple mutation efficiently reduced the cAMP-induced increase in phosphorylation of SIK3 as well as the increased 14-3-3 binding, back to basal levels or below (Fig. 4A–B).

Wild type mouse SIK1 over-expressed in adrenal cells undergoes nucleocytoplasmic shuttling in response to cAMP elevation, whereas SIK1 in which the PKA-site S577 has been substituted for an alanine, remains in the nucleus [15]. This previous SIK1 finding, along with the heavy phosphorylation of SIK3 on PKA consensus sites and the fact that the number 14-3-3 binding sites (often controlling localization) on SIK3 increase in response to cAMP-elevation, prompted us to speculate whether the subcellular localization of SIK3 might be regulated by increased cAMP levels. However, when expressed in HEK293 cells and analyzed in a confocal fluorescence microscope, both wild type and T221A/S469A/S551A/S674A mutant SIK3 (which is devoid of 14-3-3 binding) appeared to be localized mainly in the cytoplasm of non-stimulated cells and this did not change following stimulation with forskolin (Fig. 4C). We included human wild type and S575A SIK1 (corresponding to S577 in mouse) as a control, and as expected, wild type SIK1 translocated to the cytoplasm in response to forskolin, whereas the S575A mutant (corresponding to mouse S577) did not (Fig. 4C).

### *3.4. cAMP-induced effects on SIK3 are mediated by Protein kinase A (PKA)*

As protein kinase A (PKA) is the kinase suggested to be responsible for cAMP-induced regulation of SIK1 in adrenal cells [15] and as mutation of the residues detected in the phosphopeptide mapping of human SIK3 appeared to reduce the overall phosphorylation of PKA consensus motifs on SIK3, we investigated a potential role for PKA in the cAMP-mediated regulation of SIK3. We found that in addition to

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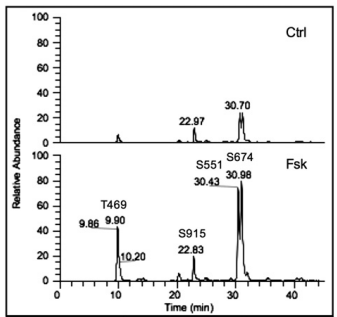


| Phosphopeptide | Time | Phosphorylation site | Induction upon forskolin-treatment |
|---|---|---|---|
| RHpTVGVADPR | 9.9 min | T469 | 9-fold |
| QLpSADSAEAHSLNVNR | 22.8 min | S915 | Minor |
| RApSDGGANIQLHAQQLLK | 30.5 min | S551 | 4-fold |
| RFpSDGAASIQAFK | 31.1 min | S674 | 4-fold |

D

| Phosphopeptide | Phosphorylation site |
|---|---|
| pTWCGSPPYAAPELFEGK | T221 |
| GPSPLVTMTPAVPAVTPVDEEpSpSDGEPDQEAVQR | S592, S593 |
| DSNTLHLPTERFpSPVR | S668 |
| QPpSNpSPPPMSSAMIQPHGAASSSQFQGLPSR | S794, S796 |
| IQPSpSPPPNHPNNHLFR | S779 |
| GISIpSPSAGQMQMQHR | S884 |
| GSPSSYSPSTGVGFpSPTQALK | S963 |
| HQQVPHILQGLLpSPR | S1022 |
| DGFQDSKpSSSTLTK | S1161 |
| NKVPpSREPVIGNCMDR | S1217 |
| pSpSPGQAVELPDHNGLGYPAR | S1229, S1230 |

**Fig. 3.** Phosphopeptide mapping of SIK3. HEK293 cells were transfected with constructs encoding HA-tagged wild type SIK3 (sequence obtained from http://kinase.com) or kinase inactive SIK3 (not shown), and treated with or without the cAMP-elevating agent forskolin (Fsk, 50 μM, 30 min) (A, B, C). SIK3 was immunoprecipitated with pre-coupled HA-agarose and subjected to SDS-page gel electrophoresis followed by Coomassie blue staining (B) or western blotting with indicated antibodies (A). SIK3 excised from the Coomassie gel was subjected to phosphopeptide mapping (C, D). The four most abundant cAMP-induced phospho-peptides are depicted in C, and additional phospho-peptides, that were either 10–20× less abundant, or not induced by cAMP, are listed in D. The data shown is representative of two separate experiments.

qualifying as 14-3-3 binding sites, the three prominent phosphorylation sites that were induced upon cAMP-elevation (T469, S551 and S674) all qualified as PKA consensus sites. In order to establish a potential requirement for PKA in the regulation of SIK3, we pre-incubated SIK3-expressing HEK293 cells with the PKA inhibitor H89, prior to stimulation with forskolin. As seen in Fig. 5A, inhibition of PKA resulted in a reduction of the cAMP-induced phosphorylation of SIK3, suggesting that PKA indeed has a role in the cAMP-mediated regulation of SIK3. Similarly, when we analyzed co-immunoprecipitated 14-3-3, the amount of 14-3-3 bound to SIK3 was reduced (Fig. 5A).

As other kinases have similar consensus motifs to that of PKA and as the PKA inhibitor H89 is not entirely specific [26], we also investigated a potential participation of protein kinase B (PKB) and ribosomal S6 kinase (RSK), as these kinases are reported to be regulated by cAMP/PKA signaling [27,28], and could potentially directly phosphorylate SIK3 on the cAMP-induced sites that we identified. When cells were subjected to pre-treatment with the specific PKB inhibitor MK2206 and were stimulated with forskolin or IGF-1, forskolin treatment resulted in an increase in phosphorylation of SIK3 on PKA sites, that was not observed with IGF-1 (Fig. 5B), strengthening our finding that insulin did not regulate SIK3 in adipocytes (Fig. 1A). Furthermore, the PKB inhibitor did not affect the forskolin-induced PKA phosphorylation, suggesting that the increase in phosphorylation is not mediated by PKB (Fig. 5B). Phosphorylation of the activity-controlling PKB site S473 served as a control for PKB-activation and inhibition.

To study the possible involvement of RSK, a target of MEK/ERK signaling, we pre-treated SIK3-expressing cells with the specific MEK inhibitor PD0325901 and stimulated the cells with forskolin or epidermal growth factor (EGF), an activator of the MAPK pathway. As seen in Fig. 5C, forskolin treatment resulted in an increase in phosphorylation of SIK3 that could not be observed with the MAPK pathway activator EGF. Additionally, the MEK inhibitor did not affect the forskolin-induced phosphorylation, demonstrating that active RSK is not required for the cAMP-induced regulation of SIK3. Phosphorylation of ERK, a substrate for MEK, served as a control for successful stimulation with EGF, and the inhibition of MEK.

