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Is The $G_{\alpha i}$-GPCRs Machinery Working in Primary Rat Skeletal Muscle Cells?

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ABSTRACT

GPCRs are the largest family of proteins in the human genome and a target for huge numbers of therapeutic drugs, although the role of skeletal muscle in the action of these drugs is unclear. The purpose of this research was to identify genes encoding G_i-GPCRs highly expressed in skeletal muscle and in cultured preparations thereof and investigate whether G_i-coupling working in primary skeletal muscle cells.

Skeletal muscle cells were cultured from vastus lateralis obtained from male Wistar (180-200 g) rats according to [1]. mRNA expression, cAMP assay and western blot were examined by conventional methods [2-4]. Data were reported as means of triplicate or quadruplicate wells generated from two animals, except where indicated. Statistical analysis was conducted using one or two-way ANOVA with Bonferroni's multiple comparisons test.

mRNA encoding G_i- (*adora1*, *adra2a*) receptors were detected using gene microarray (Agilent, all ranked <24000 out of 41090). QRT-PCR (Taqman) identified α_{2A} receptor and CB_1 cannabinoid receptor mRNA expression with Ct values of 28-31 and 26.5-27.5 in myoblasts, myotubes and skeletal muscle tissue, respectively. Neither basal nor forskolin (1 µM)–evoked elevation of cAMP was altered in the presence of the A_1-selective agonist S-ENBA (10^{-7} M). In myotubes, forskolin (1 µM) –evoked elevation of cAMP (17 ± 1) was significantly inhibited in the presence of

UK14304 (10^{-7} M) (12 ± 0). However, rauwolscine (10^{-7} M) did not prevent this effect. Treatment with UK14304 increased phosphorylation of extracellular signal-regulated kinase 1/2 (25 ± 1.6 compared to basal ERK phosphorylation of 13 ± 3.5 P-ERK/ERK); these responses were significantly inhibited by rauwolscine (11 ± 1). Interestingly, rauwolscine showed also a significant inhibition of phosphorylation of ERK (3.6 ± 0.24). In cultured myotubes, forskolin (1 μM)–evoked elevation of cAMP was unaltered in the presence of ACEA (10 nM) or rimonabant (100 nM) for 10 minutes. Treatment with ACEA (10 nM) for 10 minutes increased activation of extracellular signal-regulated kinase 1/2 (~400%); these responses which were significantly inhibited by rimonabant (100 nM).

In summary, we observe expression and functional responses to example members of the GPCRs in rat skeletal muscle preparations. However, these findings did not provide direct evidence for G_i-coupling for G_i-GPCR tested, including the A_1 adenosine receptor.

Keywords: GPCRs, G_i-Coupling, Skeletal Muscle, Myotubes

INTRODUCTION

Skeletal muscle is a heterogeneous tissue since it contains a variety of fibres that differ in contractile, functional, metabolic and molecular characteristics [5, 6]. Moreover, skeletal muscle is the largest organ in the human body and represents ~40% of the human body mass and 35-40% of the total body weight in the rat [7, 8]. Indeed, skeletal muscle utilizes the majority (70-80%) of ingested glucose since it is the main site for insulin-dependent glucose uptake [9]. Therefore, it is generally considered a major site of peripheral insulin resistance.

G protein coupled receptors (GPCRs) are the largest family of proteins in the human genome. Indeed, GPCRs are the richest targets for pharmaceutical drugs on the market today; it is estimated that they are the targets of 30-50% of all medications due to their vast and varied roles in regulating the body processes, metabolism and signal transduction and their involvement in key biological functions [10, 11]. GPCRs are expressed in every tissue and play a major role in many diseases.

cAMP is an important second messenger in skeletal muscle. It was found to increase the expression of members of the orphan family of nuclear receptors, subfamily 4 (NR4A), compared to other nuclear receptors in skeletal muscle

[12]. These transcription factors regulate the gene expression of proteins responsible for fat and glucose metabolism through up-regulating the mRNA expression of pyruvate dehydrogenase kinase 4 (PDK4), forkhead box protein O1 (FOXO1), peroxisome proliferator-activated receptor-γ coactivator (PGC-1α), phosphatidate phosphatase LPIN1 (lipin-1α), GLUT4 and muscle phosphofructokinase (Pfkm) [13-15].

There are, however, very few studies about the expression of G_i-GPCRs and their signalling and the diversity and roles of these receptors in skeletal muscle. There has yet been no comprehensive analysis carried out of GPCRs and GPCRs signalling pathways in skeletal muscle published in the literature. This study utilized microarray technology to identify the identity and relative levels of GPCRs expressed in skeletal muscle.

Myotubes are primary skeletal muscle cells sharing the morphological, metabolic and biomedical characteristics and properties of adult skeletal muscle [16, 17]. Our study showed that mRNA for many G_i-GPCRs were expressed in skeletal muscle tissues; notably A_1 adenosine receptors, NPY Y_1 receptor, CB_1 receptor, α_{2A}-adrenoceptors. Since there has been little evaluation of GPCRs, in particular G_i-GPCR signalling in myotubes, and it was not possible to investigate G_i-GPCR apparently expressed in skeletal muscle, some examples were chosen. From the top 38 highly ranked GPCRs list detected using the microarray and GPCRs detected using the QRT-PCR (Taqman) in skeletal muscle, α_{2A}-adrenoceptors, A_1 adenosine, NPY Y_1 and CB_1 cannabinoid receptors were selected as G_i-coupled. This group (G_i-GPCRs) were tested by investigating phosphorylation of ERK. Moreover, this group (G_i-GPCRs) were tested by quantifying levels of cAMP.

AIMS

The purpose of the investigations in this study was to characterize the mRNA expression of G_i-GPCRs in rat mixed fibre-type skeletal muscles using DNA microarray and QRT-PCR (Taqman) techniques. The purpose of the investigations in this study was also to assess the downstream signalling of G_i-GPCRs in rat primary skeletal muscle cells using cAMP assay and western blot.

