

# Adenosine Receptors Machinery and Purinergic Receptors in Rat Primary Skeletal Muscle Cells

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## ABSTRACT

GPCRs are the largest family of proteins in the human genome. One family of GPCRs is adenosine receptors, which are divided into  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$  adenosine receptors based on pharmacology and coupling to cAMP production. Another family of receptors is P2Y receptors, which are divided into P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>. Indeed, GPCRs are targets 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 adenosine and purinergic receptors highly expressed in skeletal muscle and in cultured preparations thereof. Skeletal muscle cells were cultured from vastus lateralis obtained from male Wistar (180-200 g) rats as Blau and Webster method with slight modification (Blau *et al.*, 1981). mRNA expression, cAMP and calcium measurement were examined by conventional methods (Akaneya *et al.*, 2006; Hudson *et al.*; Millns *et al.*, 2001). Data were reported as means of triplicate or quadruplicate wells generated from two animals. Statistical analysis was conducted using one or two-way ANOVA with Bonferroni's multiple comparisons test. mRNA encoding G<sub>s</sub>- (*adora2a*, *adora2b*), G<sub>i</sub>- (*adora1*), and G<sub>i</sub>-coupled (*p2ry1*, *p2ry2* and *p2ry6*) receptors were detected using gene microarray (Agilent, all ranked <24000 out of 41090). Treatment of myotubes with NECA (5'-N-ethylcarboxamidoadenosine) ( $10^{-7}$  and  $10^{-5}$  M) for 10 minutes elicited a significant increase in cAMP ( $18 \pm 0.33$  compared to basal levels of  $4.3 \pm 0.15$  pmol/well), which were significantly inhibited in the presence of the  $A_{2B}$  antagonist PSB603 (Fredholm *et al.*) ( $10^{-5}$  M,  $5.3 \pm 0.33$  pmol/well). Treatment of myotubes with the  $A_{2A}$ -selective agonist CGS21680 ( $10^{-7}$  M) failed to evoke a significant cAMP elevation. Neither basal nor forskolin (1  $\mu$ M)-evoked elevation of cAMP was altered in the presence of the  $A_1$ -selective agonist S-ENBA ( $10^{-7}$  M). All 748 myoblast cells (imaged from four animals; 150-250 cells/animal) showed an elevation in intracellular calcium levels in response to 1 mM ATP, with an increase in fluorescence ratio (the max of response (peak) obtained from drug minus the baseline ( $\Delta$ FR); Fura-2 340/380 nm excitation) of  $0.33 \pm 0.06$ . 575 cells responded to  $10^{-5}$  M UTP ( $0.18 \pm 0.08$   $\Delta$ FR). Responses to ATP were insensitive to the P2Y<sub>1</sub> antagonist MRS2179 ( $10^{-7}$  M). In summary, we observe expression and functional responses to example members of the adenosine and purinergic GPCR families in rat skeletal muscle preparations.

**Key words:** GPCRs, cAMP, calcium and myotubes, adenosine receptors, purinergic receptors.

## INTRODUCTION

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 (Kobilka, 2007; Tilakaratne *et al.*, 2005). GPCRs are expressed in every tissue and play a major role in many diseases.

One family, which was investigated, was adenosine receptors, which are divided into  $A_1$ ,  $A_{2A}$ ,  $A_{2B}$  and  $A_3$  adenosine receptors based on pharmacology and coupling to cAMP production.

A<sub>1</sub> and A<sub>3</sub> adenosine receptors are coupled to inhibitory G<sub>i</sub>-proteins, whereas the A<sub>2A</sub> and A<sub>2B</sub> adenosine receptors are coupled to stimulatory G<sub>s</sub> proteins. Another family of receptors, the P2Y receptors, was investigated using Ca<sup>2+</sup>-imaging. P2Y receptors are a family of plasma membrane GPCRs involved in several cellular functions and are divided into P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>.

Skeletal muscle is a heterogeneous tissue since it contains a variety of fibres that differ in contractile, functional, metabolic and molecular characteristics (Pette *et al.*, 1997; Staron *et al.*, 1999). 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 (Delbono *et al.*, 2007; Pedersen, 2011). Indeed, skeletal muscle utilizes the majority (70-80%) of ingested glucose since it is the main site for insulin-dependent glucose uptake (Toft *et al.*, 1998). Therefore, it is generally considered the most important site of insulin resistance.

Myotubes are primary skeletal muscle cells sharing the morphological, metabolic and biomedical characteristics and properties of adult skeletal muscle (Henry *et al.*, 1995; Raymond *et al.*, 2010). This paper showed that mRNA for many GPCRs were expressed in skeletal muscle tissues; notably A<sub>1</sub> and A<sub>2A</sub> adenosine receptors, P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors. Since there has been little evaluation of GPCR signalling in myotubes, and it was not possible to investigate all the GPCR apparently expressed in skeletal muscle, some examples were chosen. This selection was based on an attempt to examine signalling through the three main groups of G protein, G<sub>s</sub>, G<sub>i</sub> and G<sub>q</sub>.

From the top highly ranked GPCRs list detected using the microarray (Agilent, all ranked <24000 out of 41090) in skeletal muscle, A<sub>2A</sub> adenosine receptors were selected as G<sub>s</sub>-coupled, while A<sub>1</sub> adenosine receptors were selected as G<sub>i</sub>-coupled. This group (G<sub>s</sub> and G<sub>i</sub>-GPCRs) were tested by quantifying levels of cAMP. However, P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptor were selected as G<sub>q</sub>-coupled and were tested by assessment of levels of intracellular calcium ions.

## Aims

The purpose of the investigations in this study was to characterize the mRNA expression of adenosine and purinergic receptors that coupled to different G proteins (G<sub>s</sub>, G<sub>i</sub> and G<sub>q</sub>) in rat mixed fibre-type skeletal muscles using DNA microarray and to assess the downstream signalling of GPCRs in rat primary skeletal muscle cells using cAMP assay, calcium imaging and immunoblotting.

