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β-adrenergic signalling and novel effects in skeletal muscle

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“Ever tried? Ever failed?
No matter. Try again.
Fail again. Fail better.”

*Samuel Beckett*
Abstract
Skeletal muscles have, due to their large mass, a big impact on the whole body metabolism. There are many signals that can regulate the functions of skeletal muscles and one such signal is activation of α- and β-adrenergic receptors (α- and β-ARs) by the endogenous ligands epinephrine and norepinephrine. Such activation leads to several effects which are examined in this thesis.

Stimulation of β-AR on muscle cells induces glucose uptake, an event that both provides the muscle with energy and lowers the blood glucose levels. We discovered that the β-ARs induce glucose uptake via the glucose transporter protein GLUT4, which is known to be regulated by insulin. Interestingly, we found that the intracellular pathway is partly different from that of insulin. However, both pathways include mTOR, a kinase involved in several metabolic processes but not previously known to be activated by β-ARs.

The most classical second messenger downstream of β-ARs is cAMP. Surprisingly, we found this molecule to be only partly involved in the β-adrenergic glucose uptake and we identified GRK2 as a key molecule for this event.

A novel effect of β-AR stimulation presented in this thesis is the inhibition of myosin II-dependent contractility in skeletal muscle cells. The intracellular pathway regulating this event was different from the one regulating glucose uptake, involving both classical and novel molecules in the β-AR pathway.

We also found another stimulus activating insulin-independent glucose uptake in skeletal muscle cells: Shikonin, a natural compound used in traditional Chinese herbal medicine. Shikonin increased glucose uptake in skeletal muscle cells via a calcium- and GLUT4-dependent mechanism and improved glucose homeostasis in diabetic rats.

Taken together, we have identified new key molecules in the β-adrenergic signaling pathway as well as novel downstream effects. We conclude that glucose uptake in muscles can be activated by β-adrenergic stimulation or by Shikonin and that both improve glucose homeostasis in diabetic animals. This knowledge can hopefully be used in the search for new drugs to combat type II diabetes.
This thesis is based on the following papers, referred to in the text by their respective Roman numerals.

I. Sato M#, Nodi Dehvari#, Öberg AI, Dallner OS, Sandström AL, Olsen JM, Csikasz RI, Summers RJ, Hutchinson DS and Bengtsson T. (2013)
An insulin-independent pathway including β-adrenoceptors and mTORC2 that translocates GLUT4 and increases glucose uptake in skeletal muscle
Manuscript

II. Öberg AI, Dehvari N and Bengtsson T. (2011)
β-adrenergic inhibition of contractility in L6 skeletal muscle cells
PLoS One 6(7):e22304

Glucose uptake in skeletal muscle can be fully induced via the β2-adrenoceptor and GRK2 without cAMP-production
Manuscript

Shikonin increases glucose uptake in skeletal muscle cells and improves plasma glucose levels in diabetic Goto-Kakizaki rats
PLoS One 6(7):e22510
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Abbreviations

AC  Adenylate cyclase
ACC  Acetyl-CoA carboxylase
AR  Adrenoceptors
AKAP  A-kinase anchoring protein
AMP  Adenosine-5'-monophosphate
AMPK  AMP activated kinase
cAMP  3'-5'-cyclic adenosine monophosphate
CNG  Cyclic nucleotide-gated channels
CREB  cAMP response element binding protein
DAG  Diacylglycerol
4E-BP1  Factor 4E binding protein 1
EDL  Extensor digitorum longus
Epac  Exchange factor directly activated by cAMP
ERK 1/2  Extracellular signal regulated kinases 1/2
GEF  Guanine-nucleotide-exchange factor
GLUT  Facilitative glucose transporters
GRK  G-protein coupled kinase
GSV  GLUT4-storage vesicle
GPCR  G-protein coupled receptors
HCN  Hyperpolarisation-activated cyclic nucleotide-gated channels
HSL  Hormone sensitive lipase
IP3  Inositol trisphosphate
mTOR  Mammalian/mechanistic target of rapamycin
mTORC1  mTOR complex 1
mTORC2  mTOR complex 2
NKCC  Na⁺-K⁺-2Cl⁻-co-transporter
NMII  Non muscle myosin II
PDE  Phosphodiesterase
PKA  Protein kinase A
PKB  Protein kinase B, Akt
PIKK  Phosphoinositide 3-kinase (PI3K)-related kinase family
PIP2  Phosphatidylinositol 4,5-bisphosphate
SGK1  Serum- and glucocorticoid-induced protein kinase 1
SGLT  Sodium dependent glucose transporters
S6K1  Ribosomal S6 kinase 1
SREBP  Sterol regulatory element-binding protein
Raptor  The regulatory associated protein of mTOR
Rictor  Rapamycin-insensitive companion of mTOR
PI3K  Phosphatidylinositol 3-kinase
PIP3  Phosphatidylinositol (3,4,5)-triphosphate
PKCα  Protein kinase Cα
PKI  Protein kinase inhibitor peptide
PLC  Phospholipase C
PTX  Pertussis toxin
ROCK  Rho-activated kinase
7TMR  Seven-transmembrane receptor
VMH  Ventromedial hypothalamus
1. Introduction

The main function of skeletal muscles is to create movement, but due to their large mass they also have big impact on metabolic functions. Skeletal muscles have high energy demand and are thus important for energy expenditure. They have also a regulatory function by being a major site of glucose disposal and thereby regulating blood glucose levels (DeFronzo et al. 1981). Skeletal muscles are also suggested to have endocrine functions. The hormone irisin is for example shown to be released from skeletal muscles upon exercise, leading to changed expression of several metabolic genes in white adipocytes (Bostrom et al. 2012).

Skeletal muscles are subjected to regulation by stimuli such as internal cues, hormones from the bloodstream and neurotransmitters from nerve-endings. Hormones and neurotransmitters bind to receptors on the cell surface and thereby induce intracellular signalling cascades. One receptors found on skeletal muscle cells is the β2-adrenoceptor (β2-AR) which is activated by the hormone epinephrine and the neurotransmitter norepinephrine. There are also synthetic substances available that can interact with the β2-ARs. These can have different effects on the receptor-activity, summarised in table 1.

Table 1. The definitions of common words referring to molecules which can interact with a receptor.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>A substance that binds to a receptor</th>
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<tr>
<td>Agonist</td>
<td>A ligand that activates a receptor or a signal</td>
</tr>
<tr>
<td>Partial agonist</td>
<td>An agonist which cannot elicit the full response of the receptor at any concentration</td>
</tr>
<tr>
<td>Antagonist</td>
<td>A ligand that binds to a receptor without stimulating any activity but hinders other ligands from activating the receptor or a specific signal</td>
</tr>
<tr>
<td>Inverse agonist</td>
<td>An antagonist that in addition to having an inhibitory effect also reduces the basal activity of the receptor</td>
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Activation of the β2-ARs in skeletal muscles will induce several events, both signals inside the cells as well as various downstream effects, which are described in this thesis. To understand what is happening inside the cells after β2-AR activation, and correlating these events with different endpoints, is a very interesting field which opens up possibilities for designing new drugs.

Although the β2-AR is a well-studied receptor, its role in skeletal muscle is only poorly understood and the aim of our research is to add new knowledge in this field. My current view of the β-adrenergic signalling network and its downstream
effects in skeletal muscles is illustrated in Figure 1. Colour indicates our novel findings with light pink indicating molecules that are previously known to be activated by β-ARs, and for which we have found a new role, while dark pink items mark molecules and events that prior to our studies were unknown to be activated by β-ARs.

The main focus of my research has been on β-adrenergic stimulated glucose uptake. It was previously not known which transporter proteins are involved in this process, but in Paper I we revealed that the insulin-regulated glucose transporter GLUT4 is needed also for β-adrenergic glucose uptake. By further studying the intracellular pathway we discovered a new player in the β-adrenergic signal: mTOR, a kinase involved in several metabolic events. We found mTOR to be necessary both for β-adrenergic- and insulin-stimulated glucose uptake. In Paper III we continued to examine the intracellular pathway and found this to be only partly dependent on the classical second messenger cAMP. Instead we identified the molecule GRK2 as a key player in the β-adrenergic signal to glucose uptake.

We have also discovered a new effect of β-AR stimulation in muscles cells: inhibition of cellular contraction. That β-adrenergic signalling can attenuate contraction is a well-known phenomenon in smooth muscle cells while previous results from skeletal muscles have been ambiguous. In our system, the skeletal muscle cell line L6, activation of β-ARs clearly prevented contraction. We examined the intracellular signal regulating this event and found the second messenger cAMP and K⁺-channels to be involved (Figure 1).

Thus, we have discovered new intracellular signals, new mechanisms and new endpoints due to β-AR signalling in skeletal muscle cells. In this thesis, β-adrenergic signalling in skeletal muscles will be discussed with the main focus on our novel findings.
Figure 1. My current view of β-AR signalling in skeletal muscles cells. Oval shape indicates intracellular molecules while boxes indicate downstream events that are stimulated by β-ARs. Pink colour marks our novel findings and dark pink indicates molecules or events that prior to my studies were unknown to occur downstream of β-ARs.

2. Adrenergic signalling
Skeletal muscles are subjected to regulation from several endogenous signals e.g. hormones and neurotransmitters. The primary function of muscles is contraction which is mainly regulated by the release of acetylcholine from motorneurons, while. Other functions can be regulated by other stimuli, for example the hormone insulin which is released from the pancreas after a meal and regulates glucose uptake and glycogen synthesis in skeletal muscle cells. This thesis will however focus on the effects of sympathetic signalling, a stress signal that both activates a systemic “fight or flight”-response and regulates distinct organs in order to maintain everyday homeostasis. The sympathetic system includes release of epinephrine from the adrenal medulla as well as release of norepinephrine from sympathetic nerve endings. Epinephrine and norepinephrine bind to adrenoceptors on the cell surface and thus induce several effects such as increased heart rate, pupil dilatation, inhibition of digestion and redirection of blood flow.
2.1. Adrenoceptors
There are nine subtypes of adrenoceptors (ARs) with distinct tissue expression and pharmacological properties: $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1D}$, $\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$, $\beta_1$, $\beta_2$ and $\beta_3$. These are G-protein coupled receptors (GPCRs) meaning that they can act by activating G-proteins by exchanging the bound GDP to a GTP. The different AR subtypes couple to distinct G-proteins leading to different intracellular pathways (Figure 2).

The $\alpha_1$-ARs couple to $G_{q/11}$ that activates phospholipase C (PLC). PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2), making inositol trisphosphate (IP3) and diacylglycerol (DAG), which activate Ca$^{2+}$- and protein kinase C (PKC) respectively. The $\alpha_2$-ARs, couple to $G_\gamma G_\delta$ which inhibits cAMP-production. The $\beta$-ARs couple to $G_s$ that activates adenylate cyclase (AC) and thus increases cAMP-production. Both norepinephrine and epinephrine show approximately the same potency on the $\alpha_1$-AR and $\alpha_2$-AR. They have also the same potency on the $\beta_1$-AR, while epinephrine is much more potent than norepinephrine on $\beta_2$-AR. $\beta_3$-AR exhibits opposite properties to $\beta_2$-AR with norepinephrine as the more potent agonist. Thus the AR subtypes differ both in pharmacological profiles as well as intracellular effects, why the AR-expression pattern in a specific tissue greatly influences the tissue’s response to adrenergic agonists.

