

# Assessment of AMPK-Stimulated Cellular Long-Chain Fatty Acid and Glucose Uptake

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## Assessment of AMPK-Stimulated Cellular Long-Chain Fatty Acid and Glucose Uptake

Joost J. F. P. Luiken, Dietbert Neumann, Jan F. C. Glatz, Will A. Coumans, Dipanjan Chanda, and Miranda Nabben

### Abstract

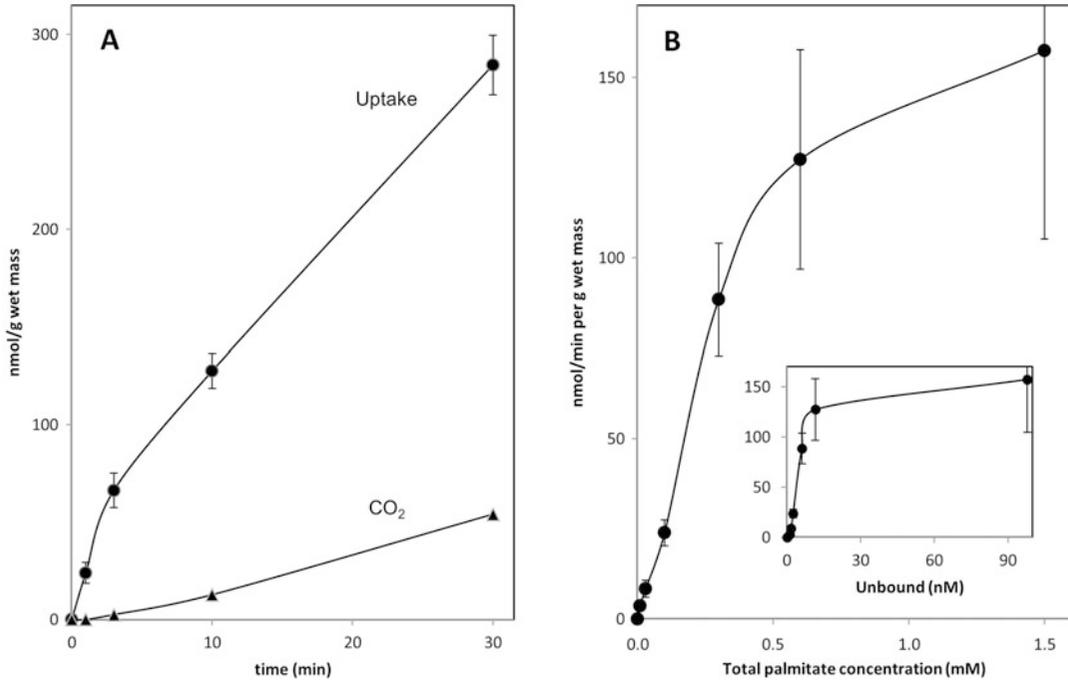
Here we describe an assay for simultaneous measurement of cellular uptake rates of long-chain fatty acids (LCFA) and glucose that can be applied to cells in suspension. The uptake assay includes the use of radiolabeled substrates at such concentrations and incubation periods that exact information is provided about unidirectional uptakes rates. Cellular uptake of both substrates is under regulation of AMPK. The underlying mechanism includes the translocation of LCFA and glucose transporters from intracellular membrane compartments to the cell surface, leading to an increase in substrate uptake. In this chapter, we explain the principles of the uptake assay before detailing the exact procedure. We also provide information of the specific LCFA and glucose transporters subject to AMPK-mediated subcellular translocation. Finally, we discuss the application of AMPK inhibitors and activators in combination with cellular substrate uptake assays.

**Key words** Long-chain fatty acid uptake, Glucose uptake, Initial uptake rate, AMPK inhibitors, AMPK stimuli, CD36 translocation, GLUT4 translocation

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## 1 Introduction

For the majority of mammalian cell types, LCFA and glucose are the most important substrates for cellular energy production. But in order to serve as energy source, both substrates need to be taken up across the plasma membrane. In the remainder of this chapter, we focus on cardiomyocytes, but the general principles of the cellular uptake process are likely to apply to most other mammalian cell types, as has been proven for skeletal muscle cells, adipocytes, hepatocytes, enterocytes, etc. Notably, as observed in cardiomyocytes and skeletal muscle cells, the crossing of the plasma membrane presents the rate-limiting step in the cellular metabolism of both substrates [1–3]. Once inside the cells, both substrates can be readily oxidized according to the metabolic needs of the cells but also stored in times of plenty. Based on its lipophobic properties,



**Fig. 1** Kinetics of palmitate uptake and utilization in rat cardiomyocytes. (a) Time course of palmitate uptake and utilization. Cardiomyocytes were incubated with palmitate bound to BSA at final concentrations of 90 and 300  $\mu\text{M}$ , respectively (palmitate/BSA molar ratio 0.3), and analyzed at the indicated time points on cellular uptake of palmitate (filled circle) and formation of  $\text{CO}_2$  (filled triangle). (b) Palmitate uptake as function of the exogenous palmitate concentration. Cardiomyocytes were incubated with varying concentrations of palmitate bound to BSA (final BSA concentration 300  $\mu\text{M}$ ) resulting in palmitate/BSA molar ratios ranging from 0 to 5. Cellular uptake of palmitate was determined at 3 min after substrate addition. In the inset, palmitate uptake is plotted against the unbound palmitate concentration, which is calculated from the palmitate/BSA molar ratio [14]. This figure is modified from Ref. [8]