## MATERIALS AND METHODS

### Materials

NECA, CGS21680, PSB603 and S-ENBA were purchased from Tocris Company. All other materials were purchased from Sigma unless otherwise mentioned. 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). TriReagent was purchased from Invitrogen. FBS (fetal bovine serum) and Ham's F10 were purchased from PAA Laboratories (Somerset, UK). cAMP Kit was obtained from Cayman Chemical (Europe). Fura-2AM was bought from Calbiochem company.

### Tissue collection

Two male adolescent Wistar rats (180-200 g, 4-6 weeks old) were killed by cervical dislocation without anaesthesia. Skeletal muscle were obtained. Skeletal muscle (mixed fibre-type from hindlimb) tissues were then 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.

### Primary cell 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  $\mu$ m 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. Images were taken for the cells using a digital camera connected to microscope (Nikon) at X40 magnification (Figure 1).

#### Microarray procedure

Agilent 4\*44K DNA one color whole genome microarrays were used to measure the expression of 41090 genes in 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

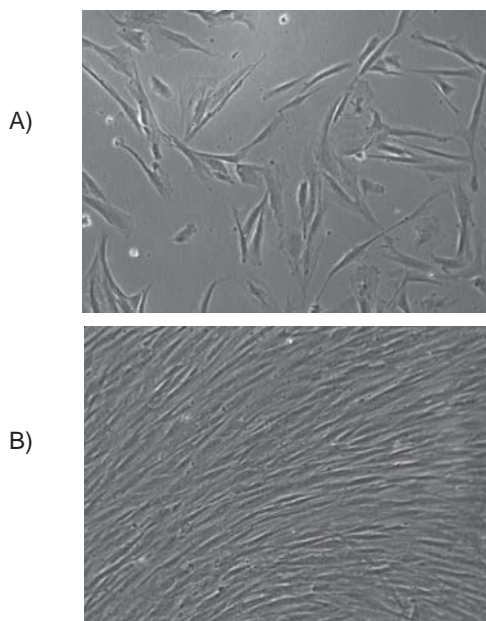
Myoblast and myotube cells were grown and differentiated as described above. The cells were collected in TriReagent, and processed according to the manufacturer's directions.

#### 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  $\mu$ l 5 M HCl was added to each well (2 ml media (Ham's F10), 6% horse serum), before cAMP measurement as described in the manufacturer's instructions.

#### Calcium imaging

Calcium imaging was performed as described previously (Millns *et al.*, 2001). In brief, satellite cells were grown on 19 mm glass coverslips and differentiated into myoblasts over around 14-21 days, myoblasts cultured onto glass coverslips were loaded with the Ca<sup>2+</sup>-sensitive fluorescent dye fura-2AM. The intracellular [Ca<sup>2+</sup>]<sub>i</sub> was quantified in single cells within myoblast clusters with the Ca<sup>2+</sup>-sensitive fluorescent dye fura-2AM using Andors IQ imaging system. Myoblast cells were washed with Ca<sup>2+</sup> buffer, then were incubated with 5  $\mu$ l 1 mM fura 2-acetoxymethyl ester (fura-2 AM) dissolved in 895  $\mu$ l buffer (NaCl, 145 mM; KCl, 5 mM; CaCl<sub>2</sub>, 2 mM; MgSO<sub>4</sub>.7H<sub>2</sub>O, 1 mM;



**Fig. 1: Representative myoblasts and myotubes derived from Wistar rat skeletal muscle. A) myoblasts taken during the third week of tissue culture and B) myotubes taken during sixth week of tissue culture**

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10 mM; glucose, 10 mM) with 100  $\mu$ l FCS for one hour and 15 minutes at 37 C°. Coverslips were fixed to a Perspex chamber using vacuum grease. A coverslip with adherent myoblast cells was positioned on the stage of a inverted fluorescence microscope and examined using Retiga chilled digital intensified charge-coupled device (CCD) camera. Myoblast cells were assessed by imaging for intracellular calcium ion levels as described previously (Chandra *et al.*, 1989; Millns *et al.*, 2001). Myoblast cells on the coverslips were exposed to UTP, ATP and ionomycin, as indicated, for one minute. In some instances, MRS2179 was perfused over the cells for 3 minutes prior to agonist addition. Peak ratios were calculated by subtracting the baseline fluorescence ratio (excitation at 340/380 and emission at 500 nm) from the fluorescence ratio obtained during drug superfusion ( $\Delta$ FR). The results are presented as means  $\pm$  standard error of mean (SEM) in change in fluorescence ratio units ( $\Delta$ FR) and mean  $\pm$  SEM of the area under the curve (AUC) in arbitrary units.

### Statistical analysis

Data were analyzed using one or two-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 were reported as means  $\pm$  standard error of mean (SEM) of triplicate or quadruplicate wells generated from two animals ( $n=2$ ). Statistics was performed from at least two repeats ( $n=2$  rats) for cAMP and western blot experiments.

## RESULTS

### mRNA expression of adenosine and purinergic receptors using Microarray

$A_1$  and  $A_{2A}$ -adenosine receptors and  $P2Y_1$ ,  $P2Y_2$  and  $P2Y_6$  receptors entities were detected in skeletal muscle tissue (ranked higher relative intensity values) than either nicotinic cholinergic alpha subunit (*chrn $\alpha$ 1*) or reference gene (TATA box binding protein (*Tbp*)) (12526 and 10220 ranking out of 41090, respectively)). These GPCR entities include three main families that coupled to

different G proteins ( $G_s$ ,  $G_i$  and  $G_q$ ) in this study. Examples of  $G_s$ -GPCRs are  $A_{2A}$ -adenosine receptors,  $G_i$ -GPCRs is  $A_1$  adenosine receptors, and  $G_q$ -GPCRs are  $P2Y_1$ ,  $P2Y_2$  and  $P2Y_6$  receptors. These examples of detected GPCR entities was investigated in this paper to examine the possible different signalling in skeletal muscle cells. Indeed, investigation of the different families ( $G_s$ ,  $G_i$  and  $G_q$ ) of GPCRs might help to confirm the expression and understand the expected signalling and functional role of these receptors.