![Figure 2](image)

**Figure 2.** Overview of the adrenoceptors and their main downstream effectors.

2.2. Expression of ARs in skeletal muscles
Skeletal muscle cells are reported to express both $\alpha$- and $\beta$-ARs (Rattigan et al. 1986; Watson-Wright et al. 1986). To further scrutinise the expression of AR-subtypes one has to consider the heterogeneity amongst muscle fibers. Skeletal muscle fibres can be divided into different fiber types depending on staining for myosin adenosinetriphosphatase (mATPase), myosin isoform or expression of metabolic enzymes. The latter classification is the most commonly used and divides muscles into type I, type IIA and type IIB fibres. Type I fibres, also called
slow oxidative fibres, contract more slowly and are more resistant to fatigue than type II fibres. They have a high content of myoglobin, mitochondria and capillaries. Type IIA fibres are fast oxidative fibers which have more glycogen and are less resistant to fatigue. Type IIB are fast glycolytic fibres with less mitochondria, myoglobin and capillaries but high amounts of phospho-creatine and glycogen, they contract rapidly and fatigue easily.

Skeletal muscles are shown to express $\alpha_1$- but not $\alpha_2$-ARs (Hutchinson et al. 2005; Martin et al. 1990; Rattigan et al. 1986). One study suggested a difference between fiber types since less $\alpha$-ARs were detected in fast-twitch muscles (Rattigan et al. 1986) while in another study (Martin et al. 1990) this difference was not found. This discrepancy may depend on the fact that muscles containing most fast-twitch fibers contain fewer blood vessels which also express $\alpha$-ARs, why there might be a difference in amount of $\alpha$-ARs in the whole tissue but not in the muscle fibres themselves.

The $\beta$-ARs, however, are considered to be the more abundant AR-form in skeletal muscles since ten times more $\beta$-ARs than $\alpha$-ARs were found with radioligand binding (Rattigan et al. 1986). The major subtype of $\beta$-ARs present in skeletal muscle cells is $\beta_2$-AR, as shown with both radioligand binding assays and pharmacological studies (Liggett et al. 1988; Roberts et al. 1998; Sillence et al. 1994; Watson-Wright et al. 1986). In fast-twitch muscles $\beta_2$-AR is the only expressed isoform while in slow-twitch muscles also $\beta_1$-AR has been detected (Kim et al. 1991; Watson-Wright et al. 1986). Slow-twitch muscles are found to have higher total density of $\beta$-ARs with a clear correlation to the proportion of type I (oxidative) fibers as well as an inverse correlation to the proportion of type IIB (glycolytic) fibers (Jensen et al. 1995; Martin et al. 1989; Watson-Wright et al. 1986; Williams et al. 1984). The skeletal muscle-like cell lines L6 and C2C12 express increasing levels of $\beta_2$-ARs during differentiation, while $\beta_1$ and $\beta_3$ mRNA are not detected (Nagase et al. 2001; Nevzorova et al. 2002; Ngala et al. 2008).

Taken together the above described results demonstrated that skeletal muscle cells express $\alpha_1$- and $\beta_2$-ARs of which the latter are more abundant. In type I fibers low levels of $\beta_1$-ARs are found while in type II fibers and cell lines, including the L6-cell which I’ve used in my studies, only $\beta_2$-ARs are expressed.

3. Adrenergic effects in skeletal muscles
Activation of ARs is reported to cause several effects in skeletal muscles. Firstly these effects will be described while the latter part of this thesis deals with the intracellular signals which regulate these events.
3.1. Muscle growth
Feeding animals with β-adrenergic agonists leads to increased muscle mass and decreased fat mass, as shown in several mammals including sheep (Baker et al. 1984), cattle (Ricks et al. 1984), swine (Jones et al. 1985) and rat (Emery et al. 1984). For this reason, the β-AR agonist clenbuterol has been used illegally by bodybuilders who want to increase their muscle-to-fat ratio. Increased tissue mass can be caused by either hyperplasia (increased number of cells) or hypertrophy (enlargement of cells). Although both effects are suggested to occur in muscles after β-agonist treatment, hypertrophy is likely to be the major effect as shown by the finding that treatment with the β-AR agonist cimaterol increased the content of both protein and RNA but decreased the content of DNA in muscles from lamb (Beermann et al. 1987). The hypertrophy has been proposed to be caused partly by increasing protein synthesis and partly by decreasing protein degradation: for example clenbuterol treatment is shown both to induce expression of genes involved in initiation of translation (Spurlock et al. 2006) and to inhibit proteolysis (Navegantes et al. 2001) in rodent skeletal muscles. In addition to the effect of increased muscle mass, β-adrenergic activation may also induce slow-to-fast twitch conversion in rats by altering the expression of myosin isoforms (Beermann et al. 1987; Stevens et al. 2000). Taken together these results state that increased muscle mass is a well established effect of β-adrenergic activation and that there may be several molecular mechanisms leading to this event.

3.2. Ion-channels and force of contraction
Various ion-channels in skeletal muscle cells are found to be affected by β-adrenergic signalling. For example is K⁺-uptake through the Na⁺-K⁺-2Cl⁻-co-transporter (NKCC) increased in rat soleus and plantaris muscles from rat due to β-AR activation (Gosmanov et al. 2002). Altered ion-transport can also affect contraction: Sympathetic stimulation can for example increase Ca²⁺ currents conducted by the voltage sensitive channel Caᵥ1, leading to increased muscle contraction. This phenomenon is well-studied in heart but isoprenaline is shown to increase Ca²⁺-flux also in cell cultures of embryonic muscles from chicken, in which the effect was additive to that of depolarisation (Schmid et al. 1985). Furthermore PKA is shown to phosphorylate the ryanodine receptor (a Ca²⁺-channel involved in muscle contraction) in rabbit skeletal muscles, indicating this to be a target of β-adrenergic signalling (Suko et al. 1993).

Since the ability to contract is the major feature of skeletal muscles it may not be surprising that adrenergic signalling can modulate this parameter. In which direction the contraction is altered may however vary between muscle types; Bowman and Zaimis showed in the 1950’s that adrenergic agonists increase force of contraction in fast-twitch muscles while having the opposite effect in slow twitch muscles (Bowman et al. 1958). Other studies have however shown increased force also in slow twitch muscles (Cairns et al. 1993).
A further clue in this issue can be obtained from our studies. We examined the effect of β-AR signalling on contractility in the L6 cell line (Paper II) which we found to contract in response to depletion of extracellular Ca$^{2+}$. Such treatment has previously been used as a model for myosin-dependent contraction in other systems (Britch et al. 1980; Ivanov et al. 2004; Ma et al. 2000; Samarin et al. 2007). Thus, to induce contraction we exposed the cells to either Ca$^{2+}$-free PBS or media containing the Ca$^{2+}$-chelator EDTA which made the cells contract and finally detach (Figure 3B). We found that the β-AR agonist isoprenaline had a pronounced inhibitory effect on cellular contraction and detachment (Figure 3C). To further examine the signalling pathway regulating this effect we developed a quantitative cell detachment assay in which we used both EDTA and trypsin which made the cells get released from each other so that the detached cells were possible to count. By this method we revealed the involvement of K$^+$-channels, thus adding these to the list of ion-channels that can be affected by β-adrenergic signalling in skeletal muscle cells.

The discrepancy between different studies regarding β-adrenergic effect on muscle contraction can have several reasons. For example, the L6-cell differs from whole muscles in the sense that the contractile machinery in the L6-cells is more similar to that found in embryonic than in fully differentiated muscle cell (Whalen et al. 1979). There are also several different myosin isoforms which are under distinct regulation. In addition to the muscle-specific myosins, muscle cells also express non-muscle myosins, which are found in all eukaryotic cells and by using the Ca$^{2+}$-depletion we activate the non-muscle myosin rather than the skeletal muscle specific isoform. Thus it is possible that the effect observed in our study is more likely to occur during development or in satellite cells than in fully differentiated muscles. One can however conclude that β-ARs can modulate the contractility in muscle cells, but in which direction may depend both on model system and on how the contraction is induced.

Figure 3. The effect of β-adrenergic stimulation on cellular contractility shown with fluorescence microscopy of L6-myotubes stained with FITC-phalloidin which binds to filamentous actin. (A) L6-myotubes grown in culture media. (B) After 3 min exposure to EDTA the cells contracted to finally detach. (C) When kept in EDTA in the presence of 1 µM isoprenaline, the cells were unable to contract.
3.3. Glycogen metabolism
Furthermore, there are several studies dealing with the effect of β-adrenergic agonists on glycogen metabolism. The level of glycogen in a muscle is determined by the rate of glycogenolysis and glycogen synthesis, processes that are catalyzed by the enzymes phosphorylase and glycogen synthase, respectively. Norepinephrine can reduce glycogen levels by increasing the activity of phosphorylase as well as decreasing the activity of glycogen synthase, as shown in both prefused rat hindlimb and in biopsies from human (Dietz et al. 1980; Raz et al. 1991). In the rat system this effect was blocked with propranolol, indicating the involvement of β-ARs (Dietz et al. 1980).

The role of β-adrenergic signalling in the glycogenolysis occurring during exercise has been investigated in several studies with somewhat ambiguous conclusions; while the β-blocker propranolol was unable to block glycogen breakdown in plantaris or soleus in rats after 55 min of tread-milling (Juhlin-Dannfelt et al. 1982), Gorski et al. showed propranolol to block glycogen breakdown in fast-twitch-muscles during low intensity exercise and in slow-twitch muscles during both high and low intensity exercise (Gorski et al. 1982). Furthermore, biopsies from human thigh muscles show that propranolol decreases the rate of glycogenolysis during dynamic exercise but not during static contraction (Chasiotis et al. 1983). Thus β-adrenergic signalling can influence glycogenolysis during exercise but this depends on fiber type as well as intensity of exercise. In contrast to the results about glycogenolysis, results from L6-myo-tubes show that treatment with β-agonist may lead to increased glycogen synthesis (Yamamoto et al. 2007). Since this effect was observed after 2 h of stimulation, it is possible that glycogenolysis is the acute effect while longer treatment leads to glycogen synthesis.

3.4. Lipolysis
Although muscles store energy as glycogen rather than lipids, there are several studies showing the presence of both triglycerides and free fatty acids in skeletal muscles from several species (review by (Gorski 1992)). Lipolysis is increased by adrenergic stimulation which is shown in human where injection of β-AR agonists induces a concentration-dependent increase in glycerol levels (Hagstrom-Toft et al. 1998). Furthermore the hormone sensitive lipase (HSL) is activated by adrenergic signalling in skeletal muscles (Langfort et al. 1999). Thus one can conclude that β-adrenergic signalling can alter also lipid metabolism in skeletal muscle cells, although the physiological role for this remains to be proven.