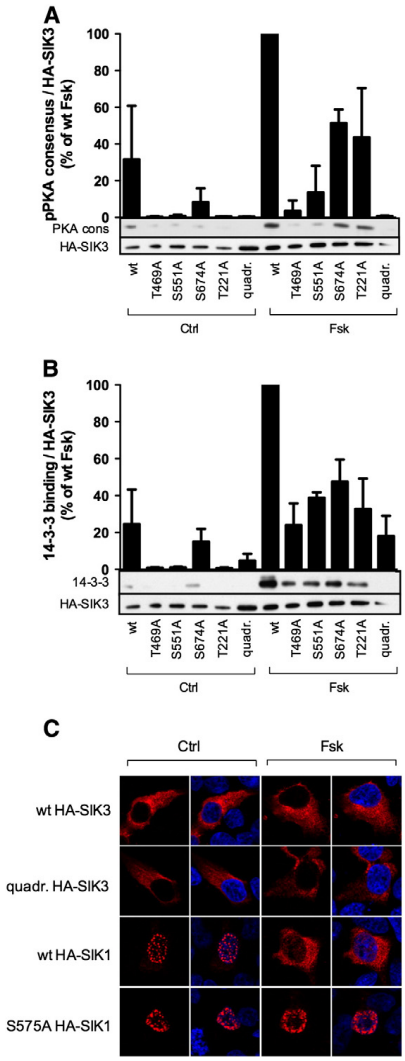


**Fig. 4.** Identification of residues involved in cAMP-mediated regulation of SIK3. HEK293 cells were transfected with constructs encoding HA-tagged wild type or indicated mutants of SIK3 (T221A/S469A/S551A/S674A; quadr.), and stimulated with the cAMP-elevating agent forskolin (Fsk, 50 μM, 30 min) or left untreated (Ctrl). Lysates or immunoprecipitates of HA-SIK3 were analyzed by western blotting for PKA phosphorylation (A) and 14-3-3-binding (B) as indicated in the figure. The graphs illustrate mean values + SD of quantified blots (corrected for differences in expression levels detected by anti-HA antibody) from three separate experiments, shown together with representative blots. HEK293 cells were transfected with wild type or S577A HA-SIK1 and wild type or T221A/T469A/S551A/S674A (qSIK3) HA-SIK3 cDNA constructs. The SIK1 or SIK3 isoforms were visualized using an Alexa 594 anti-HA antibody (red signal) and confocal imaging using a 63 × 1.4 oil DIC objective (C). Nuclei are visualized in blue (Hoechst stain). Cells shown in the experiment are representative of > 200 visualized cells from two separate experiments.

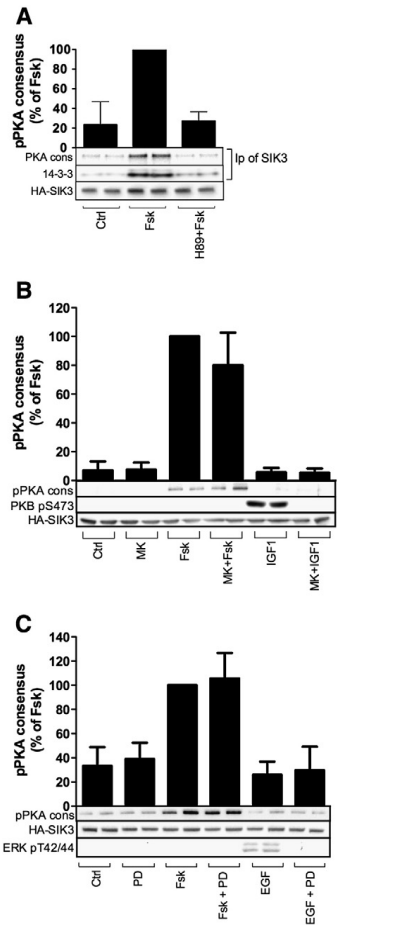


**Fig. 5.** PKA-mediated regulation of SIK3. HEK293 cells were transfected with constructs encoding HA-tagged wild type SIK3, and stimulated with the cAMP-elevating agent forskolin (Fsk, 50 μM, 30 min) (A, B, C), IGF-1 (50 ng/ml, 10 min) (B), EGF (EGF, 0.1 μM/ml, 10 min) (C) or left untreated (Ctrl), as indicated in the figure. Some samples were pre-incubated with H89 (50 μM, 20 min) (A), the PKB inhibitor MK2206 (MK, 5 μM, 1 h) (B) or the MEK inhibitor PD0325901 (PD, 0.2 μM, 1 h) (C). Lysates or immunoprecipitates were analyzed by western blotting. The graphs illustrate mean values + SD of quantified blots from three separate experiments, shown together with representative blots.

Taken together, our findings suggest that the additional 14-3-3 binding sites seen with cAMP-elevation are most likely directly phosphorylated by PKA.

## 4. Discussion

As LKB1 is a constitutively active kinase, a major challenge concerning its substrates in the AMPK-related kinase family is to understand how these respond to extracellular stimuli. For AMPK it is clear that, rather than affecting upstream kinases, the majority of activating extracellular signals act at the level of AMPK itself, through changes in

its binding of AMP, which protects it from de-phosphorylation. In this paper we have identified the cAMP/PKA pathway as a new upstream regulatory mechanism for SIK3, resulting in its inhibition in response to β-adrenergic signals in adipocytes.

Even though a recent paper suggested that insulin could regulate an SIK isoform in Drosophila through PKB [5], insulin had no effect on SIK3 phosphorylation in rat adipocytes. Therefore, to our knowledge, this is the first demonstration of regulation of endogenous SIK3 by extracellular signals in mammalian cells.