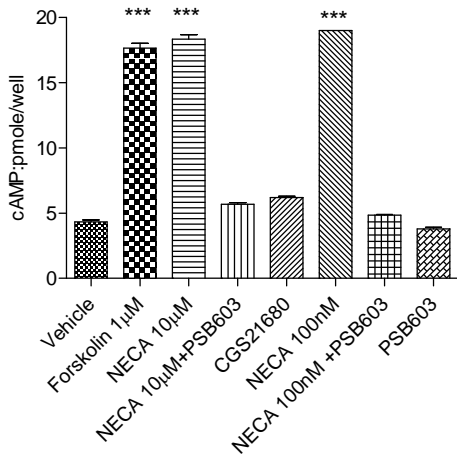
### Effect of adenosine receptor ligands on cAMP levels

mRNA encoding  $A_{2A}$  and  $A_1$  adenosine receptors was detected in skeletal muscle in this paper, where  $A_{2A}$  adenosine receptors were anticipated to elevate cAMP levels and  $A_1$  adenosine receptors would likely inhibit cAMP production.

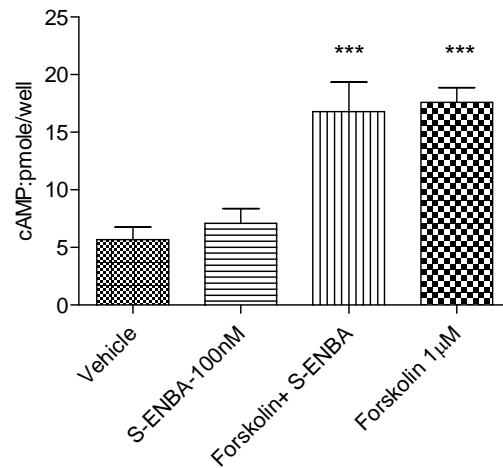
Treatment of myotube cells with the non-selective adenosine receptor agonist NECA (Castanon *et al.*, 1994; Klotz *et al.*, 1998) at concentrations anticipated to activate  $A_{2A}$  and  $A_{2B}$  adenosine receptors (100 nM and 10  $\mu$ M, respectively) for 10 minutes showed significant increases in cAMP compared to vehicle (0.01% DMSO). Treatment of myotube cells with the selective  $A_{2A}$  adenosine receptor agonist CGS21680 (100 nM) (Ongini *et al.*, 1999), however, did not induce any increase in cAMP. The stimulatory effect of NECA was significantly inhibited by the selective  $A_{2B}$  adenosine receptor antagonist PSB603 (10  $\mu$ M) (Borrmann *et al.*, 2009),  $p < 0.001$  (Figure 2).

The potential coupling of  $A_1$  adenosine receptors to inhibition of cAMP was investigated using the  $A_1$  receptor-selective agonist S-ENBA (Haynes *et al.*, 1998; Hussain *et al.*, 1995). However, neither basal nor forskolin (1  $\mu$ M)-evoked elevation of cAMP in myotube cells was altered in the presence of S-ENBA (100 nM) (Figure 3).

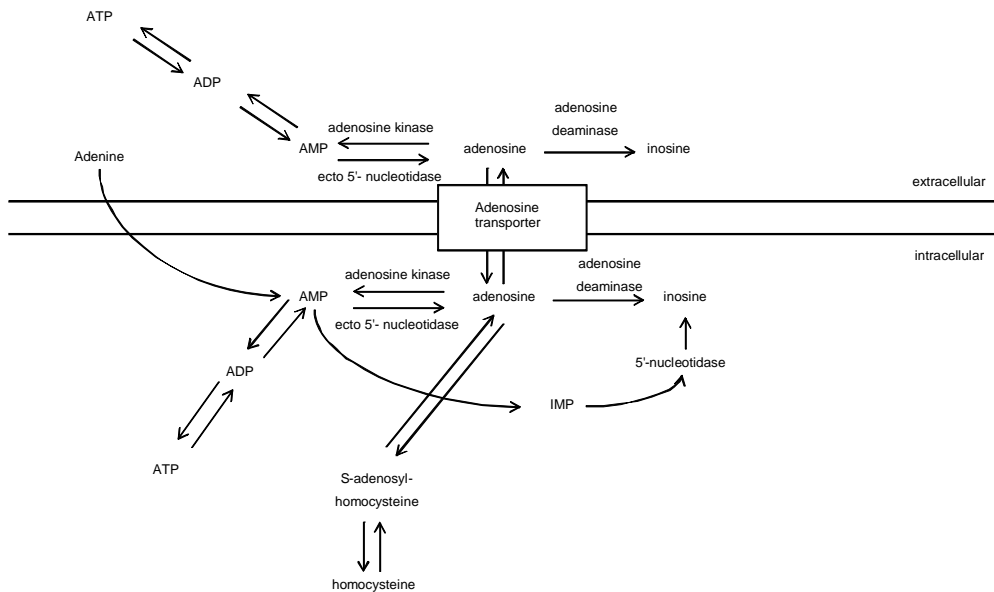
In order to investigate the presence of other components of the adenosine system (Figure 4), the expression for the enzymes and transporters required for adenosine cycling was assessed.