Abbreviations

(GPCRs); G protein coupled receptors, (CB_1); cannabinoid receptor 1, (Adora1); adenosine A1 receptor,

(NPY); neuropeptide Y, (AC); adenylyl cyclase, (cAMP); cyclic adenosine monophosphate, (ACEA); Arachidonyl-2-chloroethylamide, (AEA); N-arachid--onoylethanolamine, anandamide, (NECA); 2-(4-[2-carboxyethyl]-phen- -ethylamino) adenosine-5′-N-ethyluron amide, (S-ENBA); (2S)-N6-(2-endonobanyl) adenosine, (ICI 118,551); erythro-dl-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol, (UK14304); 5-Bromo-6-(2-imidazolin-2-ylamino)q uinoxaline, (MAPK); Mitogen activated protein kinase, (ERK 1/2); extracellular signal-regulated kinase 1/2, FBS; (fetal bovine serum), (PBS); phosphate buffered saline, (SEM); standard error of mean, (ANOVA); analysis of variance, (APS); ammonium persulphate, (cDNA); Complementary DNA, (Ct); Cycle threshold, (DMSO); Dimethyl sulfoxide, (mRNA); messenger ribonucleic acid, (PCR); Polymerase chain reaction; (P-value); Probability, (RT-PCR); Reverse transcription polymerase chain reaction, (SDS-PAGE); Sodium dodecyl sulfate polyacrylamide gel electrophoresis, (SEM); Standard error of the mean, (TBST); Tris-buffered saline with Tween 20, (*cnr1*); cannabinoid receptor 1.

MATERIALS AND METHODS

Materials

NECA, S-ENBA, UK14304 and rauwolscine were purchased from Tocris Company. All other materials were purchased from Sigma unless otherwise mentioned. TriReagent was purchased from Invitrogen. RNeasy Mini Total RNA Purification kits were purchased from Qiagen (West Sussex, UK). Reagents for reverse transcription of RNA to cDNA were purchased from Invitrogen (Paisley, UK). Taqman reagents were purchased from Applied Biosystems (CA, USA). Primers and probes were purchased from Eurofins MWG GmBH (Ebersberg, Germany). 30% Bis-acrylamide was purchased from Severn Biotech (Kidderminster, UK). Seeblue Protein Ladder was purchased from Fermentas (York, UK). Nitrocellulose membrane was purchased from GE Healthcare (Amersham, UK). Goat 680 anti-rabbit, goat 680 anti-mouse, goat 800 anti-rabbit and goat 800 anti-mouse secondary antibodies were purchased from Li-Cor Biosciences (Cambridge, UK). P-ERK and ERK primary antibodies were purchased from Cell-Signalling company. FBS (fetal bovine serum) and Ham's F10 were purchased from PAA Laboratories (Somerset, UK). Horse serum was purchased from Invitrogen.

cAMP Kit was obtained from Cayman Chemical (Europe).

Tissue Collection

Two male adolescent Wistar rats (180-200 g, 4-6 weeks old) were killed by cervical dislocation without anesthesia. Liver, adipose (mixed from subcutaneous, epididymal and omental) and skeletal muscle were obtained. Skeletal muscle (mixed fibre-type from hindlimb) tissues were separated bilaterally: tissue was immediately frozen in liquid nitrogen and stored at -80 C° with liver and adipose tissue, muscle tissue was cultured after the isolation of satellite cells in gelatine-coated flasks.

Tissue Culture

Muscle culture was performed as Blau and Webster method with slight modification (Blau *et al*., 1981). Vastus lateralis muscles from Wistar rats were removed and immersed in phosphate buffered saline (PBS), washed to remove the remnants of blood, and minced finely with scissors and scalpel blades on a Petri dish. Then, the minced muscle was transferred to a 50 ml flask containing a "flea" and 5-10 ml of 0.25% (W/V) trypsin/EDTA (1X) for incubation at 37 C° for 15 minutes. After that, the supernatant was transferred to a 50 ml flask and neutralised with an equal volume of medium (streptomycin, penicillin, foetal bovine serum and Ham's F10), then centrifuged at 1700 rpm for 5 minutes. The collected cells were filtered through 100 μm nylon mesh ("cell strainers") to purify the cells from the debris, and centrifuged for 10 minutes at 17,000 rpm (g=26) at room temperature. The supernatant layer was removed and the cell pellet (satellite cells) was re-suspended in Ham's F10 growth medium, pre-plated on uncoated flasks for 10 minutes at 37 C° to purify these satellite cells from fibroblasts present in the extract, and then transferred to culture flasks coated with 0.2%) W/V) gelatin. The satellite cells were then grown to confluent myoblasts and differentiated into myotubes in growth medium; 20% (V/V) fetal bovine serum (FBS) and 5 ml of penicillin and streptomycin (10,000 units penicillin and 10 mg streptomycin/ml in 0.9% NaCl) were added to Ham's F10. After one day, the cells were fed with fresh medium, cells require fresh medium every 48 hours. The cells were fed with 20% (V/V) FBS fresh medium for three weeks, then reduced to 10% (V/V) FBS fresh medium for two weeks and then changed to 6% horse serum and 10 mM

glucose Ham's F10 for two to three days.

Microarray Procedure

Agilent 4*44K DNA one color whole genome microarrays were used to measure the expression of 41090 genes in liver, adipose tissue and mixed fibre-type of skeletal muscle from rat hindlimb. The microarray experiment was carried out according to manufacturing instructions. After assessing the quality control criteria generated from feature extraction software, extracted data were further processed with GeneSpring GX 11.