Previous studies of SIK1 in adrenal cells and 3T3L1 fibroblasts, suggest that the PKA-dependent phosphorylation of S577 (corresponding to S551 in SIK3 and S587 in SIK2) governs SIK1 function by causing a translocation away from its substrates in the nucleus [15,29]. The fact that this site is conserved in SIK2 and SIK3, has led to the suggestion that all SIKs are regulated in the same manner, but such a regulation of SIK3 has not been addressed until now. Data generated in our laboratory has revealed that in SIK2, S358 rather than the previously reported S587, is the main site induced by cAMP, and that this phosphorylation governs increased 14-3-3 binding and relocalization, but not a change in the kinase activity of SIK2 [16]. In the present study, we demonstrate a clear impact of cAMP-elevating agents on the phosphorylation of SIK3 in adipocytes, and this phosphorylation was accompanied by an inhibition of SIK3 intrinsic kinase activity. Although it was not investigated at the endogenous level, this did not appear to be the case for SIK1 [15]. It has been demonstrated that the 14-3-3 binding to the T-loop of exogenous SIK3 in non-stimulated HeLa cells expressing wild type LKB1, regulates its subcellular localization, as the T221A mutant re-distributed from punctate structures to a more even cytosolic dispersion [13]. Although we did not observe any immediate punctate structures in the basal state, or apparent subcellular re-distribution of SIK3 in response to cAMP-elevation in HEK293 cells, we do not rule out the possibility that a small sub-set of SIK3 molecules is subject to relocalization, or that cAMP-induced 14-3-3-binding results in the formation of multi-protein complexes, that may affect SIK3 function. The idea that only a portion of all SIK3 kinase molecules may be regulated by cAMP is supported by the fact that cAMP is released in regional subcellular pools [30]. Moreover, this notion is also in line with the moderate, yet consistent, decrease in SIK3 kinase activity that we observed in response to β-adrenergic stimulation in adipocytes. In a previous study, in vitro dissociation of 14-3-3 from the T-loop of SIK3 purified from non-stimulated cells, was shown to reduce its kinase activity [13], leading to the conclusion that 14-3-3-binding to the T-loop stimulates SIK3 activity in vivo. Our data suggest that 14-3-3 binding to PKA sites outside the kinase domain of SIK3 has the opposite effect on activity, at least in adipocytes, revealing a complex or potentially cell type specific mode of regulation of SIK3 kinase activity.

As mentioned above, an additional difference regarding how cAMP regulates the SIK isoforms, appears to be which individual amino acids are phosphorylated and mediate the effect of cAMP. We have addressed this directly, by performing in vivo phospho-peptide mapping followed by site-directed mutagenesis of SIK3. Surprisingly, mutation of T221 resulted in a reduced PKA phosphorylation of SIK3, as measured by western blotting with the PKA consensus antibody. However, based on the fact that phosphorylation of T221 was not induced by cAMP in our phospho-peptide mapping or pT221 western blotting, and that T221 does not conform to a typical PKA consensus site, we do not believe that T221 is a target for PKA phosphorylation. More likely, this result may be a consequence of cross-reactivity of the PKA consensus antibodies. S551, the site in SIK3 corresponding to SIK1 S575 (mouse S577) — the mutation of which largely reversed the nucleocytoplasmic translocation of SIK1 [15], only partially mediated the cAMP-induced 14-3-3-binding of SIK3. Instead, T469 was the site most strongly induced by cAMP, and its mutation had a more dramatic effect on the total PKA phosphorylation and 14-3-3-binding than that of S551. The fact that single mutation of both S551 and T469 resulted in a reversal of the cAMP-induced phosphorylation back to basal levels, may indicate that these phosphorylations influence/are dependent on each other. Although to a much lesser degree, mutation of S674 also affected the cAMP-induced regulation of SIK3. A site similar to T469 in SIK3 can be found in SIK2 (T370 in human SIK2) but not in SIK1, whereas S674 is unique for SIK3. T469, S551 and S674 are however all conserved among different mammalian species of SIK3. Mutation of all three sites, as well as T221, was required for total abolishment of PKA phosphorylation. This supports the notion that SIK isoforms might differ significantly in how they are regulated and that SIK3 in particular, might not share the same regulatory features as SIK1 and SIK2, in which mainly one site appears to mediate the effects of cAMP. Interestingly, we found the far C-terminal part of SIK3, which is not present in other SIK isoforms, to be phosphorylated at several residues in non-stimulated cells. The physiological relevance of these phosphorylations, as well as how and by what kinases they are regulated, remains to be investigated.

## 5. Conclusions

In summary, we conclude that the relatively unstudied protein kinase SIK3 can be regulated by β-adrenergic stimulation in adipocytes. We demonstrate that cAMP-elevation induces a PKA-dependent phosphorylation of SIK3 and that this coincides with increased binding to 14-3-3 proteins and a subsequent decrease in SIK3 kinase activity. By means of phosphopeptide mapping and site-directed mutagenesis we find that the cAMP-mediated regulation of SIK3 appears to depend on three residues, T469, S551 and S674, that all play a role in the cAMP-induced phosphorylation and 14-3-3-binding. The biological role of SIK3 in adipocytes, and most other cell types, is not yet understood. However, our finding that its activity can be regulated by cAMP-elevation, suggests that SIK3 may mediate some of the effects of this second messenger on cellular function. Investigating a potential role for SIK3, for example in the context of lipid metabolism in adipocytes, will be of future interest.

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# Paper III

# Evaluation of the role of AMPK in adipocytes using the AMPK activator A769662

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# Abstract

AMP-activated protein kinase (AMPK) is a sensor and regulator of cellular energy, and its activation constitutes a strategy for the treatment of insulin resistance. We employed A769662, a direct allosteric AMPK activator, to evaluate the role of AMPK in adipocyte metabolism. Treatment of rat adipocytes with A769662 resulted in a similar degree of AMPK activation as compared to the previously used activators AICAR and phenformin. However, in contrast to these activators, which in line with previous findings reduced insulin-induced glucose uptake and lipogenesis, as well isoproterenol-induced lipolysis, A769662 did not significantly affect any of these pathways. We did however observe a dose-dependent reduction in insulin-induced de novo fatty acid synthesis with all activators, including A769662. Collectively, our results raise the possibility that some effects of AICAR and phenformin are AMPK-independent, and challenge our previous understanding of the role of AMPK in adipocytes.


# Abbreviations

AMP-activated protein kinase (AMPK), AICAR (5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside), Tubulin folding cofactor D (TBCD), Phosphoinositide 3 kinase (PI3K)


# Key words

AMPK, A769662, adipocyte, lipogenesis, lipolysis, glucose uptake


# High-lights

- A769662 results in activation of AMPK in adipocytes.
- A769662 does NOT inhibit hormone-induced lipolysis, lipogenesis and glucose uptake in adipocytes.
- A769662 dose-dependently inhibits insulin-induced fatty acid synthesis in adipocytes.

