《Original Articles》

Expression of purinergic receptors on mouse brown adipocytes

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Abstract

Neurotransmitter receptors on brown adipocytes and sympathetic nerve fibers contribute to thermogenesis by mediating Ca^{2+} dynamics among brown adipocytes. We investigated the functional expression of purinergic receptor subtypes on brown adipocytes of mouse interscapular fat. Ca^{2+} imaging showed that applied 10 μM ATP, 10 μM BzATP (a $P2X_1$ and $P2X_7$ agonist), 1 μM 2MeSATP (a $P2Y_1$ and $P2Y_{11}$ agonist) or 100 μM UTP (a P2Y agonist) increased intracellular Ca^{2+} concentration. RT-PCR suggested the expression of $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{13}$ and $P2Y_{14}$ among the seven P2X subtypes and seven P2Y subtypes examined. Immunoblotting confirmed the expression of $P2X_1$ and $P2X_7$. These results showed the functionally expression of $P2X_1$ and $P2X_7$ on mouse brown adipocytes. The roles of purinergic receptor subtypes in the thermogenesis are discussed.

Keywords: brown adipocytes, ATP, receptor, Ca^{2+}

Introduction

Brown adipocytes are thermogenic organ. In response to cold exposure, heat is produced by uncoupling of respiration from ATP synthesis by the activation of uncoupling proteins under the enhanced hydrolysis of neural lipids by the β-adrenergic activation of noradrenaline which is released from sympathetic nerves [1, 2, 3]. Simultaneously, sympathetic nerves are thought to release ATP [4]. Extracellular ATP also evokes a number of responses in brown adipocytes [5, 6]. Brown adipocytes respond to both noradrenaline and ATP. Adrenergic stimulation increases membrane conductances [7], elevates intracellular Ca^{2+} concentration [8, 9, 10], and activates thermogenesis [11]. Purinergic stimulation by ATP also increases membrane conductances and intracellular Ca^{2+} concentration and initiates increases in heat production [12]. In addition, ATP but not noradrenaline increases membrane trafficking [6].

Mammalian purinergic receptors consist of seven P2X subtypes (ionotropic receptors: $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$) and eight P2Y subtypes (G protein-coupled receptors: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$). Omatsu-Kanbe et al. reported that they detected mRNA of $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor subtypes among seven P2X subtypes ($P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_6$ and $P2X_7$) and five P2Y subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) by using RT-PCR analysis in rat brown adipocytes [5]. However, the expression of mRNA of other subtypes, typically that of $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$ mRNA and the expression of receptor proteins in mice, remains to be identified. In the

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present study, we show the functional expression of $P2X_1$ and $P2X_7$ on mouse brown adipocytes, with a variety of techniques including Ca^{2+}-imaging, reverse transcriptase-mediated polymerase chain reactions (RT-PCR) and Western blotting. It is known that $P2X_1$ is mainly expressed in smooth muscle and has a role of synaptic transduction between neurons for sympathetic vasoconstriction in small arteries, arterioles and vas deferens [13]. $P2X_7$ is involved in apoptosis in addition to synaptic transduction [14]. These purinergic receptors contribute to important roles in physiological condition in various cells. The roles of these purinergic receptors are discussed in terms of Ca^{2+} dynamics in mouse brown adipocytes.

Method

Cell isolation

Preparations and solutions are essentially similar to those in the previous study [10]. In brief, mice (C57BL/6J supplied from SLC Japan: Male, 4 weeks old) were killed by cervical dislocation after being anesthetized with intraperitoneal pentobarbital administration. All procedures were performed in accordance with the Animal Experimentation Guide lines of Nagoya University of Arts and Sciences. Interscapular brown adipose tissues were isolated. Brown adipocytes were isolated by treatment with collagenase type-2 and DNase-I and cultured for 7 days. Culture medium was composed of D-MEM, 10% fetal bovine serum, penicillin and streptomycin. Adipocytes cultured from mice for 2–7 days were used for imaging of intracellular Ca^{2+} concentration ($[Ca^{2+}]i$).