glucose does not readily cross the plasma membrane, and membrane transporters have long been recognized to mediate the bulk of glucose uptake [4]. These transporters are mainly members of the GLUT family: integral membrane proteins that contain 12 - membrane-spanning helices. In contrast, due to their lipophilic properties, LCFA have been postulated to cross the plasma membrane solely via passive diffusion [5, 6]. Only in the late 1980s of the previous century, transporters emerged as key mediators of bulk LCFA uptake (e.g., see [7]). The latter notion followed from several lines of kinetic evidence, including saturation of cellular LCFA uptake (Fig. 1), and sensitivity to competitive substrates and to inhibitors of protein-mediated membrane transport [2]. However, LCFA uptake also displays a non-saturable component, which at physiological LCFA concentrations contributes only modestly to the uptake process, but increases in relative contribution at exceedingly high nonphysiological concentrations [7]. The uptake procedure described below is carried out at low and physiological LCFA concentrations as measured in human plasma [8].

## 1.1 Considerations of the Uptake Assay

### 1.1.1 Applicability

This protocol of measurement of cellular substrate uptake is dedicated to primary cells in suspension and is suitable for investigation of the short-term regulation of substrate uptake. Working with primary cells in suspension has the advantage to prevent major cell loss accompanying their maintenance in culture. When one is interested in long-term regulation of substrate uptake, as exerted by transcription factors and subsequent *de novo* synthesis of substrate transporters, use of primary cells attached to laminin-coated wells is a more appropriate model of study. In that case, we advise to use a modified version of this uptake protocol [9]. On the other hand, cultured cells can be detached from their plates and undergo the uptake protocol as described below.

### 1.1.2 Radioactive Tracers

The most sensitive method to measure substrate uptake is via radioactive tracers. Uptake is measured as cell-associated radioactivity, which can be determined upon centrifugation and subsequent washing of the cells after a fixed time of incubation with the radioactive substrates. The most commonly used LCFA tracer is [1- $^{14}\text{C}$ ]palmitate, but also [ $^{14}\text{C}$ ] or [ $^3\text{H}$ ] derivatives of oleate are often used, although these have the disadvantage of being considerably more expensive. The radiolabeled LCFA will be taken up into the cells mainly via LCFA transporters. Thereafter, they are intracellularly transported by small cytoplasmic fatty acid-binding proteins (FABP) to the cytoplasmic leaflet of the outer mitochondrial membrane for conversion into CoA esters by fatty acyl-CoA synthetase. This activation is needed for further metabolism, being oxidation within mitochondria, storage as triacylglycerols within lipid droplets and/or conversion into various other lipid species [3]. Depending on cell type and metabolic state, a variable portion of the fatty acyl-CoA will be destined for  $\beta$ -oxidation. Importantly, palmitate uptake needs to be measured during the initial uptake phase, when substrate uptake kinetics proceeds linear with time. Namely, beyond the initial uptake phase, efflux of the  $^{14}\text{C}$ -label will gradually increase as a result of equilibration of [1- $^{14}\text{C}$ ]palmitate with the endogenous non-labeled palmitate pools [8]. Hence, during the initial uptake phase, measurement of uptake provides unbiased information on unidirectional influx of palmitate. Another reason for a rapid measurement of palmitate uptake relates to a progressive loss of  $^{14}\text{C}$ -label due to  $\beta$ -oxidation. Specifically using [1- $^{14}\text{C}$ ]palmitate, the  $^{14}\text{C}$ -label at the C1 position will already disappear within the first round of  $\beta$ -oxidation, yielding a radioactive acetyl-CoA and a non-labeled myristoyl-CoA, of which the latter can no longer be traced. The radiolabeled acetyl-CoA subsequently enters the TCA cycle leading to  $^{14}\text{CO}_2$  production. This  $\text{CO}_2$  diffuses away from the cells and the liquid medium into the gas phase. Hence, the portion of  $^{14}\text{CO}_2$  produced from [1- $^{14}\text{C}$ ]palmitate escapes the radioactive detection and leads to underestimation of the uptake rates. In metabolically active primary

cardiomyocytes, this initial uptake phase amounts to 3–5 min after addition of the radiolabeled substrates (Fig. 1a) [8]. In most cardiac cell lines, the initial uptake rate is lower compared to primary cardiomyocytes and in the linear phase for up to 30 min [9, 10].

To obtain information about cellular glucose uptake, non-metabolizable glucose analogs such as radiolabeled 2-deoxyglucose (2-deoxy-D-[<sup>3</sup>H]glucose) are often applied, so that glucose uptake can be assessed independently of metabolism. Glucose and 2-deoxyglucose display similar uptake kinetics in cardiomyocytes (Luiken, unpublished). Whereas glucose is mostly used in uptake studies at physiological millimolar concentrations, it is advisable to use 2-deoxyglucose at submillimolar concentrations in order to minimize undesirable accumulation of 2-deoxyglucose-6-phosphate. The use of non-metabolizable analogs would also be advantageous for LCFA uptake studies. Yet, non-metabolizable LCFA analogs as 2-bromopalmitate [11] display much slower uptake rates due to the bulky bromo group and therefore do not reflect their naturally occurring counterparts. For the same reason, also iodo-fatty acids would display nonphysiologically low uptake rates. These iodo-fatty acids (e.g., 15-(p-iodophenyl)3(R,S)-methylpentadecanoic acid) are, however, suitable for PET imaging of myocardial metabolism [12].