**Fig. 2: Effect of NECA, CGS21680 and PSB603 on cAMP accumulation in myotubes (n=2 rats). cAMP production was measured following incubation of cells for 10 minutes with vehicle (0.01% DMSO), NECA (100 nM and 10 μM), PSB603 (10 μM), CGS21680 (100 nM) and forskolin. (\*\*\*) p < 0.001 versus vehicle or NECA+PSB603. Data were analyzed using one way ANOVA test followed by Bonferroni post-hoc.**



**Fig. 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.**



**Fig. 4: Schematic summary of the regulation of extra- and intracellular adenosine and inosine concentrations. ATP: adenosine tri-(di-, mono-) phosphate; ADP; adenosine diphosphate; AMP; adenosine monophosphate. IMP; inosine monophosphate (Marshall, 2000; Spielman *et al.*, 1991).**

Adenosine deaminase, adenosine kinase and S-adenosylhomocysteine hydrolase mRNA expression were detected in the skeletal muscle tissue in this study. In addition, ecto-5' nucleotidase and ectonucleotide pyrophosphatase/phosphodiesterase 1, 2 and 3 mRNA were also detected in the skeletal muscle. Nucleoside transporters mRNA were detected as well (Table 1).

#### Elevation of intracellular calcium ion levels in myotubes

mRNA encoding P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors was detected using the microarray in this paper. As the majority of P2Y receptors are G<sub>q</sub>-coupled, Ca<sup>2+</sup>-imaging techniques were employed to investigate their functional coupling.

748 myoblast cells were imaged from four animals. ATP (1 mM) elevated intracellular calcium ion levels in all 748 myoblast cells (imaged from four animals; 150-250 cells/animal), with a peak increase in fluorescence ratio ( $\Delta$ FR; Fura-2 340/

380 nm excitation) of  $0.33 \pm 0.06$  after approximately one minute, while 575 cells responded to 10  $\mu$ M UTP ( $0.18 \pm 0.08$  FR). Thus, around 77% and 100% of cells responded to UTP and ATP, respectively (Figure 5) and (Figure 6).

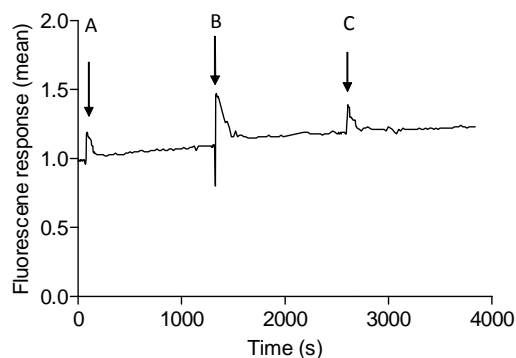
Two sets of myoblast cells were imaged from 4 different rats. One set (713 cells) were investigated for responses to ATP alone, while ATP responses in the other set (815 cells) were assessed after 3 minutes exposure to MRS2179, a selective P2Y<sub>1</sub> receptor antagonist (Boyer *et al.*, 1998). There was no significant difference in the response between the two sets either in AUC or in Peak (Figure 7).

#### DISCUSSION

GPCRs expression was examined in this paper using Agilent microarray. Adenosine and purinergic receptors entities were found to be expressed in skeletal muscle in this paper. These GPCRs are somewhat less well-known in terms of

**Table 1:** RNA transcript intensity values for genes related to adenosine machinery in mixed skeletal muscle from 3 replicates generated from two male Wistar rats. The number represents the relative intensity values for these genes which are all above *tbp* (TATA box binding protein) reference gene.

Gene	Gene name	Rat A			Rat B		
		A	B	C	A	B	C
Ada	Adenosine deaminase	2.1	1.7	1.8	1.6	1.7	2.0
Adk	Adenosine kinase	2.7	3.8	3.1	3.3	3.4	3.1
Ahcy	S-adenosylhomocysteine hydrolase	3	2.8	2.3	4.1	3.7	4.1
Slc28a1	Solute carrier family 28 (nucleoside transporters) 1	-1	-1.2	-1.2	-2.7	-3.1	-2.5
Slc28a2	Solute carrier family 28 (nucleoside transporters) 2	3.2	3	3.5	0.5	0.2	0.8
Slc29a1	Solute carrier family 29 (nucleoside transporters) 1	5	4.2	5.4	4.9	5	4.9
Nt5e	5' nucleotidase, ecto	-0.7	-0.7	-0.9	-0.5	-0.3	-0.7
Enpp1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	1.7	1.2	1.2	1.7	1.7	2
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	0.6	0.3	0.7	0.6	1.1	0.9
Enpp3	Ectonucleotide pyrophosphatase/phosphodiesterase 3	1.4	1.2	2.1	1.6	2.3	2.3
Tbp	TATA box binding protein	-0.2	0.0	0.3	0.3	0.8	-0.2