3.5. Glucose uptake
For long adrenergic signalling was considered to inhibit glucose uptake since activation of ARs is shown to antagonize insulin-mediated glucose uptake in skeletal muscles from both rats and human (Chiasson et al. 1981; Jamerson et al. 1993; Lembo et al. 1994).
However, noradrenergic stimulation can by itself lead to an increase of glucose uptake in skeletal muscles, as shown in several model systems: Electrical stimulation of the ventromedial hypothalamus (VMH, an area in the brain previously shown to be involved in adrenergic regulation of brown adipose tissue) increased glucose uptake in skeletal muscles and heart without affecting insulin levels (Minokoshi et al. 1994; Shimazu et al. 1991; Sudo et al. 1991). A stimulatory effect was seen in both quadriceps, gastrocnemius, soleus and extensor digitorum longus (EDL). This effect was also mimicked by stimulation of VMH via microinjection of L-glutamate (Sudo et al. 1991). The increase in glucose uptake upon electrical stimulation of VMH was blocked by guanethidine which inhibits norepinephrine release from nerve ending, but was not altered by adrenomedullation (removal of adrenal medulla), indicating norepinephrine from sympathetic nerves, rather than epinephrine from the adrenal gland, to be responsible for the observed glucose uptake (Minokoshi et al. 1994). Furthermore, injection of the β-AR agonist BRL37344 in anesthetized rats increased glucose uptake in muscles (Abe et al. 1993; Liu et al. 1995). This injection augmented insulin-levels, but BRL37344 can also act directly on muscles since it stimulates glucose uptake in a dose-dependent manner in isolated muscles (Abe et al. 1993). BRL37344 is however not a specific β2-AR agonist. It was firstly designed as a β3-AR agonist and later found to also act on skeletal muscles and β2-ARs. Furthermore, very low concentrations of BRL37344 can have effects which are not dependent on any AR; BRL37344 is found to stimulate glucose uptake in rat soleus and EDL (which does not express β3-ARs) and when used at the concentration 10^{-11} M the BRL37344-effect could not be blocked by the β2-antagonist ICI 118551 (Liu et al. 1996; Ngala et al. 2008; Ngala et al. 2009). These results was confirmed in studies using soleus from KO-mice: 10^{-11} M BRL37344 increased glucose uptake in soleus from both β2-KO and β-less mice (Ngala et al. 2009). However, the effect of higher concentrations (10^{-6} M and 10^{-8} M) was blocked by ICI 118551 and the effect of 10^{-6} M BRL37344 was abolished in the KO-models, showing these concentrations of BRL37344 to increase glucose uptake in skeletal muscles by acting on the β2-AR (Ngala et al. 2009).

There are also contradicting results regarding the effect of β-adrenergic signalling in skeletal muscle cells showing epinephrine to decrease glucose uptake in ex vivo rat soleus (Sloan et al. 1978). This could however be a methodological issue since another paper showed that isoprenaline treatment decreases glucose uptake in the rat epitrochlearis muscles when Krebs-Henseleit Buffer is used as assay buffer, but when supplemented with 1 % BSA, the glucose uptake is increased (Young et al. 1985). Also in our studies, isoprenaline treatment increased glucose uptake in ex vivo muscles and in these studies media containing BSA was used (Paper I). The outcome may also differ with the ligand: while BRL37344 increased glucose uptake in mouse soleus, in the same study clenbuterol and salbutamol had the opposite effect (Ngala et al. 2008). In any case norepinephrine is found to increase glucose uptake in perfused rat gastrocnemius and soleus (Han
et al. 1998) and in our studies we found isoprenaline to stimulate glucose uptake both ex vivo in soleus from Sprague-Dawly rats and in vivo in mice (Paper I). A further indication of the physiological relevance of adrenergic stimulation of glucose uptake is that injections with β-AR ligands stimulate glucose uptake in muscles in living mice (Paper I, Paper III). In most of our studies we have used muscle-like cell lines and treatment with β-agonists is shown to increase glucose uptake in both C2C12 and L6 (Nevzorova et al. 2002; Nevzorova et al. 2006; Ngala et al. 2008; Tanishita et al. 1997). The latter cell line is used as a model system in Paper I-IV and in all our experiments we found the β-adrenergic ligand isoprenaline to induce glucose uptake to the same extent as insulin (Figure 4, Paper I, III, IV). Thus, there is a convincing body of evidence showing that β-adrenergic stimulation leads to increased glucose uptake in skeletal muscle cells.

![Figure 4](image)

**Figure 4.** (A) L6-cells differentiated into myotubes were used as a model system in Paper I-IV. (B) These cells responded to the β-adrenergic agonist isoprenaline by increasing glucose uptake to the same level as induced by insulin.

### 4. Adrenergic effects on glucose transporters

Of the diverse β-adrenergic effects which are described in chapter three, the increase in glucose uptake is the effect which our research has mainly focused on. An increase in glucose uptake can be achieved through different molecular mechanisms. The proteins involved in glucose transport, as well as the different ways known to alter the transport through these proteins, will be presented in the following sections along with our novel results concerning β-adrenergic effects on the glucose transporters.

#### 4.1. Glucose transporters

Although it has been suggested that glucose may enter erythrocytes via a volume activated “Cl-channel” (Kirk et al. 1992), the generally accepted mechanism for glucose uptake is via transporter proteins belonging to either of the sodium dependent glucose transporters (SGLT) or the facilitative glucose transporters
(GLUT). The SGLTs transport glucose via a secondary active, Na\textsuperscript{+}-coupled mechanism. The main function of these proteins is to transport glucose into the cells from the small intestine, the renal proximal tubules and salivary gland ducts (Sabino-Silva et al. 2010). However, low levels of SGTL5 are found in other tissues, including skeletal muscles from cow (Zhao et al. 2005).

The GLUTs transport glucose along a gradient via facilitative diffusion. The GLUT family comprises 14 isoforms divided into 3 classes dependent on structural similarities (Augustin 2010). Class I comprises GLUT1-4 together with GLUT14, a duplication of GLUT3 only found in testes (Wu et al. 2002). GLUT5, GLUT7, GLUT9 and GLUT11 belong to Class II and can transport both glucose and fructose. Class III includes GLUT6, GLUT8, GLUT10, GLUT12 and the proton driven myoinositol transporter HMIT (Augustin 2010).

4.2. Glucose transporters in skeletal muscle cells
In skeletal muscle biopsies from humans the following glucose transporters are found: GLUT1, GLUT3, GLUT4, GLUT5, GLUT8 and GLUT12 (Stuart et al. 2000; Stuart et al. 2006) with GLUT4, GLUT5 and GLUT12 as the most abundantly expressed (Stuart et al. 2006). On mRNA level also GLUT11 was detected, but this transporter was not found at protein level (Stuart et al. 2006). Immunohistochemistry studies reveal a difference between fiber types: While GLUT4 and GLUT12 are expressed at higher levels in type I than type II fibers, GLUT5 have higher expression in type II fibers (Stuart et al. 2006).

The L6-cell line used in our studies (Paper I-IV) expresses decreasing levels of GLUT1 and increasing levels of GLUT4 during differentiation (Mitsumoto et al. 1991). GLUT3 is reported to be expressed in L6-myoblasts and GLUT12 and GLUT5 are found in both myoblasts and myotubes (Hajduch et al. 2003; Stuart et al. 2009; Taha et al. 1995).

The function of the GLUT-expression-pattern in muscles is not fully understood. The most well-studied of the current transporters are GLUT1 and GLUT4. GLUT1 was the first cloned isoform (Mueckler et al. 1985) and is ubiquitously expressed (with the exception of some hepatocytes) while GLUT4 is expressed in skeletal muscles and adipose tissues where it mediates glucose import upon insulin stimulation. GLUT3, which belongs to Class I as do GLUT1 and GLUT4, is highly expressed in neurons and is thus the predominant GLUT-homolog in the brain. In addition to glucose, GLUT3 can also transport other types of sugar like galactose, mannose, maltose, xylose, as well as dehydroascorbic acid (Colville et al. 1993). GLUT5 belongs to Class II and is more prone to transport fructose than glucose and is primarily expressed in the small intestine. GLUT8 and GLUT12, which belong to Class III, are both suggested to be involved in insulin-stimulated glucose uptake; GLUT8 is found to be insulin-responsive in blastocysts from mice (Carayannopoulos et al. 2000), but not in primary rat adipocytes, 3T3L1, HEK293
and CHO or CHO-cells (Augustin et al. 2005; Lisinski et al. 2001). Insulin stimulation of human skeletal muscles induces an increase of GLUT12 in plasma membrane comparable to the insulin-stimulated GLUT4 translocation in both L6-myoblasts and humans, as shown by muscle biopsies taken before and after insulin-infusion (Stuart et al. 2009).

4.3. Mechanisms for increasing glucose uptake
The rate of glucose uptake in a cell is regulated by several mechanisms. For example, the amount of GLUT can be up-regulated via enhanced transcription or translation or by reduced degradation. It may also be possible to increase the activity of the already present GLUTs, via conformational changes in the proteins, due to re-location to distinct compartments in the plasma membrane or ologomerisation with other GLUTs. The possibility of altered intrinsic activity is still somewhat controversial and is not proven to happen in muscle cells.

Since little was known about which end mechanism is responsible for the β-adrenergic regulated glucose uptake, we chose to investigate this (Paper I). By using different pharmacological inhibitor we narrowed down the number of possible GLUT-isoforms and concluded that GLUT4 must be involved, a finding that was confirmed by siRNA silencing of GLUT4. After ruling out the possible involvement of transcription and translation, we formulated the hypothesis that β-adrenergic stimulation might induce translocation of GLUT4, the same mechanism as used by insulin. By immunohistochemistry staining we showed that β-adrenergic stimulation promotes GLUT4-translocation to the same extent as insulin does (Figure 5). Interestingly, this finding was repeated in human skeletal muscle cells indicating this to be happening also in humans.

![Figure 5](image)

**Figure 5.** β-adrenergic signalling stimulates GLUT4-translocation. Confocal imaging of L6-myoblasts revealed that GLUT4-translocation occurs not only after treatment with insulin but also in response to stimulation with the β-adrenergic agonist isoprenaline (Paper I).

That β-ARs can induce GLUT4-translocation is a completely novel finding, but GLUT4-translocation due to other stimuli is a well-described event. Translocation
of GLUTs from intracellular storages to the plasma membrane was first described in rat adipocytes in response insulin (Cushman et al. 1980; Suzuki et al. 1980) and was later demonstrated also in skeletal muscles (Wilson et al. 1994). In the basal state, the intracellular pool of GLUT4 can be found in either circulation endosomes, the trans-Golig-network or special GLUT4-storage vesicles (GSVs) of which the latter is believed to be responsible for the major part of insulin-stimulated glucose uptake (Bryant et al. 2002). In muscle cells, translocation may not only occur to the plasma membrane but also to the t-tubules (Ploug et al. 1998). There are also stimuli other that insulin that can induce GLUT4-translocation in skeletal muscle cells such as the AMP activated kinase (AMPK) and muscle contraction (Douen et al. 1990; Kurth-Kraczek et al. 1999).

Our next question was if the β-adrenergic signal may converge with any of other pathways reported to cause GLUT4-translocaiton. According to previous studies from our lab, AMPK is not activated by β-ARs (Hutchinson et al. 2006) while there are indications of β-adrenergic signalling to share features with the insulin-pathway (Nevzorova et al. 2006). Therefore we wanted to compare the β-adrenergic pathway with that of insulin. Insulin signalling has been subjected to thorough investigations since loss of insulin response in peripheral tissues is a hallmark of type II diabetes. The insulin signal is a complex process involving several pathways including phosphatidylinositol 3-kinase (PI3K), phosphatidylinositol (3,4,5)-triphosphate (PIP3) and protein kinase B (PKB, also known as Akt). How this signal is transduced to the point GLUT4-translocation is not fully understood but one key molecule is AS160 which is phosphorylated by Akt.

We found that the β-AR signal to glucose uptake does not involve the insulin activated molecules PI3K, PIP3, Akt or AS160. However, the two pathways are still not completely different since we discovered the involvement of a more recently described player in the insulin cascade: the mammalian target of rapamycin (mTOR, also known as the mechanistic target for rapamycin). Both pharmacological studies and knock-down with siRNA showed dependence on mTOR for both insulin and β-adrenergic stimulated glucose uptake in L6-myotubes. Furthermore, immuncytochemistry staining in the presence of an mTOR-inhibitor revealed mTOR activation to be necessary for the GLUT4-translocaiton. Thus, the findings in Paper I establishes that activation of β2-ARs induces glucose uptake via mTORC2-dependent GLUT4-translocation.