### 1.1.3 LCFA-Albumin Ratios

Another complication with respect to measuring LCFA uptake is that this substrate is virtually insoluble in aqueous solutions [13]. In the mammalian circulation, LCFA are almost completely bound to albumin. In most cellular systems, high-affinity albumin binding sites have been found, which brings the LCFA-albumin complex in close proximity to the plasma membrane. Subsequently, the LCFA gain access to their membrane transporters. Hence, the cellular uptake of LCFA from the LCFA-albumin complex is an entire protein-mediated process. Conversely, dissolving radiolabeled LCFA in lipophilic solvents, such as DMSO, would lead to the partitioning of LCFA into the outer plasma membrane monolayer rather than being taken up by a physiological process involving membrane-binding proteins. Albumin has 6–8 binding sites for LCFA. When palmitate will be mixed with albumin at ratios exceeding 8:1, palmitate micelles will be formed. Such micelles will readily incorporate into membranes so that nonphysiologically high uptake rates will be recorded. An important point of consideration is that palmitate is taken up as a function of the “free,” i.e., non-albumin-bound palmitate concentration and not the total palmitate concentration [8]. At least in media with albumin concentrations >4 μM, the free palmitate concentration is entirely governed by the palmitate-albumin ratio [14]. Therefore in studies on fatty acid uptake regulation, the respective Materials and Methods sections (describing the uptake assay) should detail not only the total LCFA concentration but also the LCFA-albumin ratio. The physiological

LCFA-albumin ratio roughly varies between 0.3 and 3. Of further note, possible changes in LCFA uptake would be most sensitively detected at palmitate-albumin ratios well below the ratio at which the rate is half of  $V_{\max}$ , thus reflecting an apparent  $K_m$  (in analogy to Michaelis-Menten kinetics of enzyme reactions). As an example, in cardiomyocytes, palmitate-albumin ratios should be used below a ratio of 1.45, which amounts to the apparent  $K_m$  of palmitate uptake in this cell type (Fig. 1b) [8].

#### 1.1.4 Stop Procedures

Several stop procedures have been applied to terminate the uptake process, for instance, centrifugation of the cells through a layer of silicon oil [5]. This latter stop protocol can be applied to primary hepatocytes but is not suited for every cell type. For studies with cardiomyocytes, we have adopted a stop procedure from Sorrentino et al. [7]. This method brings together three different means contributing to stop radiolabeled substrate uptake: (1) dilution with excess cold substrate, (2) removal of LCFA that are loosely attached to the outer leaflet of the plasma membrane (but have not been taken up) by inclusion of albumin in the stop buffer, and (3) addition of phloretin, a nonselective inhibitor of carrier-mediated membrane transport processes [15].

#### 1.1.5 Limitations

The substrate uptake rates to be calculated from these single-cell suspensions will definitely differ from the *in vivo* uptake rates, because the cells are incubated in the absence of blood-delivered hormonal and any mechanical stimuli. In primary culture, cardiomyocytes are not stimulated to contract and, at the most, will display some irregular spontaneous contractions. But these occasional contractions do not compare with the metabolic demands of the *in vivo* contractions. Hence, *in vitro* uptake rates are expected to be at least a magnitude lower. But nonetheless, single-cell suspensions offer the opportunity to investigate the kinetics and influence of stimulating/inhibiting agents in multiple parallel incubations.

### 1.2 Regulation of Cellular Substrate Uptake by AMPK

The described protocol of cellular substrate uptake is designed for cells in suspension, which can be maintained in viable shape for periods up to 2 h. Hence only short-term regulation of substrate uptake by AMPK needs to be taken into account. Theoretically these would include posttranslational modification (e.g., phosphorylation) of substrate transporters or, alternatively, translocation of substrate transporters from intracellular stores to the plasma membrane. In particular, there is a wealth of evidence that AMPK modulates substrate uptake through the translocation of substrate transporters [3, 16, 17].

With respect to glucose uptake, mostly GLUT1 and GLUT4 have been investigated in relation to AMPK. Whereas AMPK regulation of GLUT1 has been reported to occur via direct activation at

the plasma membrane in clone-9 cells [18], GLUT1 activity in muscle cells is generally regarded as non-inducible, involved in merely basal glucose uptake [19]. On the other hand, there is ample evidence that GLUT4 translocation is the main mechanism by which AMPK regulates changes in glucose uptake [20]. In case of LCFA uptake, a number of structurally unrelated proteins have been proposed to facilitate LCFA uptake. These proteins include the peripheral membrane protein FABPpm, the channel-forming family of the FATPs, and the scavenger receptor CD36, also referred to as SR-B2, supposedly acting as a flippase [3]. Although FABPpm, FATP1, and FATP4, but not FATP6, appear to translocate to the cell surface upon AMPK activation in skeletal muscle cells [21], this has not been reproduced in cardiomyocytes [22]. It is now well established that regulation of LCFA transport by cardiac AMPK relies entirely on the translocation of CD36 to the cell surface, with a very similar molecular mechanism to that of GLUT4. Of note, the same upstream and downstream signaling components appear to be involved, thereby further reinforcing the similarity of both translocation processes. The upstream and downstream mechanisms involved in AMPK-mediated GLUT4 translocation have been intensively studied (e.g., *see* Ref. [20]). In short, upstream of AMPK, the kinases LKB1 and CaMKK- $\beta$  have been implicated, dependent on the cell type (LKB1 in the heart and CaMKK- $\beta$  in skeletal muscle). Downstream of AMPK, AS160 is the most important AMPK target leading to GLUT4 translocation via de-inhibition of specific Rab proteins [23]. Remarkably, AMPK-stimulated CD36 translocation in the heart and muscle shares the same upstream and downstream signaling components as AMPK-stimulated GLUT4 translocation, including LKB1, CaMKK- $\beta$ , and AS160 [24–26]. Hence the AMPK signaling pathway similarly regulates LCFA uptake and glucose uptake.