# Introduction

AMP-activated protein kinase (AMPK) is a major sensor and regulator of cellular energy levels, and is activated by a wide array of stressors that result in increased AMP/ADP to ATP ratio. Once active, AMPK promotes ATP-generating pathways, while inhibiting those that consume ATP, in order to maintain cellular energy homeostasis [1].

AMPK is a heterotrimeric complex comprising three subunits; the catalytic α-subunit and the regulatory β- and γ-subunits. Two isoforms of the catalytic subunit (α1, α2) exist in mammals, two isoforms of the β-subunit (β1, β2) and three isoforms of the γ-subunit (γ1,γ2 and γ3) [2]. AMPK is activated by upstream kinases LKB1 [3-5] and Ca2+/calmodulin-dependent protein kinase kinase β (CAMKKβ), both means of activation resulting in phosphorylation of T172 in the α-subunit [6, 7]. AMPK is also sensitive to allosteric regulation by the intracellular AMP/ATP ratio, and binding of AMP or ADP to the regulatory γ-subunit results in a conformational change that renders AMPK less susceptible to dephosphorylation by phosphatases [8].

The metabolic function of AMPK has been extensively studied in skeletal muscle and liver and to a lesser extent in adipose tissue. When it was discovered that metformin [9], the biguanide widely used as the primary drug of choice for type 2 diabetes treatment [10], resulted in both activation of AMPK and downregulation of gluconeogenic genes in the liver, AMPK was considered responsible for many of the positive effects of the drug on metabolic defects associated with obesity and type 2 diabetes. Yet recent findings in mice with liver-specific knockout of the AMPK catalytic subunits α1 and α2 reveal that the inhibitory effects of metformin, as well as that of the AMP-mimetic 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) on the expression of gluconeogenic genes were maintained even in the absence of functional AMPK [11]. This, as well as a number of other studies [12, 13], suggests that AMPK is not the sole target of these agents. Whereas metformin and its related substance phenformin function by suppressing mitochondrial respiration and hence increasing the AMP to ATP ratio [14, 15], AICAR permeates cells, is converted into ZMP and binds directly to the γ-subunit, mimicking the effects of AMP [16]. A769662, a small molecule belonging to the thienopyridone family [17], has emerged as a more specific AMPK activator, increasing kinase activity in an allosteric, reversible manner via binding to an unknown residue on the β1-subunit, as well as by to some extent protecting it from T172 dephosphorylation [18-20].

In adipocytes, the activity of metabolic pathways is governed primarily by insulin and catecholamines. Previous studies addressing the role of AMPK in adipocyte metabolism have mainly employed AICAR, phenformin or different over-expression strategies. In stark contrast to the positive effect of AMPK activation on glucose uptake in muscle, AICAR treatment in fact leads to a reduction in insulin-induced

glucose uptake both in 3T3L1- and rat adipocytes [21, 22]. Moreover, AICAR was also shown to inhibit basal and insulin-induced lipogenesis in primary rat adipocytes – as measured by the reduced incorporation of glucose into triglycerides [22]. Regarding lipolysis, AICAR- and phenformin-induced activation of AMPK as well as adenoviral overexpression of constitutively active AMPK in primary rat adipocytes, resulted in markedly reduced isoproterenol-induced lipolysis [23]. On the same note, overexpression of a dominant-negative version of AMPK and knockout of AMPKα1 in mice resulted in increased lipolysis [24]. However, in light of the discovery that both metformin and AICAR have metabolic effects in the liver that are AMPK-independent, and the fact that A769662 has emerged as potent and more specific tool for AMPK activation, we wondered if existing data on the role of AMPK in adipocyte metabolism could stand to be re-examined.

In conclusion, we found that although A769662 activated AMPK to a similar extent as did AICAR and phenformin, the effects of A769662 on adipocyte metabolism was markedly different to that of previously used AMPK activators.

# Materials and methods

## Materials

Sprague-Dawley rats were obtained from Charles River laboratories and complete protease-inhibitor tablets were purchased from Roche (Mannheim, Germany). Pre-cast Novex SDS Polyacrylamide Bis-Tris gels, dithiothreitol (DTT) and lauryl dodecyl sulphate (LDS) sample buffer were all purchased from Invitrogen (Carlsbad, USA). Protein-G-Sepharose was purchased from GE Healthcare Biosciences (Uppsala, Sweden) and P81 phosphocellulose cation-exchange paper was purchased from Whatman (Dassel, Germany). $^{32}$Pγ-ATP, $^{3}$H-acetic acid and $^{3}$H-glucose were all obtained from Perkin Elmer (Boston, USA). AMARA peptide was synthesized by GL Biochem (China) and enhanced Chemiluminescense Super Signal Reagent was obtained from Thermo Scientific (Schwerte, Germany). Isoprenaline and phenformin were purchased from SIGMA (St. Louis, USA) and insulin was supplied by Novo Nordisk (Målöv, Denmark). AICAR was purchased from Toronto Research Chemicals (Toronto, Canada) and A769662 was purchased from Abcam (Cambridge, UK). The antibody used for immunoprecipitation and kinase assay of AMPKα1 was a kind gift from Professor D. Grahame Hardie (University of Dundee, UK), whereas total- and phosphoprotein antibodies directed against AMPK, ACC and Raptor were obtained from Cell Signaling Technologies (Danvers, USA), and beta-actin antibodies were from Sigma (St. Louis, USA).

## Isolation and stimulation of primary rat adipocytes

Primary rat adipocytes were isolated from the epididymal adipose tissue of male 36-38 day old Sprague-Dawley rats [25], according to a protocol that was approved by the Lund University ethical review committee (approval no. M92-13). Adipocytes were suspended in 2-10 % cell suspensions (depending on the subsequent method of analysis), in Krebs-Ringer medium containing 25 mM HEPES pH 7.4, 200 nM adenosine, 2 mM glucose and 1 % BSA. Modified forms of Krebs Ringer medium were utilized for the glucose uptake assay and the lipogenesis assay, see below.