Imaging with Fura-2 and analyze

Mouse adipocytes were loaded with Fura-2/AM (5 μM) for 40 min at 37°C. Changes in $[Ca^{2+}]i$ were measured from adipocytes with conventional Ca^{2+}-imaging system (CCD camera, C4742–12R, Hamamatsu photonics, Shizuoka, Japan) set on an inverted microscope (ECLIPSE Ti with an objective, 40 x water, numerical aperture 1.15, NIKON, Tokyo, Japan). Fura-2 was excited alternatively at 340 nm (D340, Chroma Technology Corp., Vermont, USA) and 380 nm (D380, Chroma Technology Corp.) using a filter changer (EFC-1, Nikon, Tokyo, Japan) for the inverted microscope. Fura-2 fluorescence was recorded through a band-pass filter (D535/30, Chroma Technology Corp.). Fluorescence intensity was averaged over the contour of each cell by software (Aquacosmos, Hamamatsu photonics) in the experiments of single cell culture, while it was averaged over the area containing several numbers of cells in the experiments of adipose tissue. The ratio of fluorescence excited at 340 nm to that at 380 nm, F340/380, was converted to a $[Ca^{2+}]i$ value using the dissociation constant of 145 nM, the ratio of the maximum F340/F380 to the minimum (14.1) and the fluorescence ratio of the free to the Ca-bound form (9.59).

RNA isolation and RT-PCR

Total RNA was extracted with the RNeasy Lipid Tissue mini kit (Qiagen, Germany) from brown adipose tissues isolated from 4 weeks mice under anesthesia and homogenized at 20,000 rpm (Ultra-Turrax T-25). After incubation at room temperature for 5 min and vortexed with addition of chloroform (0.2 ml homogenate /ml QIAzol, Qiagen, Germany) for 15 sec, samples were centrifuged at 12,000 rpm and 4°C for 15 min. The upper aqueous phase of centrifuges was mixed with 70% ethanol, incubated at room temperature for 10 min, and centrifuged in RNeasy Min Spin Column at 20 °C and 12,000 rpm for 15 sec, adsorbing RNA to the silica gel membrane. After addition of 350 μl RW1 buffer, the column was recentrifuged at 20 °C and 12,000 rpm for 15 sec. 10 μl DNase I and RDD buffer were added to the silica gel membrane of the column. The column was washed with 350 μl RW1 buffer with centrifugation at 20 °C and 12,000 rpm for 15 sec, then washed twice with 500 μl PRE buffer with centrifugation at 20°C and 12,000 rpm for 15 sec and 2 min, and finally centrifuged at 15,000 rpm for 1 min for drying the silica gel membrane. For extraction of RNA, 20 μl RNase free water was added to the silica gel membrane, then left for 2 min and finally

centrifuged at 15,000 rpm and 20°C for 1 min. After repetition of this procedure twice, the collected RNA in aqua solution was stored at -80°C.

Reverse transcription was performed with the First-Strand cDNA Synthesis kit (Applied Biosystems, Inc., California, USA). cDNA was stored at -20°C and subjected to the standard RT-PCR. For the standard RT-PCR, 0.5 μl First Strand cDNA reaction mixture was added to a 49.5 μl PCR reaction mixture consisting of 0.5 μM of each primer, PCR buffer (provided in the kit), 2 mM $MgCl_2$, 200 μM each dNTP mix, and 1.25 units Taq DNA polymerase. The sense and antisense oligonucleotide primers specific for different purinergic receptor subtypes are listed in Table 1. Each fragment of cDNA was amplified in a DNA thermal cycler (model 2400 GeneAmp PCR system; California, USA). The mixture was first denatured at 94°C for 2 min and then cycles of denaturation at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 30 sec were repeated for 40 cycles. This was followed by a final extension at 72°C for 2 min to ensure complete product extension. The PCR products were electrophoresed through a 2% agarose gel, and stained with ethidium bromide.