4.4. mTOR
The finding of mTORC2 to be a key player in β-adrenergic regulated glucose uptake settles a new role for this molecule since mTORC2 was not recognized to be activated by adrenergic signalling, neither in skeletal muscle cells nor in any other system (Figure 1). This is an exciting discovery and, from what we have previously known about adrenergic signalling, also unexpected. mTOR is a
serine/threonine protein kinase belonging to the phosphoinositide 3-kinase (PI3K)-related kinase family (PIKK) and is regulated by several extra- and intracellular cues reflecting local and whole body nutritional homeostasis. It has been described as a key sensor of energy and nutritional status and is involved in several metabolic processes.

mTOR occurs as a part of either of the two distinct complexes named mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Both complexes contain mTOR and the associated proteins mLST8 (also known as GβL), DEPTOR, and TriI/Tel2. In mTORC1 also PRAS140 as well as the regulatory associated protein of mTOR (raptor) are found (Laplante et al. 2012). Complex 1 is the better characterized of the two complexes and is shown to integrate input from different stimuli such as growth factors, energy status, oxygen-levels and amino acids. There are several intracellular signals that can activate mTORC1, for example Akt, Erk, Wnt and AMPK (Laplante et al. 2012). mTORC1 can also be activated by amino acid, probably via a different mechanism than the other stimuli, and a sufficient amount of amino acids are crucial for all activation of mTORC1.

There are numerous downstream effects of mTORC1 including protein and lipid synthesis as well as inhibition of autophagy. It can affect protein synthesis via direct phosphorylation of the eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and the ribosomal S6 kinase 1 (S6K1), two factors that promote protein synthesis. Also lipid synthesis is unregulated by mTORC1 via activation of the sterol regulatory element-binding protein (SREBP), a transcription factor regulating various genes involved in cholesterol and fatty acid synthesis.

However, when the involvement of mTOR in glucose uptake was investigated, it was not mTORC1 but mTORC2 that was found to be crucial for both insulin and β-ARs to induce glucose uptake (Paper I).

The mTORC2 contains the rapamycin-insensitive companion of mTOR (riplot) along with mSin1 and Protor1/2. The name rictor comes from the fact that the mTOR-inhibitor rapamycin, a naturally occurring compound from the bacterium Streptomyces hygroscopicus, blocks the kinase activity only of mTORC1 and not of mTORC2. However, long-term treatment can inhibit the assembly of mTORC2, thus blocking also this complex in several, but not all, cell types (Sarbassov et al. 2006).

In contrast to mTORC1, mTORC2 is not regulated by local energy/nutritional homeostasis but is instead activated by external signals, such as insulin. mTORC2 regulates several downstream targets including Akt, the serum- and glucocorticoid-induced protein kinase 1 (SGK-1) and PKCα. Downstream effects of Akt is glucose uptake, cell survival and proliferation. mTORC2 can also regulate cell shape due to its effect on PKCα (Sarbassov et al. 2004). SGK-1 is implicated in several cellular processes including transport of ions and glucose.
Fewer signals are identified to induce activation of mTORC2 than of mTORC1, but one major activator seems to be insulin, which is shown to cause PI3K-dependent autophosphorylation of mTOR at S2481 in both mTORC1 and mTORC2 (Soliman et al. 2010). Furthermore mTORC2 is shown to phosphorylate Akt (Sarbassov et al. 2005), a key hallmark of insulin signalling. The physiological role of mTOR for the insulin signalling is further illustrated by the finding that chronic rapamycin treatment can induce glucose intolerance, hyperinsulinemia and hyperglycemia in rats due to impaired Akt-phosphorylation and glucose uptake in skeletal muscles (Deblon et al. 2012).

In Paper I we used phosphorylation of mTOR as an indicator of activation. Insulin induces phosphorylation of the two sites S2481 and S2448 while β-adrenergic signalling only caused phosphorylation of S2481, indicating β-AR to operate via a unique activation of mTOR. S2481 was phosphorylated also by the cAMP analogue as 8-Br-cAMP, which suggests the involvement of cAMP, the classical second messenger downstream of β2-AR, in the regulation of mTOR.

5. cAMP-dependent signalling
The following sections will deal with the different intracellular signalling pathways that can be activated by β-ARs and their role in skeletal muscle cells. There are several pathways known to be activated by β-ARs and the most well studied of these is the second messenger cAMP. Thus, cAMP and its downstream targets (Figure 6) will be discussed first.

5.1. cAMP in skeletal muscles
The main G-protein linked to β2-ARs is the stimulatory G-protein Gs which upon receptor activation dissociates into its α- and βγ-subunits of which the former stimulates production of 3’-5’-cyclic adenosine monophosphate (cAMP) via activation of adenylate cyclase (AC). cAMP was the first second messenger to be found and characterized as described in several papers by Sutherland and colleagues (Rall et al. 1958; Sutherland et al. 1958), for which he was rewarded the Nobel prize in 1971. Increased cAMP-levels can affect several downstream targets in the cell including protein kinase A (PKA), Epac and ion-channels (Figure 6).

Activation of β-AR is shown to increase intracellular levels of cAMP in several model systems for skeletal muscles including skeletal muscle slices, perfused muscle, muscle cell membranes and L6-cells (Han et al. 1998; Nagase et al. 2001; Nevzorova et al. 2006; Roberts et al. 1998; Schubert et al. 1976; Silence et al. 1994; Paper III). However, less is known about how cAMP regulates specific endpoints.
Figure 6. The classical second messenger downstream of β₂-ARs is cAMP, which can act via PKA, Epac, ion-channels and possibly also other targets.

Several adrenergic effects in muscle cells, including gene-expression, glucose uptake, inhibition of proteolysis, ion-transport as well as modulation of contractility is mimicked by cAMP-analogues (Cairns et al. 1993; Nagase et al. 2001; Navegantes et al. 2001; Nevzorova et al. 2002; Schmid et al. 1985; Paper II). According to these results, cAMP seems to be a central second messenger in β-adrenergic signalling in skeletal muscles. However, the caveat of using cAMP-analogues is that this may not fully mimic what is happening when the receptor is activated by an agonist. Most cAMP-analogues will not be degraded to the same extent as endogenous cAMP and adding extracellular cAMP will also give a different concentration gradient over the plasma membrane compared to receptor activation. Furthermore, external addition of cAMP will not induce the compartmentalisation inside the cell that occurs after stimulation of the receptor. Thus, a more reliable way to show cAMP-dependence might be to inhibit AC. The problem with this approach is that there are few pharmacological inhibitors of AC that sufficiently block cAMP-production. In L6-myobutes the AC-blocker ddA only inhibited a third of the isoprenaline induced cAMP-production at the timepoint 15 minutes (Paper I). The SQ2256 is more efficient, but to keep cAMP-levels down for a longer time very high concentrations are needed (Paper III). Because of these problems it is more common to show cAMP-involvement by using the AC-activator forskolin or cAMP-analogues. This may however not lead to the same conclusions, as is the case in glucose uptake: Addition of different cAMP-analogues or regular cAMP induces glucose uptake in L6-cells (Nevzorova et al. 2002; Nevzorova et al. 2006; Paper III), while pharmacological inhibition of cAMP-production only partly inhibited the β-adrenergic stimulation of glucose uptake (Nevzorova et al. 2006; Paper III).

From the above presented data it can be concluded that cAMP is indicated to play a role in several events in skeletal muscles. However, since this was the first β-
adrenergic second messenger described, many papers have examined only the cAMP-pathway and not looked for other possible effectors. In combination with the methodological problems, this fact makes it difficult to determine the precise role of cAMP in β-adrenergic effects in skeletal muscles.

5.2. PKA
Protein kinase A (PKA) was the first described effector downstream of cAMP (Walsh et al. 1968) and is often considered to be the main mediator of cAMP signalling. PKA is a serine/threonine kinase composed of two catalytic (C) and two regulatory (R) subunits. When cAMP-levels increase within the cell, cAMP-molecules will bind to the R-subunits, causing dissociation of the holoenzyme and leaving the C-subunits free to phosphorylate substrates. There are four isoforms of the R-subunit (RIα, RIβ, RIIα and RIIβ) and four isoforms of the C-subunit (Ca, Cβ, Cy and Prkx). Cβ has also six splice variants, giving rise to even further versions of PKA (Orstavik et al. 2001). However, the different PKA holoenzymes are divided into PKAI and PKAII depending on the R-subunit isoform (with PKAI containing either RIα or RIβ and PKAII containing RIIα or RIIβ). Both PKAI and PKAII are expressed in mammalian skeletal muscles with RIIα as the dominant R-isofrom (Burton et al. 1997; Hoover et al. 2001; Imaizumi-Scherrer et al. 1996).

The simultaneous presence of both isoforms in most mammalian cells could argue for divergent functions, but it is not clear whether there are any functional differences between the PKA-isoforms. There are however differences between the isoforms regarding subcellular localisation since PKAII is more prone to associate with membranes while PKAI is more prone to be localized in the cytoplasm (Reviewed in (Scott 1991)). In skeletal muscle from rats, the two subunits are found to have partly different localisation along the sarcomeres (contractile units) (Perkins et al. 2001).

To understand PKA signalling, one has to consider its interaction and regulation by the A kinase anchoring proteins (AKAPs), which serve as scaffolds for PKA. By binding the regulatory subunit of PKA, the AKAPs can localize PKA to specific sites in the cell as well as creating signalling complex by binding to other proteins such as phosphatases and phosphodiesterases. Interestingly, one of the AKAPs called gravin is found to associate also with the β2-AR (Lin et al. 2000). The gravin-β2-AR- complex interacts with phosphatases in the absence of agonists while agonist-stimulation induces association with PKA, PKC, GRK2, β-arrestin and clathrin (Lin et al. 2000; Shih et al. 1999), indicating a role for this AKAP in β-AR signalling.

There are remarkably few articles that show PKA-activation downstream of β-ARs in skeletal muscles. One reason for this may be that β-adrenergic activation of PKA is considered evident, another is methodological problems. One of the few findings of PKA activity in skeletal muscles is from rat muscles where
infusion of norepinephrine leads to increased PKA activity (Dietz et al. 1980). This was shown by a kinase-activity-assay with extracted PKA. The risk with using extracted enzymes is that this may not totally mimic what is happening within the cell. Another method to detect PKA-activity is to examine phosphorylations of known PKA-substrates in whole cell lysate. The disadvantage of this approach is that these may be phosphorylated also by other kinases. PKA can phosphorylate several downstream targets of which the most well-known are the cAMP response element binding protein (CREB, a transcription faction) and acetyl-CoA carboxylase (ACC, an enzyme regulation fatty acid oxidation). Also other kinases can phosphorylate CREB, but PKA is considered to be the main kinase for this phosphorylation.

To investigate PKA-activation in the L6-myotubes, we performed western blot using antibodies recognizing the phosphorylated form of either PKA-phosphorylation consensus site or CREB. Both antibodies showed an increase in phosphorylation in samples from cells treated with isoprenaline (Figure 7), indicating that PKA is actually activated by β-adrenergic signalling in these cells.