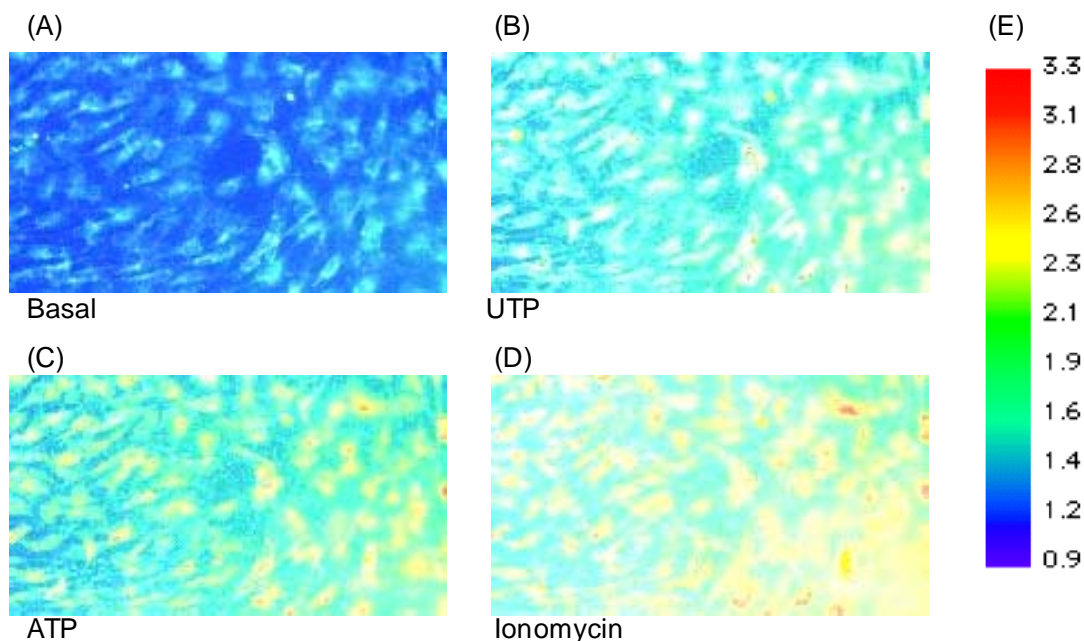


**Fig. 5:** A representative trace showing changes in 340:380 nm excitation wavelength ratios in myoblast cells, in response to UTP, ATP and ionomycin. Recording of 340:380 ratio were made at various time points, where at point A, the cells were exposed to UTP (10  $\mu$ M) for one minute, twenty minutes later, at point B, the cells were exposed to ATP (1 mM), twenty minutes later, at point C, the cells were exposed to ionomycin (4  $\mu$ M); (imaged from four animals; 150-250 cells/animal).

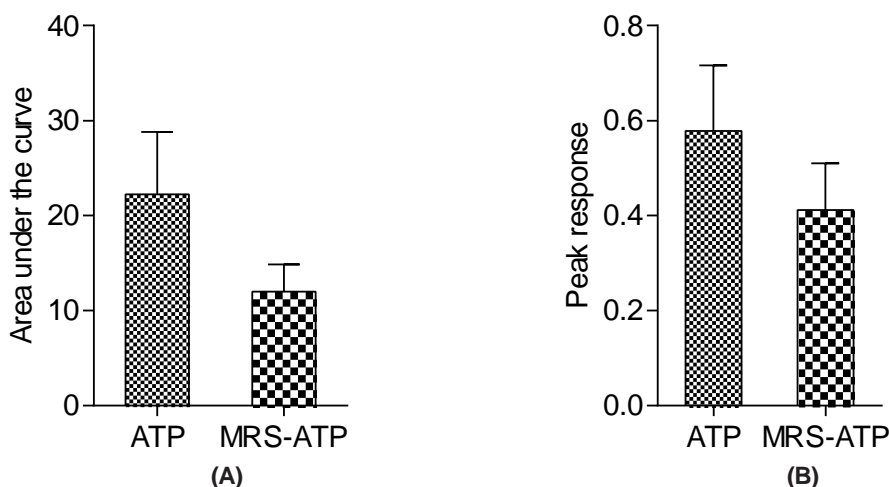
their activity in the skeletal muscle system. Relatively little information in the literature is reported in both normal and disease state about the role of these GPCRs in functional activities and in signal transduction of skeletal muscle tissues. Regarding 38 CPCR detected in skeletal muscle in this paper, three main families of GPCRs ( $G_s$ ,  $G_i$  and  $G_q$ ) were detected in skeletal muscle tissue using the microarray. Examples include  $G_s$ -GPCR,  $A_{2A}$  adenosine receptors),  $G_i$ -GPCR ( $A_1$  adenosine receptors) and  $G_q$ -GPCR ( $P2Y_1$  and  $P2Y_2$  receptors). These GPCRs were investigated in this paper for further signalling and functional roles in skeletal muscle.

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  $Ca^{2+}$ ) were conducted to assess their functionality.

cAMP is an important second messenger in skeletal muscle. It was found to increase the



**Fig. 6.** Ratiometric pseudocolour images of myoblast cells. A; basal, B, myoblast cells following exposure to 10  $\mu$ M UTP; C, myoblast cells following exposure to 1 mM ATP; D, myoblast cells following 4  $\mu$ M ionomycin; E, colour scale, arbitrary numbers representing 340:380 ratios.



**Fig. 7. Pre-exposure of myoblast cells to the P2Y<sub>1</sub> receptor antagonist (MRS2179) (10  $\mu$ M, for 3 minutes) on the effects of ATP (1 mM)-evoked peak response (A) and AUC (in arbitrary units) (B) in the whole population of cells. Myoblast cells showed an elevation in intracellular calcium levels in response to 1 mM ATP; imaged from four animals; 150-250 cells/animal. Data were analyzed using Paired t test**

expression of members of the orphan family of nuclear receptors, subfamily 4 (NR4A), compared to other nuclear receptors in skeletal muscle (Maxwell *et al.*, 2005). 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- $\gamma$  coactivator (PGC-1 $\alpha$ ), phosphatidate phosphatase LPIN1 (lipin-1 $\alpha$ ), GLUT4 and muscle phosphofructokinase (Pfk<sub>m</sub>) (Chao *et al.*, 2007; Kanzleiter *et al.*, 2010; Lessard *et al.*, 2009). The implication of this is that activation of NR4A receptors might improve glucose glycolysis, glucose transport and lipid oxidation in skeletal muscle, and consequently NR4A receptors might be a therapeutic target for diabetes and obesity. Another critical second messenger is calcium, which transduces extracellular signals into numerous intracellular events in many cell types. Indeed, the functions of calcium range from short-term responses, such as contraction and activation of some enzymes (such as adenylyl cyclase), to longer-term responses such as gene expression (Berridge, 1997). In skeletal muscle, calcium has a crucial role for contraction (Berchtold *et al.*, 2000). When calcium is released from sarcoplasmic

reticulum, it binds to troponin and pulls tropomyosin allowing the myosin to bind to the actin, consequently, contraction occurs (Berchtold *et al.*, 2000). Calcium might also improve contraction-stimulated glucose uptake through activating GLUT4 translocation, calmodulin-dependent protein kinases, calmodulin and protein kinase Cs (Ihlemann *et al.*, 1999; Jessen *et al.*, 2005; Wright *et al.*, 2004; Youn *et al.*, 1991). However, the mechanism is still unclear.