Protein isolation and western blot analysis

Brown adipose tissues isolated from mice were homogenized in 20 mM Tris buffer (pH 7.4) with 1 mM EGTA containing protease inhibitors (mix tablet; Roche Diagnostics, Indiana, USA) with Potter-Elverhjem homogenizer and then centrifuged at 4°C and 25,000 rpm (50,000 g) for 60 min. After removing the supernatant including lipid fraction, the precipitate was placed in 1 ml TE buffer. Precipitated fractions were denatured and separated by SDS-PAGE calibrated with prestained protein molecular weight markers (Bio-Rad, California, USA). Separated proteins were transferred to nitrocellulose membranes (Hybond-C, Bio-Rad). After being blocked with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 (Sigma Aldrich, Missouri, USA), membranes were stained with affinity-purified polyclonal antibodies (1/500) specific for $P2X_1$ and $P2X_7$ at 20°C for 1 hour and then at 4°C over night. After washing the primary antibody, membranes were stained with anti-rabbit horseradish peroxidase-conjugated IgG (1/10,000) for 1 hr. An enhanced luminol-linked chemical luminescence detection system (ECL; Amersham, New Jersey, USA) was used to detect each protein.

Statistical analyses

Statistical data were shown by the mean ± S.E. of the mean. Significant differences among groups were assessed by Student's t test.

Drugs

Collagenase type-2 (class 2) was obtained from Worthington Biochemical (New Jersey, USA), DNase-I from Roche Diagnostics (Indiana, USA). D-MEM (low glucose type, 11885–084), penicillin and streptomycin were from Applied Biosystems, Inc. (California, USA). Fetal bovine serum was from Thermo Electron (Melbourne, Australia). ATP, UTP and 2MeSATP were from Sigma Aldrich (Missouri, USA). BRL37344 was from Tocris Cookson (Northpoint, UK). Fura-2/AM was from Molecular Probes, Inc. (Oregon, USA). $P2X_1$ and $P2X_7$ antibodies were from Alomone (Israel).

Solutions

The composition of normal Krebs Ringer solution was (mM): NaCl, 150; KCl, 5; $CaC1_2$, 2.0; $MgSO_4$, 1.0; HEPES, 20.0; glucose, 5 (pH 7.4 adjusted with NaOH). A nominally Ca^{2+} -free Krebs solution was made by subtracting 5 mM $CaC1_2$ from Krebs solution, in which total $CaC1_2$ was replaced with 5 mM NaCl. Drugs were applied by changing a perfusing solution to the solution containing a drug(s).

Results

Ca^{2+} imaging

The application of ATP elicited Ca^{2+} responses in mouse adipocytes. The averaged intracellular calcium concentration of brown adipocytes was increased

in response to the application of 10 μM ATP (127.2 ± 36.9 nM, n=43) (Fig. 1A). Successive applications of ATP decreased the response magnitude slightly, suggesting that responding purinergic receptors were desensitized during stimulation [15]. The irrigation with the nominally Ca^{2+}-free solution suppressed the magnitude of Ca^{2+} responses (62.6 ± 22.9 nM, n=30) (Fig. 1B). This result indicates that the magnitude of Ca^{2+} responses consist of two components, Ca^{2+} influx from extracellular space and Ca^{2+} release from intracellular stores. A variety of ATP agonists also elicited Ca^{2+} responses. That is, the Ca^{2+} response to 1 μM 2-methylthio ATP (2MeSATP) (66.2 ± 8.7 nM, n=73), a $P2Y_1$ and $P2Y_{11}$ agonist [13], to 100 μM UTP (86.9 ± 9.8 nM, n=62), P2Y subtype agonist [13] and to 10 μM 3_-O-(4-benzoyl) benzoyl ATP (BzATP) (74.0 ± 21.0 nM, n=62), a $P2X_1$ and $P2X_7$ subtype agonist [16] suggest the functional expression of the respective receptor subtypes among brown adipocytes in mice (Fig. 1C-1E).

RT-PCR

The pharmacological classification of purinergic receptor subtypes is difficult because of the lack of