Taken together these results strongly indicate that PKA can be activated in skeletal muscle cells due to activation of β-ARs. The next question is if this activation is important for any downstream effects. β-adrenergic regulation of both Ca^{2+}-channels and NKCC seems to involve PKA since they are mimicked by the PKA-specific cAMP analogue (Sp-5,6-DCl-cBIMPS) and inhibited by the PKA-inhibitor H-89 respectively (Gosmanov et al. 2002; Johnson et al. 1997). Furthermore, β-adrenergic inhibition of proteolysis is likely to be mediated by PKA since it is mimicked by a PKA-selective cAMP-analogue 6-Bnz-cAMP (Baviera et al. 2010).

Another endpoint of β-adrenergic activation in skeletal muscles is activation of HSL, an effect that is more thoroughly described in adipocytes where it is clearly mediated by PKA. Epinephrine stimulation increases HSL-activity and

![Figure 7](image-url)
phosphorylation of HSL at known PKA-regulated sites in L6-cells (Watt et al. 2006), suggesting PKA-dependence also in muscle cells. Furthermore, activation of β-ARs is found to simulate the same HSL-phosphorylation in human muscles (Jocken et al. 2008).

Taken together, these results indicate that β-adrenergic regulation of both Ca^{2+}-channels, NKCC and HSL involves PKA. One has however to be aware of the methodological limitations. The available PKA-inhibitors are for example not as selective as would be desired. The PKA-inhibitor H-89 (which was used in the NKCC-study) can actually block Rho-activated kinase (ROCK) more efficiently than it inhibits PKA (Davies et al. 2000). H-89 has also been indicated to act directly on the β_{2}-AR, both agonistically and antagonistically (Baker et al. 2003; Penn et al. 1999). Also new inhibitors, such as KT 5720, can inhibit several kinases in addition to PKA (Davies et al. 2000). PKA-selective cAMP-analogues are also problematic to use since for example 6-Bnz-cAMP (used in the proteolysis study) can inhibit the cAMP-regulated K^{+}-channels bTREK-1 independent of its effect on PKA (Liu et al. 2009). The most reliable PKA-inhibitor is at the moment the protein kinase inhibitor peptide (PKI). It is however important to use more than one inhibitor in the same study in order to avoid misleading results due to the potential side effect.

From the facts described above, we can conclude that surprisingly little is known about PKA in β-adrenergic signalling in skeletal muscle cells. Although PKA is a well-known signalling molecule and skeletal muscles are an important organ in several respects, much remains to be discovered concerning this topic. The main issues at the moment are the lack of reliable methods to show PKA-activation and the need for more selective PKA-inhibitors.

5.3. Epac
For long PKA was believed to be the only effector downstream of cAMP. However, in the late 90’s another cAMP-target was discovered: the exchange factor directly activated by cAMP (Epac) (de Rooij et al. 1998; Kawasaki et al. 1998). Epac is a guanine-nucleotide-exchange factor (GEF) that can activate Rap1 and Rap2, small Ras-like G-proteins involved in cellular functions such as proliferation, differentiation, apoptosis and adhesion. Binding of cAMP to Epac induces a conformational change, so that the GEF-domain is exposed and thus able to activate the downstream G-proteins. There are three Epac isoforms: Epac1, Epac2A and Epac2B. Epac1 is ubiquitously expressed and is thus found in skeletal muscle while Epac2 is not (Kawasaki et al. 1998). Epac1 contains one cAMP-binding site while Epac2 contains two. The second cAMP-binding site in Epac2 is however not needed for activation, at least not in vitro (de Rooij et al. 2000). Another difference is that Epac1 can be translocated to the plasma membrane upon activation and although Epac2 may also be present there, this is
not dependent on cAMP (Ponsioen et al. 2009). Except these, there are no major functional differences observed between the two isoforms.

Although PKA is often described as the major mediator of cAMP-dependent signalling, there are also data showing Epac to be involved in adrenergic signalling in skeletal muscles. For example, epinephrine enhances insulin-stimulated Akt-phosphorylation in rat soleus and in EDL this is promotes also in the absence of insulin (Baviera et al. 2010; Brennesvik et al. 2005). In both systems, the Akt-phosphorylation seems to be independent of PKA since it was neither blocked by the classical PKA-inhibitor H-89 nor mimicked by the PKA-selective 6-Bnz-cAMP (Baviera et al. 2010; Brennesvik et al. 2005). Instead the Epac-selective cAMP analogue 8-CPT-2Me-cAMP was able to induce Akt-phosphorylation (Baviera et al. 2010; Brennesvik et al. 2005).

Thus, the only physiological β-adrenergic endpoint in which Epac is involved in skeletal muscle cells is inhibition of proteolysis. According to our studies, neither glucose uptake nor inhibition of contractility can be promoted by Epac-activation (Paper III, Paper II). One has however to consider that there are much fewer studies dealing with Epac- than PKA-signalling in muscles, why it is possible that Epac can be involved in other β-adrenergic endpoints which have not been examined yet. The β-adrenergic inhibition of glycogen synthase can for example be mimicked by cAMP but is not reduced by the PKA-inhibitor H-89 (Liu et al. 2000), indicating another cAMP-effector that might be Epac.

The lack of pharmacological inhibitors is a major limitation in studies of Epac-signalling, leaving selective cAMP-analogues as the best way to show Epac-dependence. There are however inhibitors for the downstream effector Rap1, but since Rap1 can be activated also by other factors, this is not a reliable method to prove the role of Epac.

Another circumstance that makes the role of Epac more difficult to elucidate is its different possible relations to PKA. One can imagine Epac and PKA to occur in the same pathway, acting either redundantly or synergistically. This seems to be the case with inhibition of proteolysis since this is induced by both Epac- and PKA-selective analogues (Baviera et al. 2010). It is also possible that these two effectors act independently of each other and regulate distinct endpoints.

A third alternative is that PKA and Epac may oppose each other, so that one of them fine-tunes the signal of the other. In skeletal muscles, there are indications of the latter mechanism: the PKA-inhibitor H-89 is found to enhance the effect of epinephrine or cAMP on Akt-phosphorylation (Baviera et al. 2010; Brennesvik et al. 2005). Inhibition of PKA is also shown to left-shift the dose response curve for glucose uptake, indicating a negative role for PKA (Nevzorova et al. 2006). Our current results indicates glucose uptake not to be Epac-dependent, but this results still indicates the possibility that different molecules downstream of the same receptor may oppose each other.
5.4. Other GEFs
It has been suggested that also GEFs other than Epac may act downstream of β-ARs. This is somewhat controversial since there are no solid proofs for such effectors. There are however some indications that it may happen: In vascular smooth muscle cells glucose uptake is activated by β-ARs independent of PKA and Epac (Kanda et al. 2007). In the same system, downregulation of Gs by long-term cholera toxin treatment inhibited the glucose uptake, indicating cAMP to be involved. Pharmacological inhibition of Rap1, the major downstream target of Epac, also blocked the glucose uptake, a finding that made the authors formulate the hypothesis of a new GEF regulating Rap1 and by this regulating glucose uptake. Of note is that in L6-myotubes, Rap1-inhibition could not diminish β-adrenergic stimulated glucose uptake (unpublished data) indicating differences in signalling between smooth muscles and skeletal muscles cells in the respect of adrenergic regulation of glucose transport.

5.5. Ion-channels
The third accepted target of cAMP, after PKA and Epac, is ion-channels which are regulated by direct binding of cAMP. This is a well characterized phenomenon in two type of ion-channels: the cyclic nucleotide-gated channels (CNGs) and the hyperpolarisation-activated cyclic nucleotide-gated channels (HCN) (reviewed in (Biel 2009)). CNGs are important for sensory signal transduction from the eye and the olfactory bulb while HCNs are present in heart and in neurons. Although neither CNGs nor HCNs are expressed in skeletal muscles, a similar mechanism may still occur; In Paper II we discovered that β-adrenergic signalling can inhibit cellular contractility in L6-myotubes (as described in section 3.2) and examination of this pathway showed that it is dependent on cAMP as it was both mimicked by 8-bromo-cAMP and the AC-activator forskolin and the effect of isoprenaline was inhibited by AC-inhibition. The β-adrenergic effect on contractility was blocked by the K+-channel inhibitor tetraethylammonium and mimicked by the K+-channel activator pinacidil (Figure 8).

Interestingly the isoprenaline effect was neither blocked by PKA-inhibitors, nor mimicked by Epac analogues, suggesting cAMP to act directly on ion-channels. There are several papers showing cAMP to induce K+-efflux from atrial smooth muscles (Ahn et al. 1995; Bieger et al. 2006; Fujii et al. 1999; Nakashima et al. 1995). In one of these papers (Bieger et al. 2006) the effect was shown to be mediated by PKA, while others only showed cAMP-dependence. Since older papers often state PKA-dependence based on the fact that cAMP is involved, the contribution of ion-channels in β-adrenergic effects may be more common than what is known today, simply because the question is too rarely asked.
K+-channels are necessary for β-adrenergic inhibition of contractility in L6-myotubes. A quantitative detachment assay was developed in which L6-cells were kept in trypsin-EDTA for 20 min and then the detached cells in the supernatant were counted. Control = unstimulated cells kept in trypsin-EDTA for 20 min, iso = cells pre-treated with 1 µM isoprenaline for 30 min before exposure to trypsin-EDTA in the continuous presence of isoprenaline. In (A) black bars represents cells pretreated with 100 mM of the K+-channel blocker tetraethylammonium (TEA) for 10 min prior to stimulation with isoprenaline of the concentration 1 µM (iso) or 1 nM (iso [10-9]). In (B), pre-treatment with the K+-channel opener pinacidil (100 µM) mimicked the effect of isoprenaline.

Of note is that cAMP can inhibit contraction in endothelial cells by acting upstream of RhoA. This effect could not be blocked by PKA-inhibitors, nor was RhoA phosphorylated, suggesting a PKA-independent mechanism (Essler et al. 2000). In the light of our results, it is possible that this mechanism is dependent on K+-channels.

Taken together there are many cAMP-dependent effects in skeletal muscles and these involve different downstream effectors: PKA affects inhibition of proteolysis, lipolysis and ion-transport (Baviera et al. 2010; Watt et al. 2006; Johnson et al. 1997) while Epac is involved in inhibition of proteolysis and direct activation of ion-channels affects contractility (Baviera et al. 2010; Paper II).

Before proceeding to the cAMP-independent signal, the phenomenon of desensitisation needs to be discussed. The reason for this is that there are molecules which participate in desensitisation of cAMP-production and at the same time start other signals.

**6. Desensitisation**

That a signal can be turned off or down-regulated, although the stimulus remains, is called desensitisation. Desensitisation is a well-studied aspect of β-AR signalling. Interestingly, molecules that are involved in ending the cAMP-signal are at the same time found to be a part of other signalling pathways, which are
described in later sections. For this reason, the desensitisation of β-ARs will be discussed before the cAMP-independent pathways.

Desensitisation of β-ARs can occur at several levels. Firstly, the receptor can, upon ligand binding, change conformation to a low-affinity state which is less prone to bind to the ligand. Secondly, there is a coupling switch from Gs to Gi, which is stimulated by PKA-mediated phosphorylation of the intracellular parts of the β2-AR. Thus the production of cAMP will decrease. There is also a constant reduction of cAMP by phosphodiesterases (PDEs) that hydrolyses cAMP into adenosine-5'-monophosphate (5'-AMP). cAMP can also be extruded from the cell, either via diffusion or via transport by members of the multidrug-resistance–associated proteins such as MRP4 (Sampath et al. 2002). Thirdly, the c-terminal tail of β2-ARs can be phosphorylated by the G-protein coupled kinases (GRKs), an event that leads to binding of β-arrestins followed by internalisation of the receptor. In addition to these effects there is a more long-term aspect of desensitisation occurring due to reduced receptor expression.