RNA Extraction and QRT-PCR (Taqman)

Myoblast and myotube cells were grown and differentiated as described above. The cells were collected in TriReagent, and processed according to the manufacturer's directions. RNA was reverse transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen). Then, QRT-PCR (Taqman) was performed as described according to the manufacturer's directions. Gene expression levels (in arbitrary units) were determined from the mean of triplicate determinants of each sample. Data from Taqman were only used if the slope of the standard curve for each plate was between -3.2 and -3.6 and R^2 values of more than 0.99. In addition, Ct values of triplicate readings for an individual sample, which were more than 0.5 Ct apart, were excluded.

The forward primer 5'CCAAAAGTG GAGAGCGACAAC3', reverse primer 5'CGTCTCGAAGGTCCCAATGT3' and 5'ATCCAGATCACCATGCCGTTCACA3' were used for *Cnr1*. The forward. Primer.5'GGCCTCAGCGGACATCCT3', reverse primer 5'CATAACCTCGTTGGCCAAAGA3' and probe 5'TGGCCACGCTGGTCATTCCCTT3' were used for *Adra2a*.

cAMP Assay

Accumulated cAMP in the myotube cells was measured by a competitive Enzyme Immunoassay (EIA) kit (Cayman Chemical). Cells were prepared as discussed above. Where indicated, cells were pre-incubated with antagonist 20 minutes prior to agonist addition. At the end of the exposure period for the drugs, 40 µl 5 M HCl was added to each well (2 ml media (Ham's F10), 6% horse serum), before cAMP measurement as described in accordance with the manufacturer's instructions.

Western Blot

The samples (20 µl) were loaded onto a 12% SDS polyacrylamide gel. 5 µl of seeblue marker (Invitrogen) was loaded with each gel as a molecular weight marker. The protein was separated by

gel electrophoresis in a Bio-Rad gel apparatus (Mini-PROTEAN) filled with SDS-PAGE running buffer. A constant current of 150 volt was supplied for around one hour. The separated proteins from the gel were transferred to pre-soaked nitrocellulose membrane in transfer buffer. A constant current of 105 volt was supplied for around one hour. The protein was stained on the membrane with Ponceau S. Non-specific antibody binding was reduced by blocking the nitrocellulose membrane in blocking buffer (3% gelatin in 0.1% TBS.T) for one hour at room temperature with continuous gentle shaking. The nitrocellulose membrane was subsequently incubated with primary antibody overnight at 4 C° on a roller. After overnight incubation, the membrane was washed with 1XTBST three times each 20 minutes. The membrane was then incubated with secondary antibody diluted 1:10,000 for one hour at room temperature. After washing three times for 5 minutes with 1XTBST, the membrane was rinsed with distilled water. Finally, the membrane was scanned using The Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE/USA), which equipped with two infrared channels. Using two detection channels, total and phosphorylated forms could be visualized and differentiated between on the same gel (same experiments) using different secondary antibodies. Band intensities were quantified by densitometry, using Odyssey software version 3. Each blot was analyzed for p-ERK to ERK ratios to assess ERK activation.

Statistical Analysis

Data were analyzed using one way ANOVA and Bonferroni post-hoc test. Analysis was performed using GraphPad Prism, version 5.03 (GraphPad Software Inc). Differences were considered significant at $P< 0.05$. Data are reported as means ± SEM of triplicate or quadruplicate wells generated from two animals (n=2). Data were represented as means ± standard error of mean (SEM).

RESULTS

mRNA Expression of GPCRs

38 GPCR entities were detected in skeletal muscle tissue (ranked higher (higher relative intensity values) than either nicotinic cholinergic alpha subunit (*chrnα1*) or reference gene (*tbp*) (12526 and 10220 ranking out of 41090, respectively)). From these 38 GPCR entities, A_1 adenosine, NPY Y_1 receptor, α_2-adrenoceptors and others were selected as shown in Table 1. These GPCR entities include three main

families that coupled to different G proteins in this study. Examples of G_i-GPCRs are A_1 adenosine receptor and NPY Y_1 receptor. These examples of detected GPCR entities will be investigated to examine the possible different signalling in skeletal muscle cells. Indeed, investigation of G_i-GPCRs might help to confirm the expression and understand the expected signalling and functional role of these receptors and to investigate the possible cross-talk between GPCRs signalling and their downstream signalling partners' genes.

mRNA Expression of GPCRs Using QRT-PCR (Taqman)

Some entities were selected to be examined by QRT-PCR (Taqman). Examining mRNA expression of these receptors (CB_1 receptor and α_2-adrenoceptors) in skeletal muscle will be helpful in this study for further investigation. In order to examine the expression of these receptors and validate the mRNA expression of GPCRs, QRT-PCR (Taqman) was performed.

mRNA for *adra2a* and *cnr1* were detected in rat skeletal muscle tissues at Ct ~ 32 and 27, respectively (ranking in the microarray; 14208 and 34463/35034 out of 41090, respectively) as shown in Table 2. There was no significant difference in the expression of *adra2a* and *cnr1* between skeletal muscle and adipose tissues using QRT-PCR (Taqman).

Effect of a NPY Y_1 Receptor Ligand on cAMP Levels

As mRNA encoding the NPY Y_1 receptor was detected in skeletal muscle in this study, the potential inhibitory effect of NPY on cAMP levels was assessed. Neither basal levels of cAMP nor forskolin (1 µM)-stimulated cAMP were altered in the presence of NPY (300 nM) (Figure 1).

Effect of Cannabinoid Receptor Ligands on cAMP Levels

Since CB_1 receptor mRNA was detected using QRT-PCR (Taqman) in this study, the potential inhibitory effect of CB_1 agonists on cAMP levels was assessed.

In myotubes, neither basal nor forskolin (1 µM)–evoked elevation of cAMP was altered significantly in the presence of ACEA, the selective CB_1 receptor agonist [18], (10 nM), AEA, endogenous cannabinoid receptor agonist [19], (10 µM) or RIM, CB_1 antagonist/inverse agonist [20], (100 nM) for 10 minutes compared to vehicle (0.01% DMSO) (Figure 2).