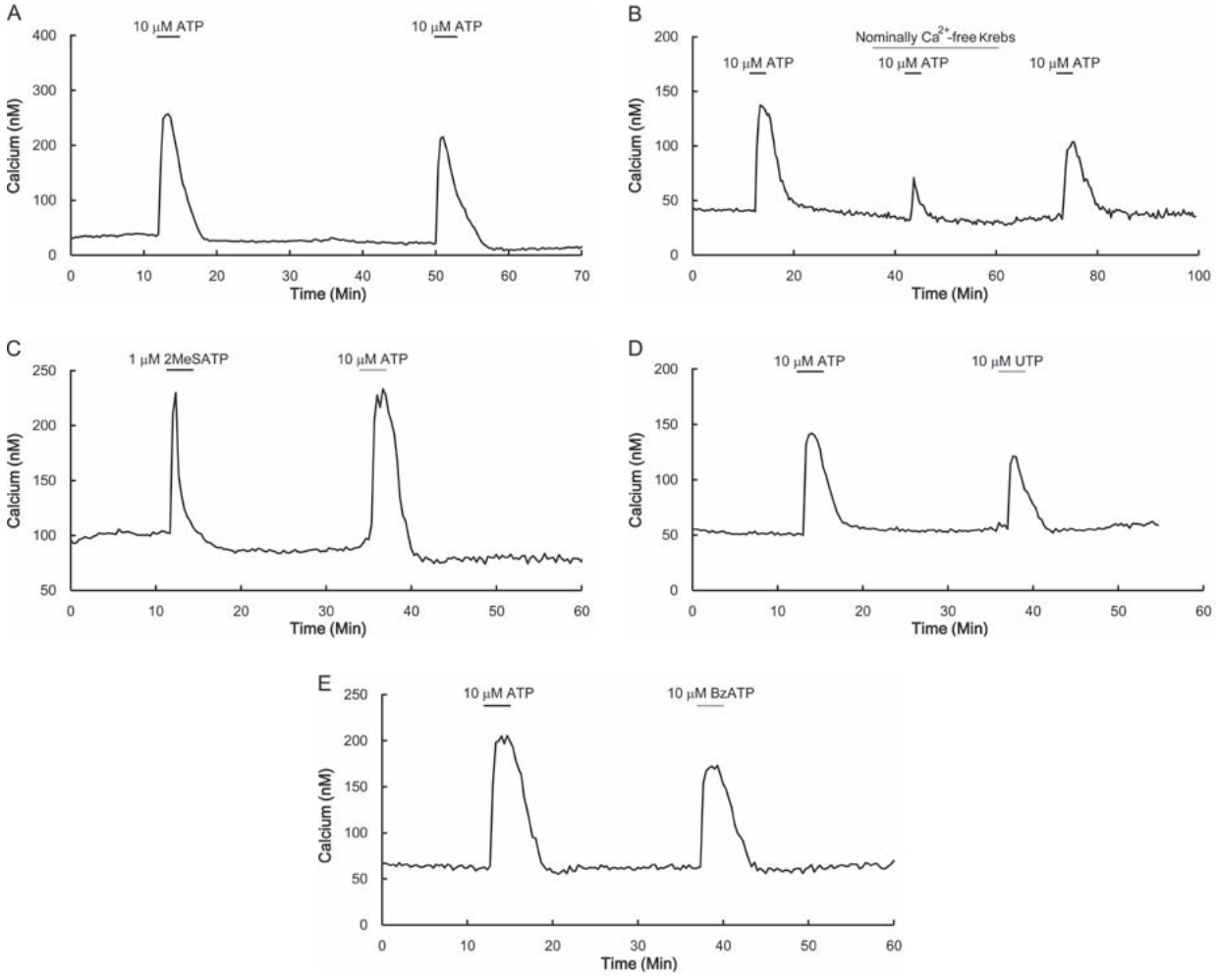

Figure 1

Ca^{2+} responses in mouse brown adipocytes by the effects of ATP and ATP analogs.

(A) Effects of successive application of ATP. ATP (10 μM) was applied during the period indicated by a black horizontal bar in Krebs solution. (B) Effects of Nominally Ca^{2+}-free Krebs solution on ATP-induced Ca^{2+} response. ATP (10 μM) was applied during the period indicated by a black horizontal bar in Krebs solution. A nominally Ca^{2+}-free Krebs solution was superfused to the bath for the period indicated by a grey horizontal bar. (C) Effects of application of 2MeSATP (a $P2Y_1$ and $P2Y_{11}$ agonist). 2MeSATP (1 μM) and ATP (10 μM) were applied during the period indicated by a black and grey horizontal bar in each. (D) Effects of application of UTP (a P2Y agonist). ATP (10 μM) and UTP (10 μM) were applied during the period indicated by a black and grey horizontal bar in each. (E) Effects of successive application of BzATP (a $P2X_1$ and $P2X_7$ agonist). ATP (10 μM) and BzATP (10 μM) were applied during the period indicated by a black and grey horizontal bar in each.