Desensitisation of β-ARs has been described in skeletal muscles: In L6-cells β2-ARs are converted into a low-affinity conformation after only minutes of agonist exposure (Pittman et al. 1980; Pittman et al. 1983) and after 10 min stimulation less β2-ARs are found in the plasma membrane, indicating receptor internalisation (Hardin et al. 1999). In the same system cAMP-levels start to decrease after 30 min of continuous β-AR agonist exposure (Nevzorova et al. 2006). An interesting finding from L6-cells is that pre-treatment with the PKA-inhibitor H89 blocks the decline in receptor density after 1 and 4 h of agonist exposure (Hardin et al. 1999) suggesting a role for PKA in receptor internalisation in this system. In whole animal systems, long term treatment with β-AR agonists leads to decreased levels of β2-AR in skeletal muscle cells of rat and guinea pigs (Elfellah et al. 1990; Kim et al. 1992; Rothwell et al. 1987; Sillence et al. 1991) and the ability to produce cAMP in response to acute β-AR agonist treatment is lost in soleus from rats after being injected with isoprenaline by osmotic pumps for 2 weeks (Roberts et al. 1998).

Thus one can conclude that desensitisation is a common feature of β2-ARs signalling also in muscle cells. The effect of this on different physiological endpoints is however more difficult to evaluate, since it is possible that a β-AR effect may decline for other reasons than receptor desensitisation. For example, the increase in muscle mass due to clenbuterol feeding in rats is attenuated after 2 weeks (McElligott et al. 1989) but this could either be due to receptor desensitisation or to other factors limiting the speed of growth. The same concern can be raised about desensitisation of β-adrenergic regulated glucose uptake. We raised the question about how the glucose uptake in muscle cells is affected by prolonged β-adrenergic stimulation and to test this,
differentiated L6-myotubes were kept for 4 days in medium with or without 1 µM isoprenaline. When glucose uptake was measured in these cells, the cells kept in isoprenaline had lost their ability to respond to acute β-adrenergic stimulation (Figure 9, unpublished data from Anna Sandström). This could potentially be due to receptor desensitisation but since the basal levels of glucose uptake were elevated by the 4-day treatment, it could just as well be that the maximum glucose uptake is already reached. One could for example imagine the numbers of GLUTs present in the cell or on the cell surface to be a limiting factor. In rats, chronic treatment with BRL37344 either decrease or totally abolish the response to acute BRL37344 injection in several muscles, but also in this system the basal glucose uptake was more or less elevated, complicating the possible interpretation of the results (Liu et al. 1995). Thus, there can be a loss of acute β-adrenergic response due to other changes in the cell than desensitisation of the receptor itself.

**Figure 9.** Chronic treatment with β-AR agonist decreases the acute effect of β-AR stimulation on glucose uptake in L6-myotubes. Cells were either kept in normal differentiation medium (basal) or in medium supplement with 1 µM isoprenaline for 4 days (4 d iso, black bars) before challenged with isoprenaline for 2 h (iso 2 h).

In conclusion, desensitisation of β-ARs in muscles is shown both in cell lines as well as in whole animals. The present results are obtained with synthetic ligands and the prevalence of this effect in the body during physiological circumstances is not known. It is possible that the endogenous ligands are rarely present long enough, in high enough concentrations, to actually induce desensitisation. In either case, the phenomenon of desensitisation has high relevance for the potential use of β-ARs as drugs targets, since it affects the possibility of long-term use.
7. cAMP-independent effects
Although cAMP for long was considered almost synonymous with β-adrenergic signalling, there is a growing body of evidence for cAMP-independent events downstream of β-ARs.

It is important to understand that there are several signals from the same receptor and that these can be either totally independent or linked in one way or another. In this thesis the term cAMP-independent will be used concerning pathways that do not directly involve cAMP, although these pathways may still be correlated to (being either modifying or modified by) cAMP.

The cAMP-independent signals can either involve G-proteins or be G-protein-independent. Examples of the former are signalling via the βγ-subunits and coupling to Gi, while the latter includes phosphorylation of the receptor by GRKs and subsequent β-arrestin binding (Figure 10). These effectors and their possible role in skeletal muscles will be discussed in the following sections.

![Diagram](image)

**Figure 10.** β2-ARs can activate signals other than cAMP, either via Gi or via totally G-protein-dependent pathways such as GRKs and β-arrestins.

7.1. Coupling to Gi
The major G-protein associated with β-ARs is Gs, but also Gi, can couple to β-ARs. This is in concordance with the general insight in this field that GPCRs tend to be somewhat promiscuous in their G-protein coupling. A switch in coupling from Gs to Gi is promoted by PKA-mediated phosphorylation of the β2-AR c-terminal tail (Daaka et al. 1997a), indicating Gi-dependent effects to occur secondary to cAMP-signalling. Coupling to Gi will both lead to inhibition of the cAMP-signal as well as inducing new effects. Examples of Gi mediated signals downstream of β2-ARs are activation of MAPK-signalling (Daaka et al. 1997a) and relaxation of endothelial cells (Ciccarelli et al. 2007). In rat cardiomyocytes the adrenergic effect on contractility can also involve Gi to bigger or smaller extent depending on ligand (Woo et al. 2009). In the same study, activation of Giα
was nicely shown by labelling active $G_\alpha$ with $^{32}\text{P-GTP}$ followed by immunoprecipitation of the different $G_\alpha$-subtypes.

The involvement of $G_\text{i}$ in $\beta$-AR signalling in skeletal muscles is indicated in rat soleus where the $G_\text{i}$-inhibitor pertussis toxin (PTX) diminished the effect of $\beta$-AR activation on ion-transport via NKCCs (Gosmanov et al. 2002). The PTX-treatment also inhibited phosphorylation of the extracellular signal regulated kinases 1 and 2 (Erk1/2), indicating this molecule to be downstream of $G_\text{i}$. In the same paper an interesting difference between muscle types was shown: in soleus both 8-bromo-cAMP and the two AC-activators forskolin and cholera toxin mimicked the isoprenaline effect while this was not the case in plantaris muscles (Gosmanov et al. 2002), indicating the heterogeneity amongst different muscles.

$G_\text{i}$ could possibly be involved in $\beta$-AR-mediated glucose uptake in muscle cells since this was blocked by PTX in L6-myotubes (Nevzorova et al. 2006). The PTX treatment did however reduce also the effect of insulin, which acts via a receptor that does not activate G-proteins, indicating that PTX might have unspecific effects. As most results on $G_\text{i}$-activation are obtained by the use of PTX, the effect on insulin signalling is troublesome since it indicates PTX to give undesired effects. Nevertheless, $G_\text{i}$ is likely to be involved in the regulation of NKCC and possibly also other effects downstream of the $\beta_2$-AR in skeletal muscles.

### 7.2. Signalling via the $\beta\gamma$-subunit

Upon activation of GPCRs, the associated G-protein dissociates into one $\alpha$- and one $\beta\gamma$-subunit (Figure 11).

The classical idea was that only the $\alpha$-subunit could signal to downstream effectors, which in the case of $\alpha$, would be AC, but there are also indications of signalling via the $\beta\gamma$-subunit. The $\beta\gamma$-subunit from both $G_\text{s}$ and $G_\text{i}$ can regulate $\text{Ca}^{2+}$- and $\text{K}^+$- ion-channels (Dascal 2001), but the major signalling effect of the $\beta\gamma$-subunit is to promote receptor phosphorylation via GRKs (which will be further discussed in the following section). Experiments performed in purified lipid bilayers show that the $\beta\gamma$-subunit can induce GRK-mediated phosphorylation of the $\beta_2$-AR. This was inhibited by the presence of the $\alpha$-subunit, suggesting that the $\beta\gamma$-subunit needs to be dissociated from the $\alpha$-subunit in order to interact with GRKs (Pitcher et al. 1992).
Direct interaction between the βγ-subunit and the c-terminal part of GRK has been shown both by pulldown assay and binding assays with radiolabelled GRK (Kim et al. 1993; Pitcher et al. 1992). Since the presence of the βγ-subunit is shown to increase presence of GRKs on the plasma membrane, both in a reconstituted lipid bilayer and in an over-expression cell system, the effect of the βγ-subunit is most likely to localize GRK to the β2-AR (Daaka et al. 1997b; Pitcher et al. 1992). This hypothesis is supported by the finding of GRK2 translocation to be inhibited by β-ARKct, a peptide corresponding to the c-terminal tail of GRK2 that blocks the interaction between βγ and GRK (Daaka et al. 1997b). Of note is that βγ-signalling downstream of β2-ARs is most often associated with G\(_i\) but there can also be effects of βγ from G\(_s\). This is for example the case in endothelial cells where βγ\(_s\) is shown to induce relaxation (Ciccarelli et al. 2007).

There is little or no evidence of βγ-dependent signalling downstream of β2-ARs in skeletal muscle cells. There are however indications that G\(_i\) and GRK, molecules often associated with βγ, could be involved in β-adrenergic signalling in muscle cells (Gosmanov et al. 2002; Nevzorova et al. 2006; Paper III). The lack of results about βγ-signalling, in skeletal muscles as well as in other tissues, can have several reasons. One is methodological issues; The only accepted βγ-inhibitor is the β-ark-ct peptide which corresponds to the GRK2 and can sequester βγ (Koch et al. 1994). Since this peptide needs to be transfected into the cells, it is not possible to use it in cells that are too difficult to transfect. In the GPCR-field, there has been more focus on G-protein independent pathways than on atypical G-protein signalling. If this mirrors what is actually happening inside the cells, or rather depends on the current interest of scientists in the field, is hard to determine.

**Figure 11.** (A) β2-ARs are, like all GPCRs, bound to a G-protein consisting of α-, β- and γ-subunits. (B) Upon agonist binding, the receptor activates the G-protein by facilitating the exchange of GDP to GTP. (C) This leads to dissociation of the subunits.
7.3. GRKs and β-arrestins

There are signals downstream of β-ARs that do not involve any G-protein. This phenomenon occurs at several GPCRs which has led to the use of other names for this class of receptors like seven-transmembrane receptors (7TMRs) or heptahelical receptors. G-protein independent signalling can be induced by phosphorylation of the receptor by the GRKs. There are seven identified GRK isoforms named GRK1-7. Of these GRK1 and GRK7 are expressed only in the retina while GRK4 has the highest expression in testes and the other isoforms are more or less ubiquitously expressed. GRK1 and GRK7 are selective rhodopsin kinases while the others can act on different receptors including on β2-ARs. Several GRK isoforms are detected in muscles: GRK2 and GRK5 (but not GRK3, GRK4 or GRK6) were detected with western blot in rat soleus and EDL (Jones et al. 2003) and in L6-cells we found expression of GRK4 and GRK6 as well when analysing this with PCR (Paper III).

The GRKs are considered to be constitutively active and are able to phosphorylate several sites on the β2-AR upon the conformational change of the receptor which occurs during agonist binding. GRK4, GRK5 and GRK6 are constitutively associated with the plasma membrane while GRK2 and GRK3 can be recruited by Gβγ-subunits. Thus GRK2 and GRK3 are linked to G-protein activity while the other GRK-isoforms are considered to be totally G-protein independent. Although PKA may increase GRK2 activity (Murthy et al. 2008) it is doubtful if cAMP/PKA has any significant role for GRK-mediated phosphorylation of β-ARs. Of the different G-protein dependent mechanisms, the βγ-subunit is the most likely to stimulate GRK-signalling, as described in the previous section.