Effect of Adenosine Receptor Ligands on cAMP Levels

mRNA encoding A_1 adenosine receptors was detected in skeletal muscle in this study, A_1 adenosine receptors would likely inhibit cAMP production.

The potential coupling of A_1 adenosine receptors to inhibition of cAMP was investigated using the A_1 receptor-selective agonist S-ENBA [21, 22]. However, neither basal nor forskolin (1 µM)–evoked elevation of cAMP in myotube cells was altered in the presence of S-ENBA (100 nM) (Figure 3).

Effect of α_2-Adrenoceptor Ligands on cAMP Levels

Adra2a expression was detected using QRT-PCR (Taqman) and microarray in this study. Functional expression of the α_{2A}-adrenoceptor as a G_i-coupled receptor was assessed by quantifying cAMP levels. Treatment of myotube cells with UK14304, an α_2-adrenenoceptor agonist [23], inhibited forskolin (1 µM)-evoked elevation of cAMP significantly. However, rauwolscine, the selective α_2-adrenoceptor antagonist [24, 25], did not prevent this effect (Figure 4).

Effect of α_2-adrenoceptor ligands on ERK phosphorylation

α_2-adrenoceptor coupling to ERK phosphorylation was investigated as an alternative coupling mechanism. Treatment of myotube cells with UK14304 for 10 minutes showed a significant increase in P-ERK1/ERK1 and P-ERK2/ERK2 ratios compared to vehicle (0.01% ethanol). The molecular weight of the bands for P-ERK1/ERK1 and P-ERK2/ERK2 was detected at 44 kDa and 42 kDa, respectively as expected. Interestingly, treatment of myotube cells with rauwolscine (100 nM), 30 minutes prior to the addition of UK14304 inhibited the effect of UK14304 significantly for both P-ERK1/ERK1 and P-ERK2/ERK2 ratio. However, rauwolscine showed a significant inhibition of basal levels of ERK phosphorylation as well (Figure 5).

Effect of ACEA, AEA, and RIM on ERK Phophorylation

In order to assess the signalling of the CB_1 receptor, ERK phosphorylation was investigated (Figure 6). Cells treated with ACEA (10 nM and 100 nM) and AEA (10 µM) for 10 minutes showed a significant increase in P-ERK1/ERK1 and P-ERK2/ERK2 ratio compared to vehicle ($P<0.001$). Pretreatment of the myotubes with 100 nM RIM for 30

minutes prior the addition of either 10 nM ACEA or 10 µM AEA inhibited these effects. However, there was no significant difference between 100 nM RIM and vehicle (0.01% ethanol). It is worth mentioning that ACEA, AEA and RIM did not activate (phosphorylate) AKT. The treatment of muscle culture with 100 nM ACEA and 100 nM RIM for 24 hours did not alter the phosphorylation for ERK1/2 compared to vehicle (Figure 6). The molecular weight of the bands for P-ERK1/ERK1 and P-ERK2/ERK2 was detected at 44 kDa and 42 kDa, respectively, as expected.

DISCUSSION

A number of G_i-GPCRs were observed to be expressed in skeletal muscle tissue using the microarray and QRT-PCR (Taqman). The signalling (functionality) associated with these receptors has not previously been investigated in detail in skeletal muscle. Therefore, conventional second messenger studies (investigating cAMP and ERK activation) were conducted to assess their functionality and/or G_i-machinary in primary rat skeletal muscle cells.

In terms of α_2–adrenoceptors, *adra2a* expression was found in rat skeletal muscle using northern blot analysis [26]. Consistent with this report, α_{2A}-adrenoceptors were also detected using the microarray and QRT-PCR (Taqman) in this study. The accepted roles for α_{2A}-adrenoceptors include acting as the major feedback regulator of noradrenaline release at nerve terminals and the regulation of insulin secretion through noradrenaline in pancreatic islets through reducing the cAMP formation [27, 28]. It is noteworthy that a mutation of α_{2A}-adrenoceptors has been shown to be associated with obesity and metabolic alterations [29]. However, there is a lack of possible roles of the α_{2A}-adrenoceptors in skeletal muscle in the literature. Therefore, a cAMP assay was performed to test the functionality of this receptor.

UK14304 was found to inhibit cAMP elevation evoked by forskolin. However, rauwolscine did not prevent this effect. The concentration which was used in this study for UK14304 is in line with that (100 nM) shown to inhibit forskolin-evoked cAMP level in rat primary superior cervical ganglionic (SCG) cells [30], and the concentration which was used in this study for rauwolscine is also in line with that (100 nM) shown to reverse the effect of UK14304 inhibition of secretin-stimulated cAMP level in purified rat bile duct-ligated (BDL) cholangiocytes

via α_2-adrenoceptors [31]. This suggests that the effect of UK14304 regarding cAMP might not function through α_{2A}-adrenoceptors since this effect was not blocked by rauwolscine or it is also possible that the concentration used for rauwolscine did not block the effect of UK14304 in these primary skeletal muscle cells. In other words, the concentration used for rauwolscine might not overcome the effect of UK14304 regarding the cAMP, in particular of the similar relative affinities for UK14304 and rauwolscine to α_{2A}-adrenoceptors (Kd=10 nM and Ki=3.5 nM, respectively) [32, 33]. Different concentration points of rauwolscine and concentration-response curve for UK14304 are required to have a clear comprehensive image of the response in these primary skeletal muscle cells regarding cAMP.