## Stimulation of primary rat adipocytes and subsequent lysate preparation

The cells were stimulated at 37°C, in a 120 rpm shaking water bath and subsequently washed twice in Krebs-Ringer medium without BSA and homogenized in 0.5-1 ml of lysis buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium-β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 mM dithiothreitol (DTT), 1 % NP40 and complete protease inhibitor (1 tablet/50 ml). The cell homogenates were centrifuged for 15 min at 13000 g and the fat layer removed. Protein concentrations were determined according to Bradford [26].

## Immunoblotting

Lysates were heated at 95°C for two minutes in LDS sample buffer and subjected to electrophoresis on Novex pre-cast SDS-PAGE gels and subsequent electrotransfer to nitrocellulose membrane. Membranes were blocked for 30 minutes in 10% (w/v) dried skimmed milk, and then probed with 1:1000 dilutions of primary antibodies for 16 h at 4°C in 5 % (w/v) BSA. Protein detection was performed with horseradish-peroxidase-conjugated secondary antibodies followed by treatment with chemiluminescence reagent. ECL signals were visualized using a ChemiDoc XRS+ camera followed by analysis of band intensities with the Image Lab software (both from BioRad).

## Immunoprecipitation and in vitro assay of AMPK kinase activity

Lysates containing 10 µg of protein were incubated at 4°C for 1-2 hours on a shaking platform with 1 µg AMPKα1 antibody conjugated to 5 µl protein G-Sepharose. The immunoprecipitates were washed twice with 500 µl lysis buffer supplemented with 0.5 M NaCl and 1 mM DTT, and twice with 500 µl of 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, and 1 mM DTT (buffer A). The kinase activity was measured in a volume of 50 µl containing 50 mM Tris-HCl pH 7.5, 0.1% 2-mercaptoethanol, 10 mM MgCl2, 0.1 mM EGTA, 0.1 mM $^{32}$Pγ-ATP (300 cpm/pmol) and 200 µM AMARA (AMARAASAAALARRR) peptide for 20 min at 30°C. The assay was terminated by applying 40 µl of the reaction mixture on p81 cation-exchange phosphocellulose paper, followed by immersion of the paper in 50 mM phosphoric acid and 5-6 subsequent washes in 50 mM phosphoric acid before liquid scintillation counting. Incorporation of $^{32}$P- phosphate was expressed as pmol ATP incorporated/mg protein/min (mU/mg).

## Lipolysis assay

400 µl of 5 % primary rat adipocyte cell suspensions were incubated at 37 °C, 150 rpm shaking, for 30 minutes with the stimuli indicated in the figures. The incubations were swiftly placed on ice, left for 20 minutes and 200 µl of the cell medium was separated from the cells. The amount of glycerol released into the medium during the assay was measured with an enzymatic method as previously described [27].

## [$^3$H]-acetate de novo fatty acid synthesis assay

After 30 min pre-incubation with indicated AMPK-activators (37 °C, 80 rpm shaking), 700 µl of 2 % primary rat adipocyte cells were suspended in Krebs-Ringer medium (see above) and incubated at 37 °C, 120 rpm shaking, for 30 minutes with [$^3$H]-acetate (1 mM (0,73 µCi/ml)) and the stimuli indicated in the figures. The assay measured incorporation of [$^3$H]-acetate into newly synthesized fatty acids [28]. Reactions were stopped with 3.5 ml of a toluol-based scintillation liquid containing 0.3 g/l 1,4-bis [5-phenyl-2-oxazolyl] benzene, 2,2-*p*-phenylene-bis [5-phenyloxazole] (POPOP) and 5 g/l 2,5-diphenyl oxazole (PPO).

## Lipogenesis assay (incorporation of D-[6-$^3$H(N)]-Glucose into triglycerides)

After pre-incubation with indicated AMPK-activators (37 °C, 80 rpm shaking) 700 µl of 2 % primary rat adipocyte cells suspended in Krebs-Ringer medium containing 25 mM HEPES pH 7.4, 200 nM adenosine, 0.55 mM glucose and 3.5% BSA, were incubated at 37 °C, 120 rpm shaking for 30 minutes with D-[6-$^3$H(N)]-Glucose (22 µCi/ml) and the stimuli indicated in the figures. The assay measures incorporation of D-[6-$^3$H(N)]-Glucose into cellular lipids as previously described [29]. Reactions were stopped with 3.5 ml of a toluol-based scintillation liquid containing 0.3 g/l 1,4-bis [5-phenyl-2-oxazolyl] benzene, 2,2-*p*-phenylene-bis [5-phenyloxazole] (POPOP) and 5g/l 2,5-diphenyl oxazole (PPO).

## Glucose uptake assay

After collagenase digestion of rat adipose tissue in a glucose-free collagenase buffer [30] cells were suspended in a 5 % solution of Krebs-Ringer medium (see above)

containing 120 nM NaCl, 4 nM $KH_2PO_4$, 1 mM $MgSO_4$, 0.75 mM $CaCl_2$, 10 mM $NaHCO_3$, and 30 mM HEPES, pH 7.4 and treated with indicated stimuli for 1 hour. Uptake of D-[$^{14}$C (U)]-glucose was measured as previously described [31].

## Statistical analysis

Results are presented as means + standard deviation (SD) of at least three independent experiments, each in which the data was normalized to a control sample (100%), to account for variations in absolute values in between experiments. Statistical analysis was performed in Graph Pad Prism, and differences were determined using one-way ANOVA followed by Holm Sidak's multiple comparison test.

# Results and discussion

## 1. A769662, AICAR and phenformin efficiently activate AMPK in primary rat adipocytes.

We first addressed whether A769662 could efficiently activate AMPK in primary rat adipocytes. Although it has been demonstrated to activate AMPK in several cell types, for example in primary hepatocytes [32, 33] and in muscle cells [32], it has never been used in primary adipocytes. When assaying the in vitro kinase activity of AMPK$\alpha$1, the predominating catalytic subunit in adipocytes [34], we found that AICAR and phenformin activated AMPK twofold and threefold respectively (Fig 1a). In line with previous reports, no change in kinase activity was observed in response to A769662, which mainly acts allosterically and only induces T172 phosphorylation to a very small extent [18-20]. Indeed, analysis of T172 phosphorylation revealed an increase in phosphorylation following AICAR and phenformin treatment, whereas no increase was observed with A769662. In order to confirm that A769662 could in fact activate AMPK in adipocytes, we monitored the phosphorylation of two well-known AMPK substrates; the lipogenic enzyme acetyl CoA carboxylase (ACC) and Regulatory-associated protein of mTOR (Raptor) – an integral part of the mTORC1 complex [10, 35]. As shown in Fig 1b and c, A769662 dose-dependently induced the phosphorylation of these two proteins, to a level similar or higher than did AICAR and phenformin. These data demonstrate that A769662 treatment leads to a robust activation of AMPK in intact rat adipocytes. This finding is in line with previous results suggesting that knockout of the $\beta$1-isoform in mice results in a reduction of AMPK activity in adipose tissue [36], whereas knockout of the $\beta_2$-isoform has no impact on AMPK activity levels in this tissue [37]. Taken together, these results imply that $\beta_1$ is the main $\beta$ subunit expressed in adipocytes, although to our knowledge this has not been investigated in detail and in a single context.