In this paper, A<sub>1</sub> and A<sub>2A</sub> adenosine receptor mRNA was detected in skeletal muscle tissue using the microarray. This is consistent with a previous study that showed that mRNA encoding for adenosine A<sub>1</sub> and A<sub>2A</sub> receptors was detected in rat primary skeletal muscle cells and tissues using northern blot (Lyng *et al.*, 2003).

In order to examine the signalling of adenosine receptors and particularly to investigate the G<sub>s</sub>-coupling GPCRs, we examined cAMP accumulation in primary rat skeletal muscle cells. NECA, a non-selective adenosine receptor agonist (Castanon *et al.*, 1994; Klotz *et al.*, 1998), was used to examine activation of the A<sub>2A</sub> or A<sub>2B</sub> adenosine receptor, while S-ENBA was used to examine activation of the A<sub>1</sub> adenosine receptor. S-ENBA



did not show any inhibition of cAMP, although the concentration of S-ENBA chosen is consistent with that (100 nM) shown to increase whole cell currents of both inner-wall and cannula-derived human Schlemm's canal cells through  $A_1$  adenosine receptors (Karl *et al.*, 2005). However, little in the literature is reported about rats. The lack of response to  $A_1$  adenosine receptor stimulation in this study is in line with a previous study that showed forskolin-evoked cAMP was not suppressed by the  $A_1$  adenosine receptor agonist, R-PIA in rat primary skeletal muscle (Lynge *et al.*, 2003).

However, NECA, a non-selective agonist for adenosine receptors (Castanon *et al.*, 1994; Klotz *et al.*, 1998), stimulated cAMP accumulation at both 100 nM and 10  $\mu$ M. The concentration of NECA which was used in this study is in line with that (100 nM) shown to increase cAMP levels in rat coronary endothelial cells (Bindewald *et al.*, 2004). To examine which adenosine receptor ( $A_{2A}$  or  $A_{2B}$ ) leads to increases in cAMP levels, CGS21680, an  $A_{2A}$  adenosine receptor-selective agonist (Ongini *et al.*, 1999) and PSB603, an  $A_{2B}$  adenosine receptor-selective antagonist (Borrmann *et al.*, 2009), were employed. CGS21680 did not alter cAMP levels. However, PSB603 blocked the effect of NECA. This is consistent with a previous study which showed that the stimulation of  $A_{2B}$  adenosine receptors by NECA activates adenylyl cyclase and increased cAMP selectively, and not the  $A_{2A}$  adenosine receptors which were not stimulated by CGS21680 in rat primary skeletal muscle cells (Lynge *et al.*, 2003). The concentration which was used in this study for PSB603 is in line with that (10  $\mu$ M) shown to inhibit adenosine-induced bicarbonate ion secretion in duodenal rats through  $A_{2B}$  adenosine receptors (Ham *et al.*, 2010). Similarly, the concentration of CGS21680 used in this study is also in line with that (100 nM) shown to elevate rabbit carotid body cAMP content (Chen *et al.*, 1997), and (100 nM) shown to enhance cAMP accumulation in hippocampal nerve terminals of aged rats (Rebola *et al.*, 2003). Therefore, the  $A_{2B}$  adenosine receptor is an active receptor with regards to cAMP modulation. Regarding  $A_{2A}$  adenosine receptor, the lack of response for  $A_{2A}$  adenosine receptors might indicate that the number of  $A_{2A}$  adenosine receptors might be too small to elicit adenylyl cyclase activation. Another

explanation for the lack of effect of  $A_{2A}$  adenosine receptor activation is the mismatch between mRNA and protein expression. Since skeletal muscle contains multiple cell types (satellites, myoblasts and myotubes), receptor expression might be restricted to a specific cell type.

The mRNA expression of adenosine receptors, together with the functional coupling of the  $A_{2B}$  adenosine receptor, suggests a role for adenosine in skeletal muscle function.  $A_{2B}$  adenosine receptor activation was found to increase NR4A expression in smooth muscle (Mayer *et al.*, 2011), it is possible, therefore, that  $A_{2B}$  adenosine receptors affect NR4A through the cAMP pathway in skeletal muscle, and consequently,  $A_{2B}$  receptors might modulate fat and glucose metabolism in skeletal muscle tissue. This is supported by the fact that 1) NR4A mRNA was found to be expressed in skeletal muscle using microarray in this study. 2) cAMP was found to be involved in increase of expression of NR4A in skeletal muscle (Kawasaki *et al.*, 2011; Pearen *et al.*, 2008; Pearen *et al.*, 2006). 3) NR4A was shown to be reduced in skeletal muscle of diabetic animals (Fu *et al.*, 2007). 4) NR4A was associated with genes related to glucose and fatty acid utilization through up-regulating the mRNA expression of PDK4, FOXO1, PGC-1 $\alpha$  and lipin-1 $\alpha$  (Pearen *et al.*, 2008). 5) NR4A null mice after high-fat feeding compared with wild-type animals was shown to exhibit decreased mRNA expression of GLUT4 and PDK4 and Lipin 1 $\alpha$  and impaired insulin receptor substrate 1 (IRS-1) phosphorylation and insulin resistance in skeletal muscle, and slower blood glucose clearance and increased body weight and decreased energy usage (Chao *et al.*, 2009). 6) In C2C12 cells, C2C12 siRNA-NR4A cells were shown to decrease mRNA expression of fatty acid translocase (CD36/fat), uncoupling protein-3 (UCP3) and GLUT4 compared to wild type native C2C12 cells (Maxwell *et al.*, 2005). 7) In C2C12 cells transfected with adenovirus-mediated NR4A expression, non-insulin glucose uptake was shown to be increased significantly compared to normal C2C12 cells (Chao *et al.*, 2007). Taken together, modulation of  $A_{2B}$  adenosine receptor by ligands might affect glucose and fatty acid utilization in skeletal muscle. Therefore, the implication of this is that  $A_{2B}$  adenosine receptor agonists should be

recommended to be investigated as a therapeutic option in diabetes or obesity.