Interestingly, UK14304 evoked a significant elevation of phosphorylated ERK1/2; an effect blocked by rauwolscine. Furthermore, the inhibitory effect of rauwolscine on basal levels of cAMP could be interpreted to mean that α_2-adrenoceptors in this tissue exhibit constitutive activity. Rauwolscine has been reported to be an inverse agonist in stable Chinese hamster ovary cell lines expressing constitutively activated porcine α_{2A}-adrenoceptors in which the suppression of cAMP production in these cells is reversed by rauwolscine [34]. However, nothing in the literature is reported about constitutively activated rat α_{2A}-adrenoceptors. It is also possible that the influence of rauwolscine on ERK activation, but not for cAMP inhibition in this study, might be ascribed to an antagonist bias (i.e. affecting one pathway and not affecting another) [35, 36]. It is also possible that α_2-adrenoceptors mediate the ERK phosphorylation through an adenylyl cyclase-independent cascade in this study. ERK phosphorylation might be mediated through $G_{\beta\gamma}$ subunit. This is in line with that overexpressed $G_{\beta\gamma}$ subunit in CHO cells was shown to activate MAP kinase [37]. As activation of α_2-adrenoceptors was shown in this study to stimulate ERK, and ERK signal transduction was traditionally suggested to growth related process [38-40], it is possible that α_2-adrenoceptors have a role in skeletal muscle growth. The implication of this is that long-term treatment of rats with α_2-adrenoceptor agonists should stimulate skeletal muscle growth and lean weight gain.

A series of investigations of cAMP levels was performed for CB_1 and NPY Y_1 receptors. It is well known that the

CB_1 receptor is coupled to G_i in many tissues [41]. In this study, the mRNA expression of CB_1 was detected in rat skeletal muscle culture and tissue using QRT-PCR (Taqman). The CB_1 receptor is very highly expressed in the brain [42], but also many studies have found that the CB_1 receptor is expressed in peripheral tissues, including adipose and skeletal muscle tissues. However, to date, no specific role(s) of CB_1 in the skeletal muscle has been fully understood.

Neither basal nor forskolin (1 µM)–evoked elevation of cAMP was altered significantly in myotube cells in the presence of ACEA (10 nM), AEA (10 µM) or RIM (100 nM) for 10 minutes. Indeed, the concentration which was used for ACEA and AEA in this study is in line with that (10 nM) and (10 µM), respectively shown to activate ERK in rat myotube cells (Figure 6), and the concentration which was used for RIM in this study is also in line with that (100 nM) shown to block ERK phosphorylation (Figure 6). However, it was shown that cannabinoid receptor ligands produce a CB_1 receptor-dependent reduction in cAMP levels in transfected CHO cells [18] and different rat brain regions [43], and treatment of rat L6 myotube cells with 100 nM RIM for 24 hour was also shown to increase intracellular cAMP production [44]. The Esposito *et al* study is not consistent with the present study. This is due to the fact that rat L6 cells are different from rat primary skeletal muscle cells, and the exposure time of RIM is also different between two studies. It is worth noting that CB_1 receptor is functionally-active receptor depending on *cnr1* was detected in skeletal muscle tissue using QRT-PCR (Taqman), and the activation of CB_1 receptor by ACEA phosphorylates ERK1/2 in CB_1 dependent manner (data was presented in Figure 6 for convenience).

NPY Y_1 receptors are expressed throughout the central and peripheral nervous systems, where the receptor mediates a variety of responses such as the regulation of metabolism and food intake [45, 46]. Several NPY Y_1 receptor antagonists were developed as potential anti-obesity agents [47]. NPY Y_1 receptor mRNA is also expressed primarily in kidney, heart and skeletal muscle and vascular smooth muscle [48]. Activation of NPY Y_1 receptors mainly inhibits adenylyl cyclase via G_i proteins [49].

Nothing is known in the literature about possible roles for the NPY Y_1 receptor in skeletal muscle. However, neither

basal nor forskolin-evoked elevation of cAMP concentration was altered after treatment of myotubes with the selective NPY Y_1 receptor agonist [Leu^{31}, Pro^{34}] Neuropeptide Y [50]. The concentration which was used in this study for NPY is in line with that (300 nM) shown to inhibit cAMP accumulation in rat slices of the dorsomedial medulla [51, 52].

Regarding signalling via G_i-GPCRs (including α_{2A}-adrenoceptors, A_1 adenosine receptor, CB_1 receptor and NPY Y_1 receptor), the activation of these GPCRs did not inhibit forskolin-evoked cAMP. The different explanations behind the lack of response for the activation of G_i-GPCRs might be due to: 1) As skeletal muscle expressed *adcy2 and adcy6*, it is possible that decrease in cAMP level by inhibition of AC2 and AC6 through G_i protein might be neutralized by activation of AC through $G_{\beta\gamma}$ subunit. This is supported by the fact that $G_{\beta\gamma}$ subunit was found to increase AC2 activity in insect ovarian Sf9 cells infected with recombinant baculovirsus (B-rACII) [53], and coexpressed G_i protein in Sf9 cells was also found to inhibit AC2 and AC6 activity [54]. 2) The receptors might not couple to G_i protein subunit, therefore, no effect was observed for ligands. 3) G_i protein might not be expressed in skeletal muscle; a potential significant influence is that the conditions for culturing myotubes are different from those *in vivo*, including intermittent innervation and variable (time, concentration, etc.) exposure to hormones. 4) The mismatch between mRNA and protein expression of these receptors, as suggested previously. As activation of any of the identified G_i GPCRs failed to decrease cAMP levels, it is strongly supported that these GPCRs did not couple to G_i subunit. However, further investigation is suggested to examine the protein expression of these receptors and G_i subunit using immunoblotting and immune cytochemistry. Moreover, investigation is recommended to test the signalling of these receptors using cAMP assay in presence of electrode to mimic the *in vivo* conditions for this primary cell culture. Further investigation is also suggested to examine the coupling of these receptors to G_i protein using pertusis toxin, for example, the effect α_{2A}-adrenoceptors of ERK phosphorlyation can be examined if ERK phosphorlyation occurred through coupling to G_i protein. Indeed, inhibition of ERK phosphorlyation should be observed in the presence of pertusis toxin. Furthermore, siRNA for

$G_{\beta\gamma}$ subunit is suggested to examine the effect of these GPCRs activation on the cAMP level.