GRK-mediated phosphorylation of the β2-AR facilitates binding of β-arrestins. This event leads to internalisation of the receptor and desensitisation of the cAMP response. Although PKA is involved in some parts of receptor desensitisation, it is only when the β2-AR is phosphorylated by GRK that β-arrestins are able to bind and inhibit the GTPase activity of the receptor (Lohse et al. 1992).

There are four arrestin isoforms of which two are found to interact with the β-ARs. These are named arrestin2 and arrestin3 or, more frequently used, β-arrestin1 and β-arrestin2. Both are considered to be ubiquitously expressed and consequently both were detected in the L6-cells by western blot (our unpublished data). Binding of β-arrestin to the receptor can in addition to the effect on internalisation act as a scaffold for other proteins, for example Src (Luttrell et al. 1999).

ERK1 and 2 are found to be regulated by β2-ARs via two mechanisms in HEK-293-cells: one fast G-protein dependent and one slower dependent of GRKs and β-arrestins (Shenoy et al. 2006). Furthermore isoprenaline treatment can induce translocation of both β-arrestin and c-Src to the β2-ARs and an interaction between β-arrestin and c-Src is necessary for phosphorylation of Erk1/2 (Luttrell et al. 1999).
The role of the different GRK isoforms for β2-AR phosphorylation and β-arrestin binding has been investigated in several papers. Silencing GRKs with siRNA in HEK-293 shows that both GRK2, GRK3, GRK5 and GRK6 can be responsible for β2-AR phosphorylation and the biggest effect was seen with GRK2 (Violin et al. 2006). In concordance with these results, Nobles et al. showed that GRK2 is responsible for phosphorylation of six amino acid residues of the β2-AR while GRK6 phosphorylates only two residues (Nobles et al. 2011).

The different phosphorylation-patterns of GRK2 and GRK6 reflect further events: silencing of GRK2 altered the conformational change of β-arrestin upon receptor interaction while GRK6 silencing did not affect this. Consequently, GRK2 had bigger effect than GRK6 on Erk-phosphorylation (Nobles et al. 2011). The GRK isoforms differ as to what extent they enhance the speed of β-arrestin recruitment. The relative contribution of the different isoforms varies between cell types: while GRK2 and GRK6 has significant effects on the speed of β-arrestin recruitment in HEK-293 cells, in U2-OS cells (a human osteosarcoma cell line) this was instead dependent on GRK2, GRK3 and GRK5, with the biggest effect of GRK5 (Violin et al. 2006). Taken together, these results show that the GRK isoforms vary in their effects on β2-AR, that this system is partly redundant and that the relative contribution of the different isoforms may differ between cell types.

When looking into skeletal muscle systems there are fewer findings of the GRK or β-arrestin signalling. Results from C2C12-cells tell however that association between β2-AR and β-arrestin occurs after 5 min of agonist stimulation and remains for over 1 h (Carter et al. 2005). Erk1/2 is phosphorylated upon β-adrenergic stimulation in both soleus and plantaris muscles from rat (Gosmanov et al. 2002; Wong et al. 2001), but whether this is due to β-arrestins is not certain since also other signals, including cAMP-dependent ones, can affect Erk-phosphorylation.

In Paper II we examine the role of GKR2 in β-adrenergic glucose uptake. When we by pharmacological inhibition of AC discovered that cAMP is not responsible for more than a part of the β-adrenergic stimulated glucose uptake, we chose to investigate the possible involvement of GRK2 since this molecule previously has been shown to regulate GLUT4 trafficking in CHO-cells (Dehvari et al. 2012). Excitingly, knocking down GRK2 in L6-myotubes significantly blocked the response to isoprenaline, indicating GRK2 to be necessary for the β-adrenergic stimulation of glucose uptake (Figure 12). There are few data concerning the physiological role of GRK2 in skeletal muscles and our current data suggest a novel role for this kinase by showing it to be involved in the regulation of glucose uptake.
Figure 12. GRK2 is necessary for β-adrenergic stimulation of glucose uptake. Measurement of glucose uptake in L6-myotubes transfected with siRNA against GRK2 (black bars) revealed that removal of GRK2 abolishes the β-adrenergic effect on glucose uptake (n=3).

In conclusion, skeletal muscle expresses both β-arrestins and several GRK-isoforms, but there are few results about their function in this system. While β-arrestins have not been associated with any β-adrenergic endpoint in skeletal muscle cells, our current results show that GRK2 is necessary for β-adrenergic stimulation of glucose uptake in myotubes.

8. Signalling bias
As described in previous chapters, there is a diversity of both intracellular signals and endpoints downstream of the β2-AR in skeletal muscle cells. The traditional view of GPCR signalling was that the receptor could switch between only two states, being either active or inactive. According to this model, the active form, which is stabilized or induced by any agonist, could promote all the downstream effects equally. There is however emerging evidence that this model is incomplete. Instead, the β2-AR is found to occur in multiple conformations and these are differently stabilized by different ligands (Kahsai et al. 2011). Consequently, ligands can differ in their ability to regulate specific pathways and endpoints. There are several expressions used for this phenomenon and the terminology is unfortunately somewhat inconsistent, making a complex matter even more confusing. It has been referred to as ligand-directed signalling, stimulus trafficking, functional selectivity, collateral efficacy, ligand bias, biased
agonism, ligand-directed signalling bias or signalling bias. In this thesis however, the term *signalling bias* will be used. A key expression in the field of signalling bias is *reversal of efficacy*. This is when the effects of two different ligands are tested for two distinct responses and one drug is found to have the highest efficacy for the first endpoint while the other drug has the highest efficacy for the second endpoint. Though reversal of efficacy is a clear version of signalling bias, there are also other types. For example, a receptor may not couple totally equally to different pathways. This means that a partial agonist may only activate the strongest coupled pathway while a full agonist can activate all pathways. How well the receptor is coupled to different downstream effectors can differ between cell types, giving rise to what is called *cell-based functional selectivity* or *conditional efficacy*. Strength of coupling may also depend on further parameters, such as levels of confluence or if the cells are grown on a surface or in suspension (Kaya et al. 2012).

A ligand may thus act as a full agonist on one pathway and at the same time be a partial agonist or even an antagonist for another. In this perspective it gets difficult to categorize ligands as agonists, antagonists etc. for the receptor. Instead these expressions should rather describe the effect of a ligand on a specific endpoint.

The phenomenon of signalling bias is of great interest from a pharmaceutical perspective. Today almost half of all prescription drugs target GPCRs. Using biased ligands which exclusively activate the intracellular pathway that gives the beneficial effect could be used to increase efficacy and reduce side effects of such drugs.

### 8.1. Signalling bias at the β2-AR

Signalling bias is shown to occur on the β2-AR at the level of G-protein coupling (Wenzel-Seifert et al. 2000; Woo et al. 2009) and, what is more well documented, on cAMP versus the β-arrestin pathway (Wisler et al. 2007). Furthermore different ligands can induce distinct changes in β-arrestin conformation (Shukla et al. 2008).

When comparing the effects of β-AR ligands on cAMP-accumulation and Erk1/2-phosphorylation, of which the latter is used as marker for β-arrestin signalling, some ligands are found to affect both pathways equally: isoprenaline and labetolol are agonists for both pathways while metoprolol, biosprolol and atenolol are inverse agonists (Galandrin et al. 2006). Other ligands exerted different or even opposing effects, being agonistic for MAPK but antagonistic (carvedilol and buncindolol) or even inversely agonistic (propanolol) for cAMP-formation (Galandrin et al. 2006). Because of its promising effects on heart failure, carvedilol is the most well studied compound with regard to biased agonism on the β2-AR. Carvedilol has been described not to affect cAMP-production while it at the same time stimulates receptor binding to β-arrestins, thus inducing receptor
internalisation and Erk1/2 phosphorylation in both HEK-cells and cardiomyocytes (Galandrin et al. 2006; Wisler et al. 2007). This feature of carvedilol is believed to contribute to its efficacy in the treatment of myocardial dysfunction. Interestingly, the two endogenous AR-ligands, epinephrine and norepinephrine, are shown to have dispersed actions on the β2-AR: in cardiomyocytes the response to epinephrine is found to be PTX-sensitive while the response to norepinephrine is not, indicating only epinephrine to promote the G_i-dependent pathway (Wang et al. 2008). In this system the β2-ARs were also found to be recycled faster after epinephrine than after norepinephrine treatment (Wang et al. 2008).

Most data concerning signalling bias at the β2-AR is obtained from non-muscle cell lines or from cardiomyocytes, but there are also data from skeletal muscle cells. In C2C12-cells, terbutaline and salbutamol stimulate cAMP-production to similar levels as isoprenaline, whereas it induces β2-AR/β-arrestin-interactions to a markedly lesser extent (Carter et al. 2005). Furthermore, salmeterol is shown to act as a full agonist for cAMP-accumulation while having antagonistic effects on the β2-AR/β-arrestin-interaction (Carter et al. 2005). In mouse soleus signalling bias is found on the physiological endpoint of glucose uptake: Treatment with 10^{-8} M BRL37344 leads to increased glucose uptake while 10^{-7} M clenbuterol has the opposite effect (Ngala et al. 2009). Both effects are blocked by β2-antagonists and are abolished in β2-KO why they indeed are confirmed to be mediated by the β2-receptor (Ngala et al. 2009).

We found BRL37344 to be of further interest since it can increase glucose uptake in rat soleus (Abe et al. 1993; Liu et al. 1995; Liu et al. 1996; Ngala et al. 2008; Ngala et al. 2009) while it in the same model system has been reported not to affect cAMP-levels (Roberts et al. 1998). Thus, the effect of BRL37344 on the L6-cells was examined in Paper III. Also in this system BRL37344 was found to be a very poor agonist for cAMP and yet being able to induce glucose uptake significantly (Figure 13).

Furthermore, in contrast to isoprenaline, BRL37344 did not phosphorylate the PKA-substrate CREB and its effect on glucose uptake could not be blocked by the AC-inhibitor SQ22563 (Paper III). By pharmacological inhibition we show BRL37344 to indeed act via β2-ARs. Furthermore, transfection with siRNA against GRK2 inhibited the effect of BRL37344. Thus, while isoprenaline activated both cAMP and GRK2-depednent signals, BLR37344 only activates the latter. From these data we concluded that β2-ARs can activate a different subset of signals dependent on which ligand is activating the receptor.
Figure 13. Isoprenaline and BRL37344 exert the same effect on glucose uptake while inducing different cAMP-responses. Measurement of cAMP (A) and glucose uptake (B) in L6-myotubes in response to increasing concentrations of isoprenaline or BRL37344 at the timepoint 2 h (n=3).

9. *In vivo* effects
There are two main questions concerning the effects of β-AR stimulated glucose uptake in skeletal muscles in a whole animal system: What is the physiological role of this and can this be used pharmacologically?

Firstly, little is known when, how or even if, β-adrenergic regulation of glucose uptake occurs in the body. Epinephrine, which is more potent on the β2-AR than norepinephrine (MacGregor et al. 1996), is released during acute physical or psychological stress. During such circumstances it is logical that the muscles need fuel for “fight or flight”. Norepinephrine is on the other hand released during every-day life to control homeostasis. As previously mentioned, glucose uptake is increased in skeletal muscles after artificial activation of sympathetic nerves (Shimazu et al. 1991; Sudo et al. 1991), showing that such nerves can indeed regulate glucose uptake in this tissue. Norepinephrine can be released with high resolution in time and space – an example of this is noradrenergic regulation of the heart that is very precise. It could thus be possible that sympathetic nerves may fine-tune glucose uptake in muscles according to their need for fuel. This hypothesis is consistent with the fact that norepinephrine output is increased in muscles during exercise, independent of increased blood flow (Savard et al. 1989). It is well known that muscle contraction in itself leads to increased glucose uptake independent of AMPK and it might be possible that norepinephrine release is a part of this phenomenon.