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## 2 Materials

### 2.1 Equipment

1. Shaking water bath (including temperature control and holders for 20-mL vials).
2. Magnetic stirrer.
3. Gas phase system of 95% O<sub>2</sub>/5% CO<sub>2</sub>.
4. N<sub>2</sub> gas.
5. Liquid scintillation counter ( $\beta$ -counter).
6. Glass  $\beta$ -counter vials.

### 2.2 Cells

7. Cell suspensions can be obtained as primary cells upon isolation from rodent organs (e.g., heart) or can be derived from cell cultures upon dis-attachment from the culture wells.

**2.3 Uptake Assay**

1. Ethanol.
2. DMSO.
3. KOH: 1 M KOH.
4. CaCl<sub>2</sub>: 100 mM CaCl<sub>2</sub>.
5. Bovine serum albumin (BSA)—Fraction V (fatty acid-free).
6. Palmitic acid.
7. [1-<sup>14</sup>C]palmitate.
8. 2-Deoxyglucose/2-deoxy-D-[<sup>3</sup>H]glucose mix: 10 mM 2-deoxyglucose and 2.2 μCi 2-deoxy-D-[<sup>3</sup>H]glucose.
9. AMPK activators and/or inhibitors.
10. OPTI-FLUOR liquid scintillation cocktail.

*Solutions to Be Prepared in Advance*

11. 10× MKR: 1.17 M NaCl, 26 mM KCl, 12 mM KH<sub>2</sub>PO<sub>4</sub>, 12 mM MgSO<sub>4</sub>, 100 mM NaHCO<sub>3</sub>, and 100 mM HEPES, pH 7.55. Use a 1 L *glass* cylinder or a *glass* beaker. Weigh the indicated amount of the following chemicals: 68.36 g of NaCl, 1.94 g of KCl, 1.63 g of KH<sub>2</sub>PO<sub>4</sub>, 2.96 g of MgSO<sub>4</sub>·7H<sub>2</sub>O, 8.4 g of NaHCO<sub>3</sub>, and 23.83 g of HEPES. Add distilled H<sub>2</sub>O to a volume of 800 mL. Mix and adjust pH to 7.55 with 1 M NaOH. Make up to 1000 mL with distilled H<sub>2</sub>O and store at 4 °C. This stock solution should be diluted in later steps to the indicated dilutions.
12. Stock [1-<sup>14</sup>C]palmitate label: 1.8 mM palmitate complexed to 0.3 mM (±2.0% w/v) BSA, 17.4 μM [1-<sup>14</sup>C]palmitate, and 1 mM CaCl<sub>2</sub> in 1× MKR. Prepare pre-warmed BSA solution in a 100 mL *glass* cylinder, by dissolving 1.0 g of BSA in 40 mL of 1,25× MKR. Place in a shaking water bath at 37 °C until further use (100 rpm). Weigh 23.1 mg of palmitic acid in a 100 mL *glass* cylinder, and dissolve in 9.5 mL of pure ethanol. Add 50 μCi of [1-<sup>14</sup>C]palmitate (*see Note 1*), and mix thoroughly. Use a 10 mL *glass* cylinder to prepare 10 mL of KOH solution by pipetting 135 μL of 1 M KOH into 10 mL distilled H<sub>2</sub>O. Add, drop by drop under constant stirring, the 10 mL of KOH solution to the 10 mL of palmitate-ethanol solution (*see Note 2*). Evaporate all ethanol at 45 °C (in water bath) under a constant stream of N<sub>2</sub> gas until the odor of alcohol can no longer be detected and the volume is less than 8 mL. Adjust the volume to 10 mL with distilled H<sub>2</sub>O. Add this 10 mL KOH-palmitate solution drop by drop with a *glass* pipette (*see Note 3*), under continuous gentle stirring (*see Note 2*) to the pre-warmed BSA solution (total volume 50 mL). Add 0.5 mL of 100 mM CaCl<sub>2</sub>. Transfer into *glass* centrifuge tubes, and spin down for 10 min at 3000 × *g* at room temperature.

Aliquot the supernatant into 2–5 mL portions in *glass* vials and store at  $-20^{\circ}\text{C}$ .