These findings did not provide direct evidence for G_i-coupling for any G_i-GPCR tested, including the CB_1 receptor. The impact of the CB_1 receptor in skeletal muscle should be the subject of further investigations.

This study gave a picture and guide for the some GPCRs detected in skeletal muscle, their signaling protein genes in skeletal muscle, which has not previously been reported, in order to be investigated later.

CONCLUSION

These findings provided evidence for functionally-active CB_1 cannabinoid receptor and (potentially) α_2-adrenoceptors in skeletal muscle which might be important for skeletal muscle fat and glucose metabolism and skeletal muscle growth. However, these findings did not provide direct evidence for G_i-coupling for G_i-GPCR tested, including the A_1 adenosine receptor.

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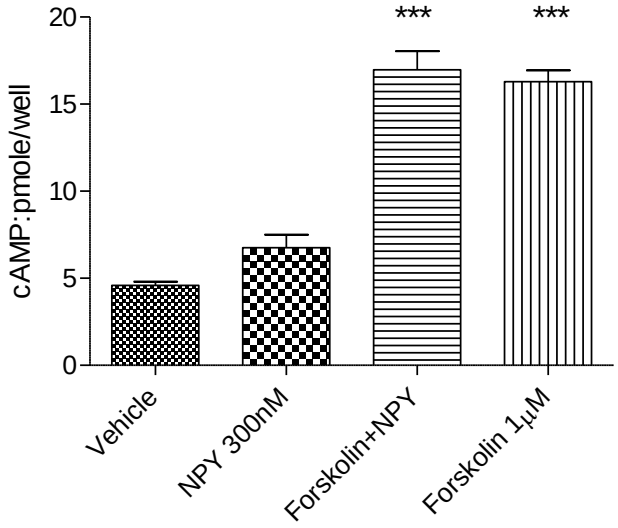

Figure 1: Effect of NPY on cAMP accumulation in myotubes (n=2 rats). cAMP production was measured following incubation of cells for 10 minutes with vehicle (0.01% DMSO), [Leu31, Pro34] Neuropeptide Y (NPY) (300 nM) and forskolin (1 µM). (***) p < 0.001 versus vehicle or NPY. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.

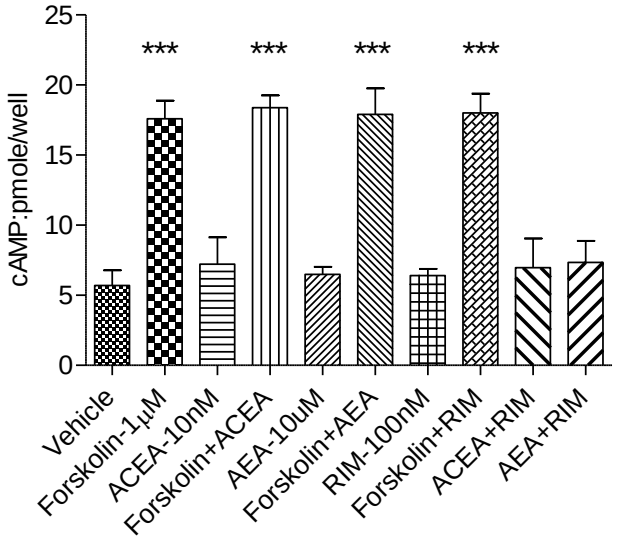

Figure 2: Effect of ACEA, AEA and RIM on cAMP accumulation in myotubes (n=2 rats). cAMP production was measured following incubation of cells for 10 minutes with vehicle (0.01% DMSO), cannabinoids and forskolin, ACEA (10 nM), AEA (10 µM), forskolin (1 µM) or RIM (100 nM) 30 minutes prior. (***) p < 0.001 versus vehicle, ACEA, AEA or RIM. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.

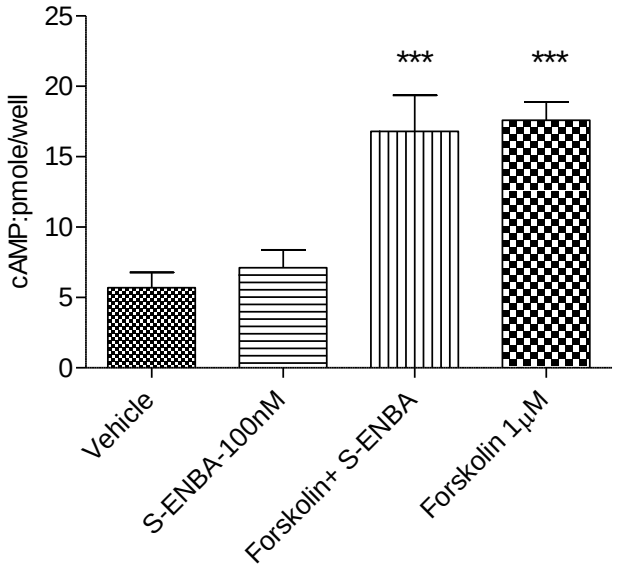

Figure 3: Effect of S-ENBA on cAMP accumulation in myotubes (n=2 rats). cAMP production was measured following incubation of cells for 10 minutes vehicle (0.01% DMSO), S-ENBA (100 nM) and forskolin. (***) p < 0.001 versus vehicle or S-ENBA. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc

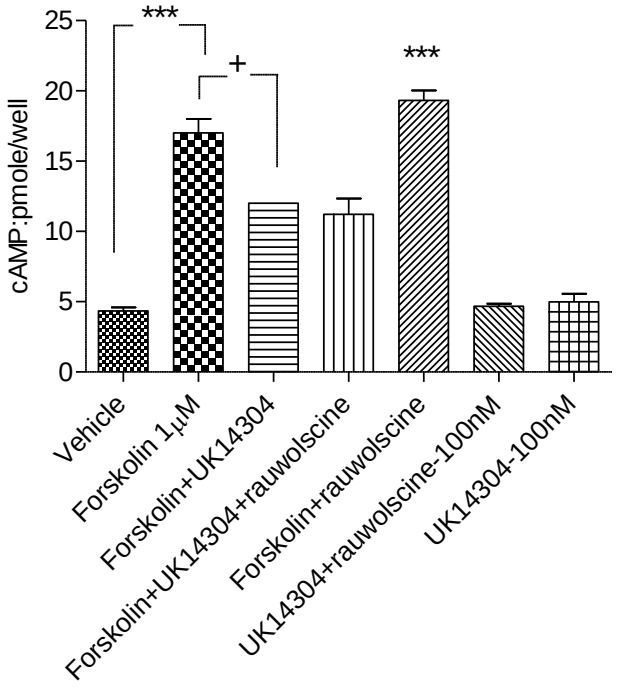

Figure 4: Effect of UK14304 and rauwolscine on cAMP accumulation in myotubes (n=2 rats). cAMP production was measured following incubation of cells for 10 minutes with vehicle (0.01% ethanol), UK14304 (100 nM) and rauwolscine (100 nM). * vs basal. + vs forskolin. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.

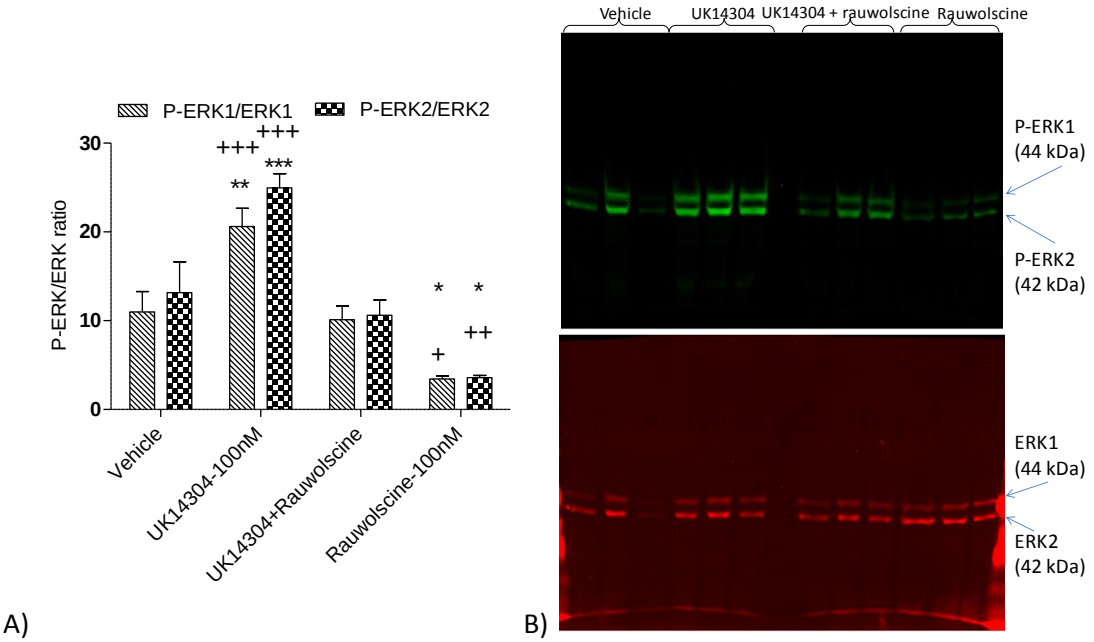

Figure 5: Effect of UK14304 and rauwolscine on phosphorylation of ERK in rat primary skeletal muscle cells (n=2 rats). A) Myotubes were treated with vehicle (0.01% ethanol), UK14304 (100 nM) for 10 minutes, UK14304 for 10 minutes + rauwolscine (100 nM) 30 minutes prior. * ± UK14304. + ± rauwolscine. B) Representative blots showing myotubes treated with vehicle (0.01% ethanol), UK14304 (100 nM) for 10 minutes + rauwolscine (100 nM) 30 minutes prior, phospho-ERK 1/2 (green bands), ERK 1/2 (red bands). Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.