## 2. Activation of AMPK by A769662 does not affect catecholamine-induced lipolysis in rat adipocytes

As shown in Fig 2, when primary rat adipocytes were pre-incubated with AICAR or phenformin, lipolysis induced by two different doses of the beta-adrenergic receptor agonist isoproterenol, was markedly inhibited. In contrast, increasing doses of A769662 did not affect basal- or isoproterenol-induced lipolysis.
Based on previous studies employing AICAR or phenformin, overexpression of constitutively active or dominant negative constructs and AMPK$\alpha$1 total knockout mice, an anti-lipolytic role has been attributed to AMPK [23, 24]. This effect of AMPK activation is thought to be mediated via phosphorylation of hormone-sensitive lipase (HSL) on the inhibitory site S565 [38], and AMPK has also been proposed to phosphorylate adipose tissue triglyceride lipase (ATGL)[39]. Our data suggest that some of these previously reported effects of AMPK modulation on lipolysis may be AMPK-independent. It would however be interesting to study potential effects of A769662 and other AMPK activators on HSL- and ATGL phosphorylation.

## 3. Activation of AMPK by A769662 has no effect on basal or insulin-induced glucose uptake in rat adipocytes

Pre-incubation with AICAR and phenformin reduced both basal- and insulin-induced glucose uptake in primary rat adipocytes (Fig 3). However, pre-incubation of the cells with A769662 revealed no changes in uptake, suggesting that AMPK is not responsible for the diminished glucose uptake seen with AICAR and phenformin. Our inhibitory effects of AICAR and phenformin on glucose uptake in adipocytes is in agreement with previous findings in adipocytes [21, 22], but in contrast to the well-known ability of these compounds, and of contraction, to increase glucose uptake in muscle. In muscle cells, the exact mechanisms mediating the effect of AMPK activation on glucose uptake are still not fully elucidated, but involve increases in the phosphorylation, and thereby inhibition, of TBC1D isoforms [40], most likely TBC1D1 [41, 42] - Rab GTPase-activating proteins and common targets of insulin- and AMPK signalling [43]. A769662 has been shown to induce glucose uptake in some muscle fibre types and in some mouse strains, but not in others [20, 44]. Surprisingly, this discrepancy could not be attributed to differences in the expression of AMPK$\beta_1$, which, although generally low in muscle [45], was found to be higher in some fibre types than others [44]. A769662-induced glucose uptake in skeletal muscle was instead concluded to be AMPK-independent and appeared to involve activation of the PI3K/Akt pathway [44]. In adipocytes, AICAR was in contract to muscle

shown to *inhibit* the phosphorylation of TBC1D4 (also known as AS160), and in particular TBC1D4 phosphorylation in the presence of insulin [46]. This effect of AICAR/AMPK activation could explain the ability of AICAR to prevent insulin-induced glucose uptake, but the mechanisms underlying the reduction in TBC1D4 phosphorylation were not identified. Although we did not monitor the phosphorylation of Akt or TBC1D4 following A769662 treatment of adipocytes, the lack of effect of A769662 on glucose uptake indicates that A769662 did not affect insulin signalling or TBC1D4 in our study.

## 4. Insulin-induced incorporation of glucose into lipids (lipogenesis) is unaffected by A769662 treatment of rat adipocytes

We initially employed an assay that utilizes 6-[$^3$H]-glucose as a substrate, to measure the insulin-induced lipogenesis after the cells had been pre-treated with the various AMPK activators. In line with previous reports [22], AICAR and phenformin both resulted in a dramatic reduction in insulin-induced lipogenesis (Fig 4). However, cells pre-treated with A769662 displayed the same level of insulin-induced lipogenesis as control cells.

## 5. A769662 inhibits insulin-induced de novo fatty acid synthesis in rat adipocytes.

Although measuring the incorporation of 6-[$^3$H]-glucose into neutral lipids is a good way to measure the amount of newly synthesized triglycerides, the majority of the glucose is incorporated into the glycerol moiety [47], and this assay is thus a poor measurement of de novo fatty acid synthesis – a process which is critically regulated by AMPK/ACC signalling, for example in the liver [48]. When we switched the substrate in our assay from 6-[$^3$H]-glucose to [$^3$H]-acetate, hence monitoring the newly synthesized population of fatty acids, we observed a dose-dependent reduction in both basal- (trend) and insulin-induced de novo fatty acid synthesis in adipocytes pre-treated with A769662. Similar results were obtained in hepatocytes, when A769662 was used to study the role of AMPK in basal lipogenesis [33, 48]. Although A769662 resulted in a phosphorylation of ACC that was at least as high as following AICAR and phenformin treatment of adipocytes, these two latter activators induced a much more dramatic inhibition of fatty acid synthesis than did A769662. This is likely to be due to the marked inhibition of insulin-induced glucose uptake by AICAR and phenformin, as this would limit glycolysis and the availability of acetyl-