*Solutions to Be Prepared Extemporaneously the Day of Experiment*

13. Uptake buffer:  $1\times$  MKR, 1 mM  $\text{CaCl}_2$ , and 0.45% (w/v) BSA. Take a 100 mL *glass* cylinder. Add 0.23 g of BSA, 49.5 mL of MKR buffer, and 0.5 mL of 100 mM  $\text{CaCl}_2$ . Place uptake buffer in a water bath at  $37^{\circ}\text{C}$ .
14. Day Label (for up to 20 incubations): 100  $\mu\text{M}$  [ $1\text{-}^{14}\text{C}$ ]palmitate, 100  $\mu\text{M}$  deoxy-glucose, and 3.3  $\mu\text{Ci}$  2-deoxy-D- [ $^3\text{H}$ ]glucose. Take a 10 mL *glass* vial, and add 9.34 mL of uptake buffer, 0.56 mL of Stock [ $1\text{-}^{14}\text{C}$ ]palmitate label, and 100  $\mu\text{L}$  of 10 mM 2-deoxyglucose/2-deoxy-D- [ $^3\text{H}$ ]glucose mix. Place the vial in a water bath at  $37^{\circ}\text{C}$ .
15. Stop solution (for  $\sim 20$  incubations): 0.2 mM phloretin and 0.1% (w/v) BSA. Prepare phloretin solution by dissolving 21.8 mg of phloretin in 400  $\mu\text{L}$  DMSO. Use a 500 mL *glass* cylinder, and add 400 mL of  $1\times$  MKR buffer, 4.0 mL of 100 mM  $\text{CaCl}_2$ , 0.40 g of BSA (Fraction V, essentially fatty acid-free), and the phloretin solution. For each condition, prepare a 15-mL centrifugation tube with 8 mL Stop solution added. Place tubes on ice.
16. Working solutions of desired AMPK stimuli (*see Note 4*) and/or inhibitors (*see Note 5*).

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### 3 Methods

1. Suspend cells in uptake buffer (50,000–500,000 cells/mL) (depending on metabolic activity of cells; *see Notes 6* and *7*), and distribute over (20-mL) *glass* vials. Add 2 mL of cell suspension per vial.
2. Preincubate the cell suspension at  $37^{\circ}\text{C}$  with a gas phase of 95%  $\text{O}_2$  /5%  $\text{CO}_2$  with inhibitors of AMPK for 20 min in a water bath (shaking at 160 rpm).
3. Subsequently, add activators of AMPK, refill the gas phase, and incubate for another 30 min at  $37^{\circ}\text{C}$ .
4. Start the uptake assay by adding 0.5 mL of Day Label to the cell suspension, and incubate for 5 min at  $37^{\circ}\text{C}$  in a water bath with a gas phase of 95%  $\text{O}_2$ /5%  $\text{CO}_2$ . Each cellular substrate uptake assay should include a “zero time control” to allow to correct for the background signal (*see Note 8*).
5. After 5 min, stop the assay, and transfer 2 mL of cell suspension from each incubation to centrifugation tubes with 8 mL of ice-cold Stop solution.

6. Spin the cells down for  $\leq 2$  min at 4 °C (*see Note 9*). Remove the supernatant and wash with 10 mL of ice-cold Stop solution. Repeat the centrifugation step.
7. Lyse the pellets in 0.5 mL of distilled H<sub>2</sub>O, and transfer the lysates to 20 mL glass  $\beta$ -counter vials containing 5 mL of OPTI-FLUOR liquid scintillation cocktail. Do not forget to include a sample with a fixed volume (e.g., 20  $\mu$ L) of Day Label for scintillation counting in order to allow for calculation of absolute uptake rates.
8. Vortex the samples, and measure the disintegration counts per minute at the  $\beta$ -counter using a combined <sup>3</sup>H/<sup>14</sup>C counting protocol. Uptake values are expressed as nmol/(g wet mass \* min), after subtracting the background signal (*see Note 8*). A representative data set of a substrate uptake assay using AMPK activators is given in Table 1.

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## 4 Notes

1. It is advised to purchase and use a radiolabeled palmitate product dissolved in ethanol, and not toluene, because toluene is extremely toxic to cells.
2. If precipitate is formed, discard the solution and start again.
3. Work as much as possible with glassware because (radiolabeled) palmitate binds non-specifically to plastic surfaces. In the uptake assay, this can lead to high background counts.
4. For selecting the appropriate AMPK activators to be used in the intended experiments, at least the following five items are relevant: (1) the cellular uptake process of interest, (2) the tissue of interest from which primary cells will be isolated, (3) the type of physiological process one aims to mimic or investigate, (4) the off-target actions of selected compound, and (5) the toxic effects of selected compound depending on the cell type.

An increasing number of pharmacological AMPK activators have been employed for the study of AMPK activation on cellular LCFA and glucose uptake. We provide here with some tips and consideration about the most commonly used compounds to investigate substrate uptake in muscle cells:

- *Oligomycin*: It is a very potent stimulator of glucose and LCFA uptake in primary cardiomyocytes and in cardiac cell lines in which the uptake of both substrates increases by ~twofold already within 15–30 min [16]. It is of note that the concentration of oligomycin tolerated by cells differs among cell types. In cardiomyocytes, oligomycin can be used at concentrations of up to 30  $\mu$ M, but this