Adenosine could reach skeletal muscle either from the bloodstream, the motor neuron innervations or the skeletal muscle itself. As skeletal muscle expressed ecto-5'-nucleotidase (ecto-5'-NT) and ecto-phosphodiesterases in this study (Table 1), it is possible that skeletal muscle mediates adenosine generation through the extracellular cAMP-adenosine pathway. This is supported by the fact that interstitial accumulation of 5'-AMP (intermediary metabolite of adenosine synthesis) and adenosine was observed after incubation newborn rat skeletal muscle cells with exogenous cAMP (Chiavegatti *et al.*, 2008). This effect was inhibited by using the ecto-phosphodiesterase (1, 3-dipropyl-8-sulfophenylxanthine (DPSPX)) and ecto-5'-NT (alpha, beta-methylene adenosine 5'-diphosphate (AMPCP)) inhibitors (Chiavegatti *et al.*, 2008; Tofovic *et al.*, 1991; Zimmermann, 1992) in which ecto-phosphodiesterase inhibitors inhibit the conversion of cAMP to 5'-AMP and ecto-5'-NT inhibitors inhibit the conversion of 5'-AMP to adenosine.

Furthermore, adenosine could be also transported into or out of the rat primary skeletal muscle cells through an equilibrative nucleoside transporter, ENT1 (SLC29A1) transporter. This may be due to the fact that when incubation rat primary skeletal muscle cells with the adenosine transporter inhibitor nitrobenzylthioinosine, the ENT1 transporter inhibitor (Ackley *et al.*, 2003; Geiger *et al.*, 1985), the rate of extracellular adenosine accumulation in the electro-stimulated muscle cells was larger compared with control cells (Lynge *et al.*, 2001).

In addition, adenosine can be metabolized in skeletal muscle through adenosine kinase and adenosine deaminase, producing adenosine monophosphate (AMP) and inosine, respectively. Inosine was shown to be implicated in cellular proinflammatory responses to ischemia in mice skeletal muscle and was also reported to activate A<sub>3</sub> adenosine receptor in mast cells (Jin *et al.*, 1997; Wakai *et al.*, 2001). However, this issue is not understood yet. AMP generated by the action of adenosine kinase, can be used to regenerate ATP

in skeletal muscle. However, intracellular adenosine and homocysteine can be produced from S-adenosylhomocysteine (AdoHcy) through S-adenosylhomocysteine hydrolase (*ahcy*). Homocysteine could be then recycled into cysteine. Indeed, low plasma cysteine level was reported to be associated with progressive loss of human skeletal muscle mass (Droge *et al.*, 1998).

Another family of receptors, the P2Y receptors, was investigated using Ca<sup>2+</sup>-imaging. P2Y receptors are a family of plasma membrane GPCRs involved in several cellular functions and are divided into P2Y<sub>1</sub>, P2Y<sub>2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub>, P2Y<sub>11</sub>, P2Y<sub>12</sub>, P2Y<sub>13</sub> and P2Y<sub>14</sub>. In this paper, mRNA for P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>6</sub> receptors were detected in skeletal muscle using the microarray. This is consistent with a previous study that these receptors were also expressed in mouse C2C12 myoblast and myotubes (Banachewicz *et al.*, 2005). However, in one study in the literature, the expression of P2Y<sub>1</sub> and P2Y<sub>2</sub> was not detected in human skeletal muscle fibre using immunoblotting and immunohistochemistry. Nevertheless, these receptors were expressed in the vasculature (Borno *et al.*, 2011). This might be ascribed to the difference in physiology between human and rat species or the disconnect between mRNA and protein levels, as suggested previously.

The vasodilatory effect of the purinergic system was shown to be ~50% lower in diabetic patients compared to control subjects, although the distribution and mRNA expression of receptors were similar in both groups (Borno *et al.*, 2011). Moreover, ATP, localized at the nerve terminal, may be released after stimulation of the prejunctional neurones, leading to activation of P2Y receptors. ATP was also shown to activate glucose uptake in mouse C2C12 skeletal muscle cells through P2 receptors (Kim *et al.*, 2002). Taken together, P2Y receptors might have a therapeutic role in skeletal muscle and diabetes. It is possible, therefore, that the activation of P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors by agonists in skeletal muscle might be therapeutic targets for diabetic patients.

Little is known about the effects of P2Y receptor activation on skeletal muscle. The effects of P2Y agonists (ATP and UTP) were tested on

levels of intracellular calcium. Intracellular calcium concentration was increased by UTP, which was mainly attributed to P2Y receptors, since UTP is a selective agonist for P2Y<sub>2</sub> receptors (El-Tayeb *et al.*, 2006). The explanation behind the fewer number of cells (77%) responding to UTP is possibly due to the fact that the P2Y<sub>2</sub> receptors expression might be different during the stage of differentiation for the cells or relative RNA expression level might not reflect the protein expression level in all cells population. Ionomycin was used as a positive control. Ionomycin is supposed to produce a maximum calcium response. However, in the protocol followed, in which I used ionomycin at the end of the protocol, it is possible that the small response of ionomycin and the differences in the background colour (Figure 6) are due to issues regarding the loading of the fura-2 dye.

As UTP was shown to increase calcium level in skeletal muscle cells in this paper, and UTP was shown to activate ERK dependant on calcium in mouse C2C12 myoblasts (Banachewicz *et al.*, 2005), and ERK was shown to have a role in myoblast proliferation (Bennett *et al.*, 1997; Jones *et al.*, 2001), it is possible that UTP through P2Y<sub>2</sub> receptor play a role in skeletal muscle growth. Moreover, as P2Y<sub>1</sub>, P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors mRNA was detected in skeletal muscle in this study, and it was also shown that at least UTP as a selective P2Y<sub>2</sub> receptor increase calcium level in skeletal muscle cells in this study, it is possible that P2Y receptor might play a role in contraction in skeletal muscle. The implication of this is that P2Y receptors might improve contraction-stimulated glucose uptake. Therefore, P2Y receptor agonists should be recommended to be investigated for diabetes.