The second question is if β-AR agonists could be used to regulate blood glucose levels, an effect that could be used in the treatment of type II diabetes. The role of skeletal muscles as a blood glucose clearing organ leads to the possibility of improving blood glucose levels in diabetic patients by inducing glucose uptake in
skeletal muscles. This idea is utilized by anti-diabetic drugs targeting AMPK which regulates several events including insulin-independent glucose uptake in skeletal muscles. Furthermore, in paper IV we show that the Shikonin, a component used in Chinese herbal medicine, can increase glucose uptake in skeletal muscle cells via GLUT4-translocation. This compound was also able to improve blood glucose levels in diabetic Goto-Kakizaki rats both in basal state and after insulin injection. These findings illustrate the role for insulin-independent glucose uptake as an adequate mechanism for glucose clearance.

Concerning the possible medical use of activating β-ARs, one need however to consider that there are effects, other than glucose uptake, that are regulated by β-ARs and that could have an impact on glucose metabolism. One such effect is increased glucose output from the liver which is affected by both β- and α-adrenergic stimulation (Vardanega-Peicher et al. 2000). Consequently, injection of epinephrine leads to elevated blood glucose levels in rat (Kansal et al. 1967). There may also be an adrenergic effect on insulin-release, although the results concerning this are somewhat inconsistent. In rats α- but not β-adrenergic, stimulation increases insulin secretion (Kansal et al. 1967; Lacey et al. 1991) while results from mice instead show that activation of β2-ARs induces insulin release (Ahren et al. 1981). Also the hormone glucagon, which acts antagonistically to insulin, is suggested to be released upon β-AR stimulation of pancreatic islets from mice (Lacey et al. 1991).

Despite that short term treatment with β-adrenergic agonist may increase blood glucose levels, long-term treatment has been found to have beneficial effects on glucose homeostasis. Oral administration of clenbuterol for 5 to 6 weeks resulted in increased glucose uptake in both muscles and fat and higher insulin and glucose tolerance in obese Zucker rats (Castle et al. 2001; Pan et al. 2001). In these studies it was not concluded if these effects were merely attributed to the change in body composition with increased muscle mass and decreased abdominal fat caused by clenbuterol treatment (Castle et al. 2001; Pan et al. 2001). However, a recent study in old C57BL/6 mice with high-fat induced diabetes showed that the β-agonist salbutamol improves glucose sensitivity (Elayan et al. 2012). In combination with exercise this treatment also leads to improved insulin sensitivity (Elayan et al. 2012). This is probably not only depending on changed body composition since exercise alone could not change glucose metabolism. Furthermore, in Paper III we injected β3-KO mice with BRL37344 twice a day for four days and found this treatment to significantly improve glucose clearance without changing body composition.

Activation of β-AR will affect several parameters in the body, which could cause negative side effects of systemic β-AR administration. Not only do the β-ARs induce growth of muscles but also of the heart which could lead to hypertrophic cardiomyopathy, meaning that the walls of the heart are thickened. Furthermore,
body builders that use β-AR agonists illegally often experience tachycardia (uncontrolled heart beating) and muscle tremor.

To overcome these problems more facts are needed about the intracellular pathway regulating the different endpoints in the distinct tissue. Such knowledge could make it possible to design drugs that activate one physiological endpoint exclusively, or at least give less undesired side effects. This could be done either by using biased ligands activating a specific function of the β2-AR or by using drugs acting on targets further down in the signalling cascade.

10. Summary and conclusions

Although skeletal muscles constitute the major part of the body and that activation of β-ARs due to sympathetic signalling occurs on daily basis, the effects of β2-AR activation in muscles have previously not been very well examined. The results presented in this thesis have added new information to this field, both concerning intracellular pathways as well as downstream effects. The main findings are illustrated in figure 14.

One important consequence of β-AR stimulation of muscle cells is glucose uptake. It has until now not been known which transporter-protein is mediating this uptake. In Paper I we discovered that β-adrenergic stimulation induces translocation of the insulin-sensitive transporter GLUT4. Furthermore, both pharmacological inhibitors and siRNA-technique showed GLUT4 to be vital for the β-AR stimulated glucose uptake. Thus the β-ARs utilize the same end mechanism for glucose transport as does insulin. Further examination of the upstream signal revealed that it shares some, but not all, features of the insulin signalling pathway. In contrast to insulin the β-ARs do not activate PI3K, PIP3, Akt or AS160. Instead we discovered that mTORC2 is a key regulator of both insulin- and β-adrenergic stimulated glucose uptake. This is very interesting since mTORC2 was previously not known to be activated by β-ARs.

In Paper II we discovered a new endpoint downstream of β2-ARs in L6-cells: inhibition of contractility. Further investigations of the signalling pathway showed that this is dependent on cAMP, but surprisingly not on the classical downstream effectors PKA or Epac. Nor was it dependent on PKG, PI3K or PKC. Instead, this specific signal involves K+-channels. There are other ion-channels that can be regulated by β-ARs, but this is the first time K+-channels are shown to be necessary for a β-AR effect in skeletal muscle cells.

Previous results concerning the possible role of cAMP in β2-AR stimulation of glucose uptake in muscle cells are somewhat ambiguous, why we aimed to investigate this further. Surprisingly, in Paper III we found that cAMP is only partly involved in β-adrenergic stimulation of glucose uptake. Instead we
discovered that the GRK isoform GRK2 is necessary for this process. This is the first time GRKs have been shown to regulate a physiological endpoint in differentiated muscle cells, why this is a very interesting finding. Furthermore, it opens up for the possibility to activate glucose uptake without simultaneously stimulating cAMP. Excitingly, we found that the β-AR ligand BRL37344 can activate glucose uptake via GRK2 without affecting cAMP. We showed that BRL37344 increases glucose uptake also in vivo in mice. Furthermore, injection of BRL37344 improved glucose clearance in glucose intolerant mice. Based on these data, it is possible that ligands with similar properties could be used to improve glucose levels in type II diabetes.

Paper IV shows that Shikonin, a compound found in the dried root of gromwell (Lithospermum erythrorhizon), used in traditional Chinese medicine, can induce glucose uptake in skeletal muscle cells. This occurred via an insulin-independent pathway involving increased levels of intracellular Ca^{2+} and GLUT4-translocation. Furthermore, we showed that Shikonin has beneficial effects in vivo by lowering blood glucose levels in diabetic Goto-Kakizaki-rats.

![Pathway Diagram](image)

**Figure 14.** In Paper I we show that activation of β_{2}-ARs can induce glucose uptake via a series of events involving cAMP, mTOR and GLUT4 (this signal is marked in green). Paper II describes how activation of β_{2}-ARs can inhibit contractility via cAMP and K^{+}-channels (pink). The work presented in Paper III shows that activation of β_{2}-ARs can activate glucose uptake also independently of cAMP and that this pathway involves GRK2 (yellow). In Paper IV we show that the compound Shikonin can increase glucose uptake in muscle cells via Ca^{2+} and GLUT4-translocation (blue).
Taken together, we have revealed new features of β-AR signalling by identifying both key molecules in the intracellular pathway as well as novel downstream effects. We have also shown that activation of glucose uptake in muscles, either via β-AR activation or via Shikonin, improves glucose homeostasis in diabetic animals. To understand what happens inside the cells and correlate this with the downstream effects is important basic knowledge, which also potentially could be used to develop new and more selective drugs. Our studies have contributed to a better understanding of this and our current knowledge is summarised in Table 2 with pink boxes indicating our novel findings.

In conclusion, β-AR signalling in muscle cells is a multifaceted phenomenon with important effects on both cellular and physiological levels. Our result gives new clues to understanding these complex events and could potentially be used in the search for new drugs to treat metabolic disorders such as type II diabetes.

**Table 2.** The correlation between different β-adrenergic effects and intracellular signalling pathways in skeletal muscle cells. Pink boxes indicate new findings presented in this thesis.

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<th>β-adrenergic effects</th>
<th>Glucose uptake</th>
<th>GLUTA translocation</th>
<th>Glycogen metabolism</th>
<th>Lipolysis</th>
<th>Inhibition of proteolysis</th>
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11. Sammanfattning på Svenska
Skelettmuskelnas viktigaste uppgift är att ge kroppen rörelseförmåga, men de är även viktiga för metabolismen. Till exempel har musklerna relativt stor påverkan på kroppens totala energiförbrukning. En annan viktig funktion är upptag av glukos (socker), vilket sänker glukoshalten i blodet. Eftersom skelettmuskulaturen är en så stor del av kroppen (viktmässigt är det kroppens största organ) kan även en liten ökning av glukosupptag leda till en märkbar sänkning av blodsockret.


I följande artiklar (Paper II och III) tittar vi närmare på vad som händer inuti en muskelcell efter aktivering av β₂-AR. Vi upptäckte att β-adrenerg aktivering hämmar kontraktionen av muskelcellerna och undersökte vilken signalväg som reglerar detta. Den vanligaste signalvägen som aktiveras av β₂-AR innehåller molekylerna cAMP och PKA. Vi upptäckte att den signalväg som påverkar kontraktionen är beroende av cAMP, men inte PKA. Istället är en viss typ av jonkanaler viktiga för just den här signalen (Paper II).

I Paper III fortsatte vi att undersöka den β-adrenergiska signalvägen till glukosupptaget. Vi upptäckte att cAMP enbart är en liten del av signalvägen och att
PKA inte är med alls. Istället fann vi att molekylen GRK2 är en nyckelapelare i den adrenerga regleringen av glukosupptag. Sammantaget visar dessa resultat att den klassiska cAMP-PKA vägen inte alls är så viktig i muskelceller som man tidigare trott. Att β-AR kan ge glukosupptag utan inblandning av cAMP är mycket intressant eftersom cAMP påverkar många olika funktioner, både i muskler och i andra organ. Till exempel kan frisättning av glukos från levern stimuleras av cAMP. Vi fann att en substans som heter BRL37344 kan aktivera β-AR på ett sätt som ger glukosupptag utan att påverka nivåerna av cAMP. Denna, eller liknande substanser, skulle alltså potentiellt kunna öka glukosupptaget i muskler utan att ge så många biverkningar. Spännande nog fann vi också att injektioner av BRL37344 förbättrade glukoshomeostasen i diabetiska möss.

I avhandlingens sista artikel (Paper IV) lämnar vi β2-AR och tittar istället på ett annat sätt att stimulera glukosupptag. Vi undersökte effekten av Shikonin, ett ämne som används inom traditionell kinesisk medicin. Tidigare studier har visat att Shikonin kan påverka glukosupptag i fettceller, men vi upptäckte att detta gäller även muskelceller och att dessa celler till och med är känsligare för Shikonin än vad fettceller är. Ytterligare försök visade att injektioner av Shikonin leder till förbättrade blodglukoshalter i diabetiska råttor.

Sammanfattningsvis har jag hittat nyckelmolekyler i den adrenerga signalvägen och har upptäckt att aktivering av β2-AR leder till flera olika effekter, exempelvis glukosupptag och inhibering av kontraktion, via distinkta signalvägar i muskelcellerna. Att förstå vad som händer inuti muskelcellerna efter β2-adrenerg aktivering och korrelera detta med olika cellulära och fysiologiska funktioner är viktig kunskap som kan vara till nytta vid exempelvis utvecklandet av nya behandlingar mot sjukdomar såsom typ II diabetes.
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