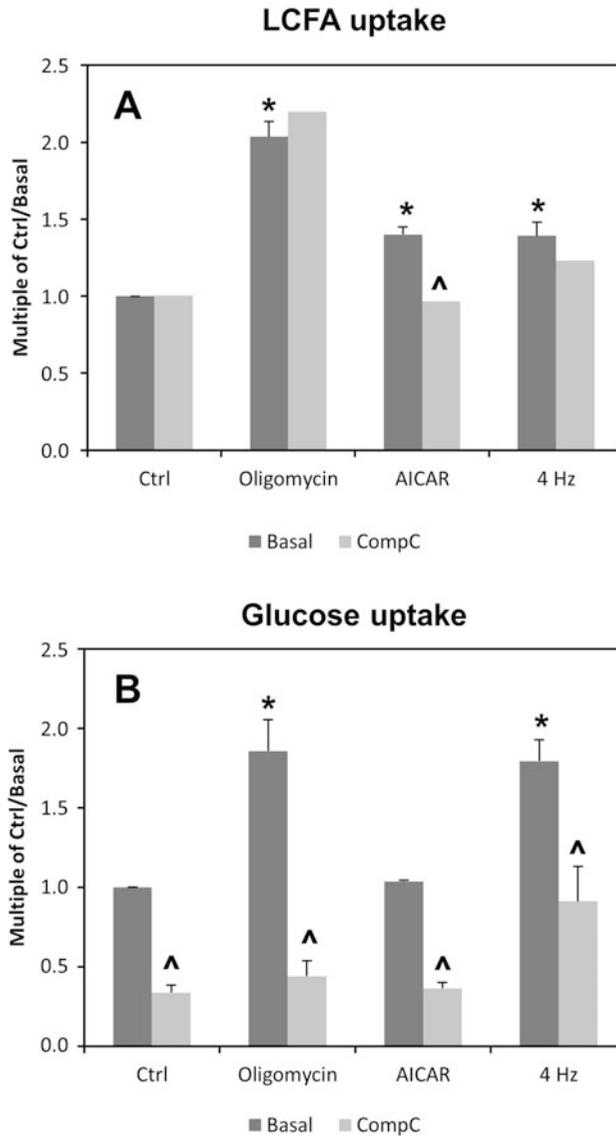
**Table 1**  
**Data obtained from an example experiment**

| Condition  | dpm <sup>14</sup> C | Minus background | Palmitate uptake | Normalized | dpm <sup>3</sup> H | Minus background | Deoxyglucose uptake | Normalized |
|------------|---------------------|------------------|------------------|------------|--------------------|------------------|---------------------|------------|
| Zero time  | 76                  |                  |                  |            | 38                 |                  |                     |            |
| Control    | 1590                | 1514             | 10.10            |            | 372                | 334              | 0.81                |            |
| Control    | 1625                | 1549             | 10.33            | 1.00       | 411                | 373              | 0.91                | 1.00       |
| Control    | 1669                | 1593             | 10.63            |            | 414                | 376              | 0.91                |            |
| Oligomycin | 3065                | 2989             | 19.94            |            | 735                | 697              | 1.69                |            |
| Oligomycin | 3093                | 3017             | 20.13            | 1.94       | 724                | 686              | 1.67                | 1.97       |
| Oligomycin | 3121                | 3045             | 20.31            |            | 790                | 752              | 1.83                |            |
| AICAR      | 2401                | 2325             | 15.51            |            | 420                | 382              | 0.93                |            |
| AICAR      | 2312                | 2236             | 14.92            | 1.47       | 406                | 368              | 0.89                | 1.03       |
| AICAR      | 2381                | 2305             | 15.38            |            | 399                | 361              | 0.88                |            |

The described method for substrate uptake measurement was applied. The following values were experimentally determined. Radioactive counts of 20  $\mu$ L Day Label were 5354 dpm (<sup>14</sup>C) and 14,705 dpm (<sup>3</sup>H). The amount of cells per incubation was 7.0 mg wet mass/mL (~70,000 cardiomyocytes/mL). The duration of the uptake assay was 5 min. Values for palmitate uptake and deoxyglucose uptake are calculated as nmol/(g wet mass  $\times$  min)

concentration is cytotoxic in cell lines, such as HL-1 cells, in which concentrations of up to 1  $\mu\text{M}$  are more advisable. Importantly, besides activation of AMPK [16], oligomycin also stimulates production of reactive oxygen species and subsequent activation of protein kinase-D1, another crucial player in oligomycin-induced GLUT4 translocation and cellular glucose uptake [27].

- *Mitochondrial inhibitors*: Uncoupling agents (such as 2,4-dinitrophenol) and respiratory chain inhibitors (rotenone) stimulate cellular glucose uptake in adipocytes and myocytes [28, 29]. However, these agents cannot be used to study cellular LCFA uptake. Unlike glucose uptake, LCFA uptake across the plasma membrane is dependent on the cellular membrane potential [30], which is destroyed by these agents since their protonophore activity is not restricted to mitochondria [28, 31]. The respiratory chain inhibitor rotenone potently impairs the oxidative metabolism of both substrates. Because uptake and subsequent metabolism are tightly coupled processes, the block in oxidative phosphorylation leads to feedback inhibition of the LCFA uptake process [8]. In contrast, the acceleration of glucose uptake upon rotenone-induced AMPK activation may be accommodated for by a concomitant acceleration in glycolysis so that all incoming glucose is efficiently phosphorylated and feedback inhibition will not occur. In case of specific interest in AMPK-stimulated glucose uptake, DNP and rotenone should preferably be used at low micromolar concentrations.
- *AICAR*: One point of concern with the use of AICAR is the increasing list of AMPK-independent actions of AICAR [32]. Nonetheless, AICAR has been used as activator of LCFA uptake in skeletal muscle [33] and glucose uptake in adipocytes [29] and skeletal muscle [34]. In cardiomyocytes, AICAR, at low millimolar concentrations, stimulates LCFA uptake by ~1.5-fold in an AMPK-dependent fashion [25, 35], but this compound is not effective in stimulation of GLUT4 translocation and cellular glucose uptake (Fig. 2b) [25, 35, 36]. Thus it appears that the stimulation of cellular glucose uptake by AICAR is tissue-specific and should be taken into consideration when studying the effects of AMPK activation on substrate uptake.
- *Metformin and phenformin*: Although these antidiabetic drugs are known to activate AMPK at low millimolar concentrations [37, 38], they failed to increase GLUT4 or CD36 translocation or cellular uptake of LCFA and glucose within 30 min in cardiomyocytes [39] (Luiken, unpublished). However, metformin has been shown to stimulate