The response for ATP was found to be higher than the response for UTP. This possibly suggests that ATP might work through the P2Y<sub>1</sub> receptor, not through the P2Y<sub>2</sub>, and that UTP might work through the P2Y<sub>2</sub>. MRS2179, the P2Y<sub>1</sub> receptor-selective antagonist (Boyer *et al.*, 1998) was investigated to block the effect of ATP. MRS2179 did not inhibit the effect of ATP.

The concentration which was used in this study for MRS2179 is consistent with that (10 µM) shown to prevent ERK activation induced by

oxygen and glucose deprivation in rat hippocampal slices (Traini *et al.*, 2011), and is also consistent with that observed to block P2Y<sub>1</sub> receptors associated with endogenous calcium activity in mouse astrocytic processes (Di Castro *et al.*, 2011). It might be that other receptors contributed to the response of ATP, such as P2Y<sub>2</sub>, P2Y<sub>6</sub> and P2X<sub>5</sub>. The concentration used in this study for ATP (1 mM) was possibly too high relative to concentration used for MRS2179 (10 µM), in particular ATP and MRS2179 has similar relative affinity for P2Y<sub>1</sub> receptors, Ki=48 nM and Ki=84 nM, respectively (Waldo *et al.*, 2002; Webb *et al.*, 1996). In other words, the concentration used for MRS2179 did not block the effect of ATP. Therefore, it is suggested to take the concentration used for ATP into consideration and perform concentration response curve for ATP or try different concentrations of both ATP and MRS2179. Further selective antagonists for P2Y<sub>1</sub> receptors, such as MRS2500 (Cattaneo *et al.*, 2004; Hechler *et al.*, 2006) could be also used to clarify the involvement of P2Y<sub>1</sub> receptors. Alternatively, siRNA investigations might allow definition of the role of particular P2 receptors in ATP- and UTP-evoked calcium responses.

As ATP increase calcium level in skeletal muscle in this study, and ATP was shown to stimulate the proliferation of cancer cells (Deli *et al.*, 2008), and to stimulate the proliferation of astrocytes via P2Y receptors (Neary *et al.*, 2009), it is possible that P2Y receptors play a role in skeletal muscle growth.

Regarding signalling via G<sub>i</sub>-GPCRs (A<sub>1</sub> adenosine 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<sub>i</sub>-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<sub>i</sub> protein might be neutralized by activation of AC through G<sub>βγ</sub> subunit. This is supported by the fact that G<sub>βγ</sub> subunit was found to increase AC2 activity in insect ovarian Sf9 cells infected with recombinant baculovirus (B-rACII) (Tang *et al.*, 1991), and coexpressed G<sub>i</sub> protein in Sf9 cells was also found to inhibit AC2 and AC6 activity (Taussig *et al.*, 1994). 2) The receptors might not couple to G<sub>i</sub> 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 immunocytochemistry. 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 pertussis toxin. Furthermore, siRNA for  $G_{\beta\gamma}$  subunit is suggested to examine the effect of these GPCRs activation on the cAMP level.

### CONCLUSION

These findings provided evidence for functionally-active  $A_{2B}$  adenosine receptors in skeletal muscle which might be important for skeletal muscle fat and glucose metabolism and skeletal muscle growth. These findings also provide evidence for  $G_s$ -coupling for the  $A_{2B}$  adenosine receptors and  $G_q$ -coupling for  $P2Y_1/P2Y_2/P2Y_6$  receptors. However, these findings did not provide direct evidence for  $G_i$ -coupling for  $G_i$ -GPCR tested, including the  $A_1$  adenosine receptors.

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### Abbreviations

(GPCRs); G protein coupled receptors, (Adora2a); adenosine A2a receptor, (Adora2b); adenosine A2B receptor, (Adora1); adenosine A1 receptor, (P2ry1); purinergic receptor P2Y, G-protein coupled 1, (cAMP); cyclic adenosine monophosphate, (ATP); adenosine triphosphate, (UTP); uridine triphosphate, (NECA); 5'-N-ethylcarboxamidoadenosine, (PSB603); 8-[4-[4-(4-chlorophenyl) piperazine-1-sulfonyl]phenyl]-1-propylxanthine, (CGS21680); 2-(4-[2-carboxyethyl]-phenethylamino) adenosine-52 -N-ethyluronamide, (MRS2179); 2'-Deoxy-N6-methyladenosine 3', 5'-bisphosphate, (S-ENBA); (2S)-N6-(2-endorbanyl) 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, (NR4A); nuclear receptors, subfamily 4, (PDK4); pyruvate dehydrogenase kinase 4, (FOXO1); forkhead box protein O1, (PGC-1 $\alpha$ ); peroxisome proliferator-activated receptor- $\gamma$  coactivator, (lipin-1 $\alpha$ ); phosphatidate phosphatase LPIN1, (PfkM); muscle phosphofructokinase, (GLUT4); Glucose transporter-4, (MAPK); Mitogen activated protein kinase, (ERK 1/2); extracellular signal-regulated kinase 1/2, (SiRNA); Small interfering RNA, ( $\Delta$ FR); fluorescence ratio units, FBS; (fetal bovine serum), Fura-2AM; (L-[2-(carboxyloxazol-2-yl)-6-amino-benzofuran-5-oxy]-2-(2'-amino-5' methyl phenoxy)ethane-N,N,N,N-tetraacetic acid pentaacetoxymethyl ester), (DMEM); Dulbecco's Modified Eagle Medium, (PBS); phosphate buffered saline, (HEPES); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (SEM); standard error of mean, (AUC); area under the curve, (ANOVA); analysis of variance, (APS); ammonium persulphate, (cDNA); Complementary DNA, (Ct); Cycle threshold, (DMSO); Dimethyl sulfoxide, (mRNA); messenger ribonucleic acid, (PBS); Phosphate buffered saline, (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.

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