CoA for fatty acid synthesis. Similarly, the inhibition of glucose uptake could also explain the strong negative effect of AICAR and phenformin on the incorporation of glucose into lipids (lipogenesis), as the availability of glycerol-6-phosphate for triglyceride synthesis would be low. Collectively, these results clearly demonstrate that AMPK inhibits de novo fatty acid synthesis in adipocytes. Moreover, AICAR and phenformin potentially elicit AMPK-independent effects on glucose uptake that may explain the dramatic inhibition of lipogenesis and fatty acid synthesis by these agents.
In summary, our study reveals marked differences in the effects of A769662 versus those of AICAR and phenformin, on adipocyte metabolism. We propose that the main reason for this is the relatively unspecific mode of action of AICAR and phenformin, as compared to A769662. We can however not rule out the possibility that the observed differences are due to the fact that A769662 reportedly activates $\beta_1$-, but not $\beta_2$-containing AMPK complexes [20], whereas AICAR and phenformin activates both isoforms. If this is indeed the case, another interpretation of our data is that AMPK$\beta_1$ is critical for the regulation of de novo lipogenesis, whereas glucose uptake and lipolysis is regulated by $\beta_2$-containing complexes. Considering that $\beta_1$ appears to be the dominant isoform in adipocytes (based for example on the lack of any detectable changes in AMPK activity after knockout of $\beta_2$),[37], as well as the fact that A769662 in addition to ACC induced the phosphorylation of Raptor to a similar degree as AICAR and phenformin, we do not favour this hypothesis. Although most studies suggest that A769662 is significantly more specific that AICAR and phenformin, AMPK-independent effects of A769662 have been reported [44, 49, 50]. An alternative explanation to the differential effects of AICAR, phenformin and A769662 in adipocytes is therefore that A769662 has off-target effects that counteract those that are mediated by AMPK activation.
All in all, the current data challenge our previous notion of AMPK function in adipocytes, and warrant further studies of AMPK in relation to adipocyte metabolism.

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# Figure legends

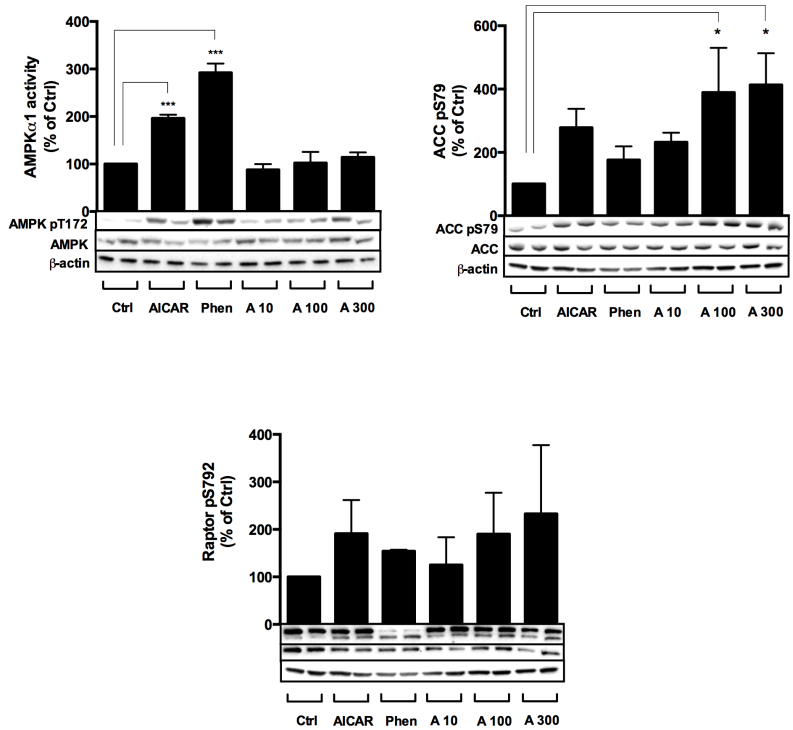


**Figure 1. AMPK in vitro protein kinase activity and in vivo substrate phosphorylation in response to treatment with AMPK activators AICAR, phenformin and A769662**

Primary rat adipocytes were isolated and incubated for 1 hour with AICAR (2 mM), phenformin (Phen, 1 mM) and A769662 (A10, 10 μM; A100, 100 μM; A300, 300 μM) as indicated in the figures. AMPKα1 was immunoprecipitated and analysed in an in vitro kinase assay toward the AMARA peptide **(a).** The graph **(a)** illustrates mean values + SD of four separate experiments. Statistical analysis was performed using one-way ANOVA, followed by Holm-Sidak's multiple comparisons test and differences were considered statistically significant when $P<0.05$ (*), $P<0.01$, and $P<0.001$ (NS, non-significant). Cell lysates were analyzed on western blot with total- and phospho-antibodies targeting AMPK T172 **(a)**, ACC S79 **(b)** and Raptor S792 **(c).** Western blot of β-actin served as a loading control (a, b and c). The blots shown in (a) are representative of four separate experiments, whereas the graphs (b, c) illustrate mean values + SD of quantified blots from three experiments respectively, shown together with representative blots.

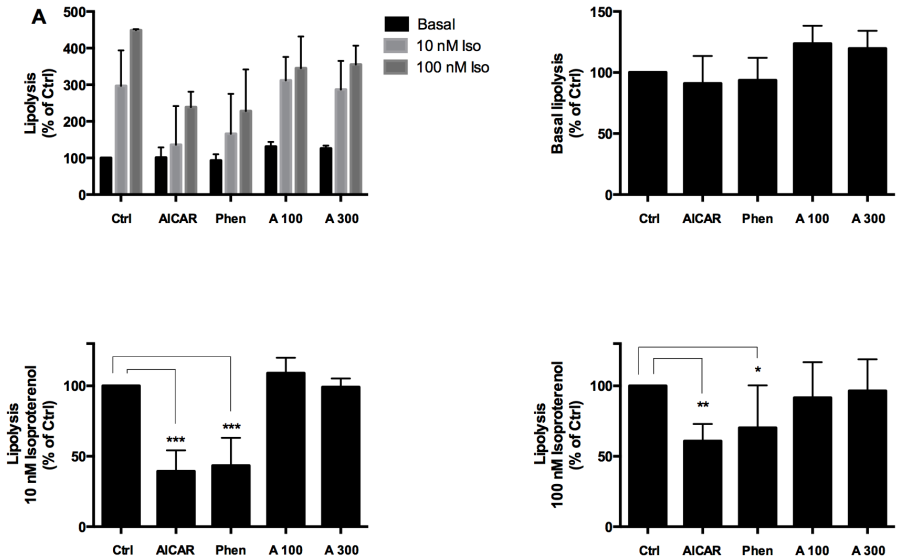


**Figure 2. Effect of pre-treatment of primary rat adipocytes with AMPK activators AICAR, phenformin and A769662 on isoproterenol-induced lipolysis**

Primary rat adipocytes were isolated and pre-treated for 30 min, with AICAR (2 mM), phenformin (Phen, 100 μM) and A769662 (A100, 100 μM; A300, 300 μM) as indicated in the figures. Lipolysis (glycerol release) was measured after the pre-treated cells had been stimulated with increasing concentrations of isoproterenol (Iso, 30 min) or left untreated (Ctrl) as indicated in the figure. The graphs illustrate mean values + SD of 5-7 separate experiments. In **(b-d)**, the data was expressed as % of its respective non pre-treated control, in order to more easily determine the effects of AMPK activators on basal- and isoproterenol-induced (10- and 100 nM) lipolysis. Statistical analysis was performed using one-way ANOVA, followed by Holm-Sidak's multiple comparison test and differences were considered statistically significant when $P<0.05$ (*), $P<0.01$, and $P<0.001$ (NS, non-significant).