**Fig. 2** Effects of Compound C on substrate uptake into rat cardiomyocytes stimulated by various AMPK activators. Cell suspensions were incubated in absence or presence of 50  $\mu$ M Compound C for 30 min, and subsequently for another 15 min without or with 5  $\mu$ M oligomycin or 1.5 mM AICAR. Alternatively, electrostimulation was performed for 7 min at 4 Hz. Then, radioactive substrates were added for measurement of (a) palmitate uptake or (b) deoxyglucose uptake during 5 min. Data are means  $\pm$  S.E.M ( $n = 4-6$ ). \*Significantly different from myocytes without additions (Ctrl) ( $p < 0.05$ ). ^Significantly different from corresponding incubations in the absence of Compound C (Basal) ( $p < 0.05$ )

GLUT4 translocation in cardiomyocytes upon long-term (18 h) treatment, indicating reliance on synthesis of specific proteins. These proteins might be involved in the negative regulation of GLUT4 endocytosis [39].

- *Small-molecule direct AMPK activators*: A screen by the Abbott Laboratories of >700,000 compounds yielded a non-nucleoside thienopyridone, A-769662, as novel small-molecule AMPK activator [40]. In skeletal muscle strips, it appeared ineffective on stimulation of glucose uptake [41]. In cardiomyocytes at concentrations between 30 and 300  $\mu\text{M}$ , during 30 min, it also failed to stimulate AMPK-Thr172 phosphorylation or LCFA and glucose uptake (Habets and Luiken, unpublished). Compared with  $\beta$ 1-containing heterotrimers,  $\beta$ 2-containing AMPK isoforms are less efficiently activated by A-769662 [42]. Given that muscle tissues mainly express the AMPK  $\beta$ 2 subunit isoform, A-769662 may be a less favorable option for studying the role of AMPK in the heart and skeletal muscle.

More recently, another small-molecule AMPK activator, 991 (also known as ex229), has been described [41]. Although it shares the same binding site on AMPK as A-769662, it showed a ~tenfold greater potency to activate AMPK [43]. Interestingly, 991 activates both AMPK  $\beta$ 1- and  $\beta$ 2-containing complexes and efficiently stimulates glucose uptake into skeletal muscle at 100  $\mu\text{M}$  for 60 min [41]. Further studies are required to investigate whether this compound is a suitable tool to study substrate uptake in cardiac cells.

- *Leptin*: This hormone is a physiological AMPK activator and stimulates CD36 translocation in an AMPK-dependent manner at 10  $\mu\text{g}/\text{mL}$  for 15–60 min, as well as LCFA uptake in skeletal muscle incubations and cardiomyocytes [44]. With respect to glucose uptake, 20 min leptin was ineffective in stimulating glucose uptake in HL-1 cardiomyocytes [45]. In contrast, 30 min leptin stimulates GLUT4 translocation in C2C12 myotubes, which was dependent on ERK2 [46] and perhaps independent of AMPK. Hence, a pleiotropy of stimulatory actions on intracellular signaling cascades makes leptin less suited as a tool to study AMPK-specific regulation of substrate uptake.
5. Genetic approaches would be the preferable manner to investigate the involvement of AMPK in substrate uptake. An excellent option would be to use primary cells from tissues derived from AMPK knockout mouse models for assessment of cellular substrate uptake. But since at least two isoforms of the catalytic  $\alpha$  and regulatory  $\beta$  subunits are expressed in mammals, one needs double-knockout mouse models for these subunits to provide full proof for the involvement of AMPK. But in case that one is only interested in the role of AMPK in a specific tissue, a single-knockout mouse will suffice, when the respective tissue only expresses one isoform. For example, in muscle

tissues, AMPK $\alpha$ 2 knockout mice would be suitable, given that the  $\alpha$ 1 subunit is only marginally expressed in muscles. Furthermore, the muscle-specific kinase-dead AMPK $\alpha$ 2 overexpressing mouse [47] provides a very suitable model for the role of AMPK in muscles. A second option would be to silence AMPK in cell lines, e.g., by siRNA approach or CRISPR/Cas9 (see Chapter 11). However, when AMPK mouse models or silencing methodologies are not available, one can consider a pharmacological approach and use small-molecule AMPK inhibitors. Below is a list of compounds previously used in substrate uptake studies:

- *5-Iodotubercidin*: 5-Iodotubercidin is not a direct AMPK inhibitor but blocks the formation of AMP from adenosine via inhibition of adenosine kinase [48]. Treatment with 5-iodotubercidin for 10  $\mu$ M at 15–30 min blocks AICAR-induced LCFA uptake into cardiomyocytes [16] and glucose uptake into clone-9 cells [18] and muscle strips [49], in agreement with its ability to inhibit ZMP formation. In contrast, 5-iodotubercidin does not alter contraction and oligomycin-stimulated LCFA uptake into cardiomyocytes [16], which is readily explained by the fact that AMP formation due to stress or increased energy demands is derived from ATP utilization and not from AMP synthesis. Hence, 5-iodotubercidin is of limited use to study AMPK actions.
- *Adenine 9- $\beta$ -D-arabinofuranoside (Ara-A)*: This AMP analog decreases AMPK activity in vitro [50]. Ara-A is used in concentrations of 2–3 mM for 15–30 min and entirely inhibits AICAR-induced glucose uptake into muscle strips [49]. It also inhibits stimulation of GLUT4 translocation and glucose uptake in neonatal cardiomyocytes by respiratory chain inhibition using sodium azide (NaN<sub>3</sub>). Furthermore, Ara-A inhibits AICAR-stimulated LCFA uptake into primary cardiomyocytes [35] and also leptin-stimulated CD36 translocation and LCFA uptake into skeletal muscle and primary cardiomyocytes [44]. Hence, as AMPK inhibitor, Ara-A has a broader applicability than 5-iodotubercidin in studying AMPK-related actions but has also numerous other inhibitory effects, such as inhibition of adenylyl cyclase [51].
- *Compound C*: As selected from a high-throughput in vitro kinase assay out of >10.000 compounds, Compound C appeared a potent reversible AMPK antagonist via competition with ATP [37]. Compound C is mostly used in concentrations of 10–50  $\mu$ M and has been shown to inhibit AICAR-induced GLUT4 translocation [52] and AICAR-stimulated glucose uptake [53]. We have used Compound C (50  $\mu$ M) to study AMPK-mediated LCFA and glucose

uptake into primary cardiomyocytes. AICAR-stimulated LCFA uptake was entirely inhibited, whereas contraction and oligomycin-stimulated LCFA uptake were not significantly altered (Fig. 2a). This inhibition pattern does not match with the presumed direct action of Compound C on AMPK. Furthermore, interpretation of the effects of Compound C on AMPK-stimulated glucose uptake was complicated by a large effect of this inhibitor on basal glucose uptake (Fig. 2b), in agreement with Merlin et al. [53]. Please note that AICAR does not stimulate glucose uptake into rodent cardiomyocytes (Fig. 2b). Finally, the list of off-target actions of Compound C on other kinases, such as ERK8 and MNK1, is growing [54]. As described in Chapter 12, there are serious doubts about the specificity of Compound C and whether it is a useful tool for studying AMPK.

- *STO-609*: *STO-609* does not inhibit AMPK directly but rather blocks upstream activation. Specifically, *STO-609* is an antagonist of  $\text{Ca}^{2+}$ /calmodulin-dependent kinase kinase (CaMKK) [55]. Together with liver kinase B1 (LKB1) and transforming growth factor beta-activated kinase 1 (TAK1) [56], CaMKK- $\beta$  is one of the several kinases able to directly activate AMPK. Hence, *STO-609* is of no use to study activation of AMPK by LKB1 and TAK1. Given that most inflammatory stimuli act via TAK1 and given that LKB1 is the major upstream activator of AMPK in several tissues, including the heart [25], *STO-609* is often of limited use in AMPK research. We observed that *STO-609* (5  $\mu\text{M}$ ; 20 min) does not block LCFA and glucose uptake stimulated by oligomycin or AICAR [35].

But still, *STO-609* may be a suitable tool to connect  $\text{Ca}^{2+}$  signaling to AMPK signaling. In skeletal muscle, the  $\text{Ca}^{2+}$  signaling activator caffeine stimulates AMPK $\alpha$ 2 activation, and also LCFA and glucose uptake. Moreover, LCFA and glucose uptake stimulated by contraction or caffeine is largely blocked by *STO-609* indicating an important role of CaMKK- $\beta$  in AMPK regulation of substrate uptake in contracting muscle [24]. In contrast *STO-609* did not inhibit contraction-stimulated LCFA and glucose uptake into primary cardiomyocytes, pinpointing to a less prominent role of  $\text{Ca}^{2+}$  signaling in contraction-regulated metabolism in the heart [35].

6. The isolation procedure raises cellular stress levels, and primary cells should preferably undergo a recovery period of ~90 min at room temperature after their isolation to allow the metabolic rate to decrease to low “basal” levels. In case of cultured cells, a ~60 min recovery period is recommended after the

dis-attachment from the plates with a low trypsin concentration ( $\leq 0.05\%$ ) to minimize damage to exposed substrate transporters at the cell surface.

7. Most primary cells can be regarded as metabolically active cells, especially since isolation procedures have been optimized for many years. When these cells are immediately used for substrate uptake assays, their metabolic activity will be highest, likely resulting in relatively high substrate uptake rates. Culturing of primary cells leads to a marked decline in metabolic activity depending on the duration and culturing conditions. Upon their dis-attachment from the culture wells, it is therefore recommended to use higher cell densities in the uptake assay, compared to their freshly used counterparts. Lowest metabolic activity is displayed by the cell lines and likely dependent on the number of passages. Therefore, relatively high cell densities should be used in order to obtain accurately detectable uptake values. Overall, it is recommended to perform a pilot measurement of substrate uptake as function of the cell density.
8. Correction for background signal: At least one incubation per experiment should serve as “zero time control” ( $t = 0$ ). In this case, the cell suspension (1.6 mL) should be transferred to the Stop solution prior to addition of the Day Label (0.4 mL). The remainder of the procedure is similar as described for the other incubations. When performing the calculation of uptake rates, the counts of this “zero time control” should be subtracted from the radioactive counts of each incubation.
9. The centrifugation speed is dependent on the size of the cells (e.g., primary cardiomyocytes are pelleted at  $25 \times g$ ; several cell lines require centrifugation speeds of  $\sim 1000 \times g$ ).

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