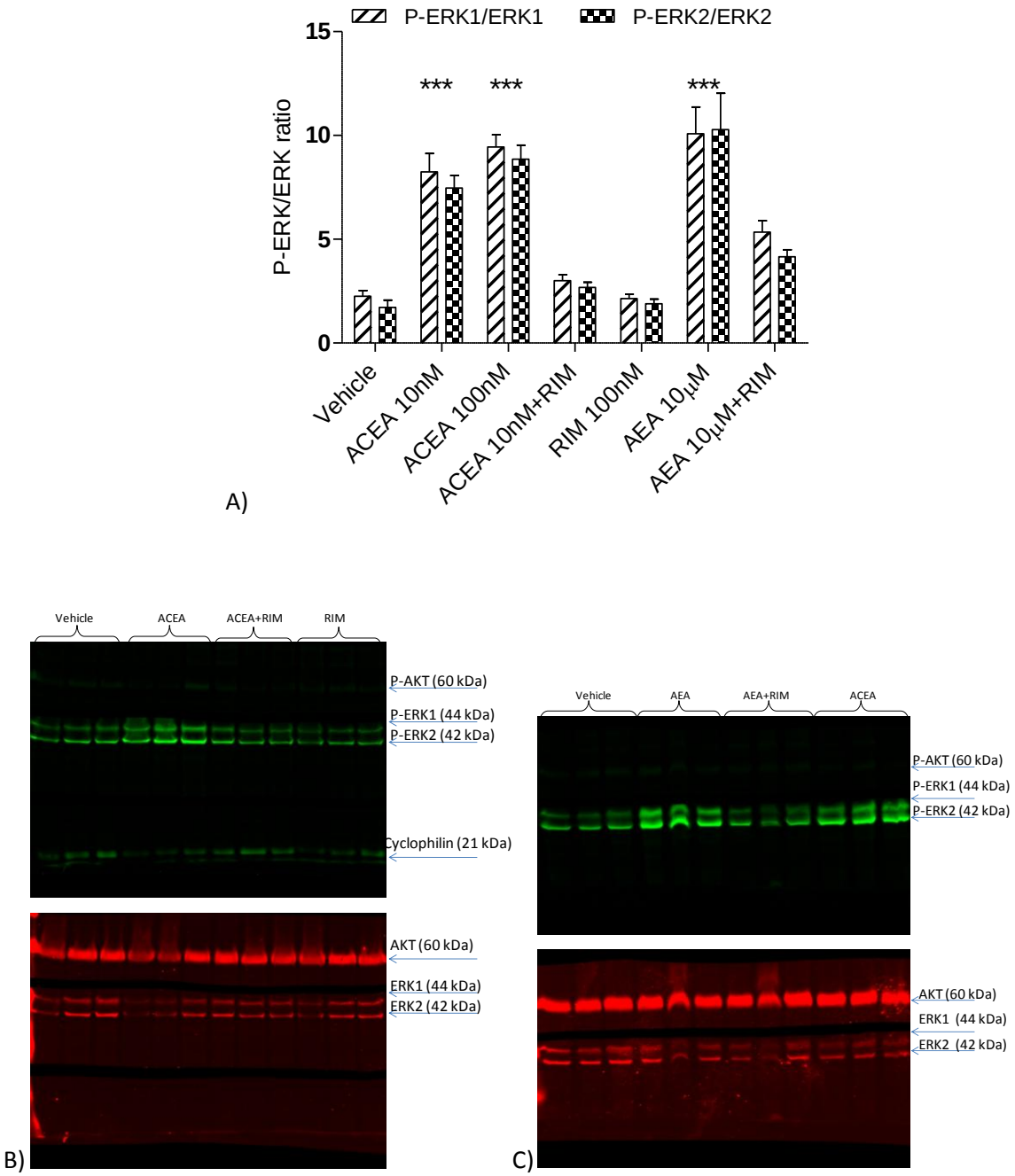

Figure 6: Effect of ACEA, AEA and RIM on phosphorylation of ERK in rat primary muscle cells (n=2 rats). A) Myotubes were treated with vehicle (0.01% ethanol), ACEA (10 and 100 nM) or AEA (10 µM) for 10 minutes; RIM only for 40 minutes; RIM for 30 minutes before the addition of either ACEA or AEA. * denotes P<0.001 when compared to vehicle, RIM and agonist+RIM conditions. B and C) Representative blots showing rat primary muscle cells treated with vehicle (0.01% ethanol), ACEA (10 nM) in B figure, ACEA (100 nM) in C figure, and AEA (10 µM) for 10 minutes and RIM for 30 minutes. Phospho-ERK 1/2 is shown in green bands and total ERK 1/2 in red bands. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.**

Table 1: Relative intensity values for selected detected GPCRs (comprising three major families of G protein signalling) in all skeletal muscle replicates. Site A is a mixture of extensor digitorum longus and tibialis anterior, site B is a mixture of soleus and plantaris, and site C is a mixture of red and white gastrocnemius muscle with roughly equal amounts of each muscle. The GPCRs was selected depending on known and available ligands local expertise, cost and above either cholinergic subunit (*chrnα1*) or *tbp* relative intensity values. NA: Not Applicable.

GPCR (gene name) [gene id]		Rat A			Rat B		
		Site					
	Principal G protein	A	B	C	A	B	C
Purinergic receptor P2Y, G-protein coupled 2 (P2ry2), mRNA [NM_017255]	G_q	1.7	1.2	0.9	1.4	1.6	1.7
Adrenergic receptor, β 2 (Adrb2), mRNA [NM_012492]	G_s	1.2	0.8	1.2	1.1	1.1	0.9
Adenosine A2a receptor (Adora2a), mRNA [NM_053294]	G_s	0	-0.2	0.5	0.7	0.5	0.3
Pyrimidinergic receptor P2Y, G-protein coupled, 6 (P2ry6), mRNA [NM_057124]	G_q	-0.3	-0.7	0.1	0	1	0
Adenosine A1 receptor. [Source:Uniprot/SWISSPROT;Acc:P25099]	G_i	-0.6	-1.3	-0.7	-0.5	-0.4	-0.6
Adrenergic receptor, alpha 2a (Adra2a), mRNA [NM_012739]	G_i	0.2	0.1	-1.2	-0.5	-1.2	-1.5
Purinergic receptor P2Y, G-protein coupled 1 (P2ry1), mRNA [NM_012800]	G_q	-1.2	-1.2	-1.9	-1.8	-1.7	-0.9
Neuropeptide Y receptor Y1 (Npy1r), mRNA [NM_001013032]	G_i	-1.2	-1.1	-2.8	-1.8	-1	-1.7
TATA box binding protein (Tbp), mRNA [NM_001004198]	NA	-0.2	0	0.3	0.3	0.8	-0.2
Cholinergic receptor, nicotinic, alpha 1 (muscle) (Chrna1), mRNA [NM_024485]	NA	0.1	-0.6	-0.7	1.1	1.5	0.3

Table 2: Ct values for QRT-PCR (Taqman) for GPCR entities and reference genes for skeletal muscle cells and tissues and for liver and adipose tissues from two replicates from two rat repeats.

Gene/Ct value	Skeletal	Myotube	Myoblast	Liver	Adipose
Cnr1	26.5-27.0	27.5	27.5	31.0	27.5
Adra2a	31.0-32.0	30.0	28.0	37.0	30.0