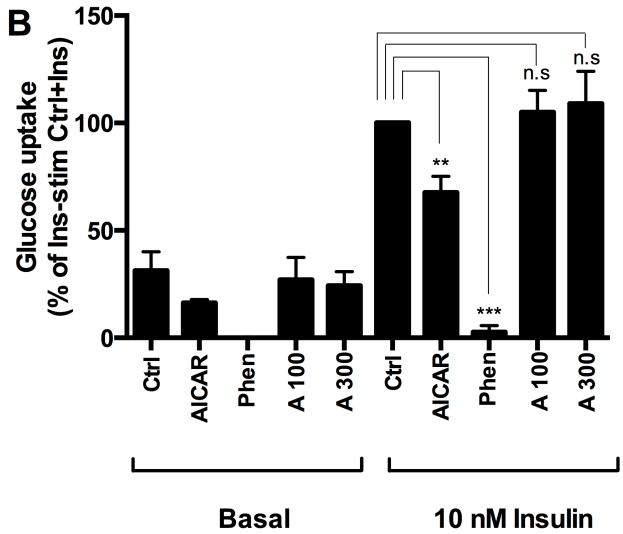


**Figure 3. Effect of pre-treatment of primary rat adipocytes with AMPK activators AICAR, phenformin and A769662 on insulin-induced glucose uptake**

Primary rat adipocytes were isolated and pre-treated for 30 min, with AICAR (2 mM), phenformin (Phen, 1 mM) and A769662 (A100, 100 μM; A300, 300 μM) as indicated in the figure. $^{14}$C-Glucose uptake was measured after the pre-treated cells had been stimulated for 30 min with insulin (Ins, 10 nM) or left untreated (Ctrl) as indicated in the figure. The graph illustrates mean values + SD of three separate experiments. Statistical analysis was performed using one-way ANOVA, followed by Holm-Sidak's multiple comparison test and differences were considered statistically significant when $P<0.05$ (*), $P<0.01$, and $P<0.001$ (NS, non-significant).

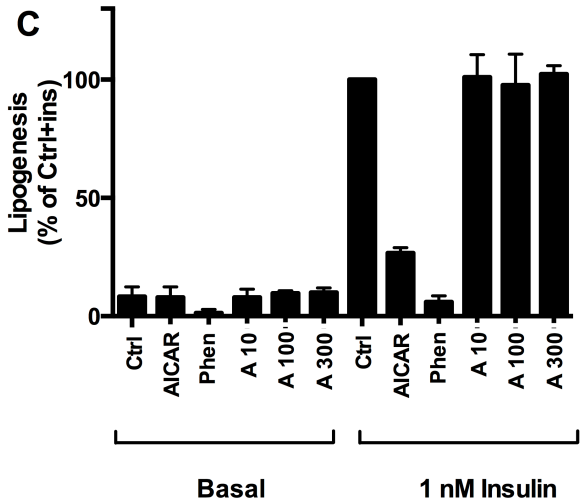


**Figure 4. Effect of pre-treatment of primary rat adipocytes with AMPK activators AICAR, phenformin and A769662 on insulin-induced lipogenesis**

Primary rat adipocytes were isolated and pre-treated for 30 min, with AICAR (2 mM), phenformin (Phen, 1 mM) and A769662 (A100, 100 μM; A300, 300 μM) as indicated in the figures. Lipogenesis was measured as the incorporation of $^{3}$H-Glucose into total lipids after the pre-treated cells had been stimulated for 30 min with insulin (Ins, 10 nM) or left untreated (Ctrl). The graph illustrates mean values + SD of three separate experiments. Statistical analysis was performed using one-way ANOVA, followed by Holm-Sidak's multiple comparison test and differences were considered statistically significant when $P<0.05$ (*), $P<0.01$, and $P<0.001$ (NS, non-significant).

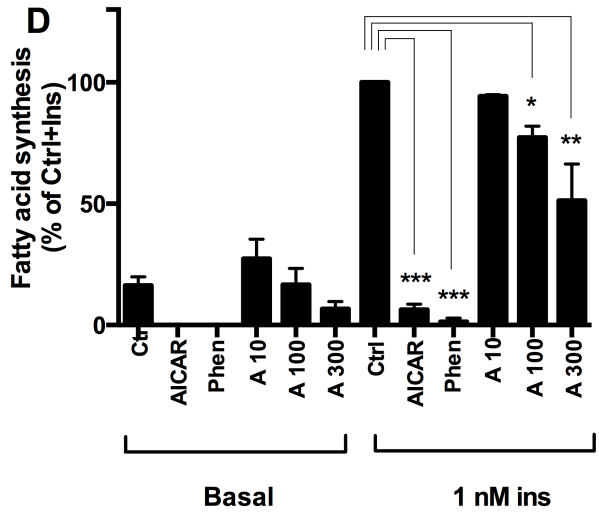


**Figure 5. Effect of pre-treatment of primary rat adipocytes with AMPK activators AICAR, phenformin and A769662 on insulin-induced de novo fatty acid synthesis**

Primary rat adipocytes were isolated and pre-treated for 30 min, with AICAR (2 mM), phenformin (Phen, 1 mM) and A769662 (A100, 100 μM; A300, 300 μM) as indicated in the figures. De novo fatty acid synthesis was measured as the incorporation of $^{3}$H-Acetate into total lipids after the pre-treated cells had been stimulated for 30 min with insulin (Ins, 10 nM) or left untreated (Ctrl). The graph illustrates mean values + SD of three separate experiments. Statistical analysis was performed using one-way ANOVA, followed by Holm-Sidak's multiple comparison test and differences were considered statistically significant when $P<0.05$ (*), $P<0.01$, and $P<0.001$ (NS, non-significant).

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