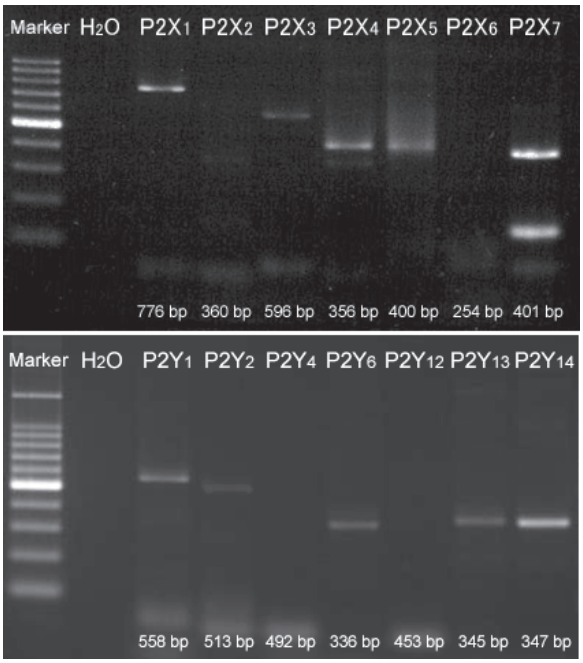

Figure 2

PCR-amplified products from cDNA reverse-transcribed from mRNA isolated from brown adipocytes

Oligonucleotide primers specific for 14 different purinergic receptor subtypes were ruin in separate reactions and the products run on agarose gel electrophoresis. The expected product size of each reaction is listed below each lane. Marker consists of 10 fragments between 100 bp and 1 kbp in multiples of 100 bp from the bottom. H_2O used in RT-PCR experiments is also examined as a negative control.

specific ligands for respective receptor subtypes. Instead, we classified them, except $P2Y_{11}$, by taking advantage of RT-PCR on brown adipocytes excised from brown fat tissue care in eliminating other tissue such as muscle and neuron. RT-PCR reactions with primers for purinergic receptor subtypes yielded cDNA fragments of the correct size for 10 out of 14 tested primers sets (Fig. 2 and Table 1). These cDNA fragments showed that expressed purinergic receptor subtypes were $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{13}$ and $P2Y_{14}$. These results suggested that mRNAs for $P2X_1$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{13}$ and $P2Y_{14}$ subtypes occurred in mouse brown adipocytes. The detection of $P2X_1$ and $P2X_7$ subtypes mRNA in mouse brown adipocytes agreed with their Ca^{2+} responses to BzATP.

Western blotting

Western blotting revealed molecules involved in

Table 1. Summary of gene-specific primer sets employed for varying purinergic receptors

Subtypes	Primer sequence 5'-sense-3' 5'-antisense-3'	Expected product size
$P2X_1$	5'-CATTGTGCAGAGAACCCAGAA-3' 5'-ATGTCCTCCGCATACTTGAAC-3'	776 bp
$P2X_2$	5'-ACGTTCATGAACAAAAACAAG-3' 5'-TCAAAGTTGGGCCAAACCTTTGG-3'	360 bp
$P2X_3$	5'-CTGTATATCAGACTTCTTCACCTACGA-3' 5'-TTATGTCCTTGTCGGTGAGGTTAG-3'	596 bp
$P2X_4$	5'-GAGAATGACGCTGGTGTGCC-3' 5'-TTGGTGAGTGTGCGTTGCTC-3'	356 bp
$P2X_5$	5'-TCCACCAATCTCTACTGC-3' 5'-CCAGGTCACAGAAGAAAG-3'	400 bp
$P2X_6$	5'-TACGTACTAACAGACGCA-3' 5'-ATATCAGGGTTCTTTGGG-3'	254 bp
$P2X_7$	5'-AAGTCTCTGCCTGGTGTC-3' 5'-GGCATATCTGAAGTTGTAGC-3'	401 bp
$P2Y_1$	5'-TGGCGTGGTGTACCCTCTCAAGTC-3' 5'-CGGGACAGTCTCCTTCTGAATGTA-3'	558 bp
$P2Y_2$	5'-CTGGAACCCTGGAATAGCAC-3' 5'-GCTGGTGGTGACGAAGTAGA-3'	513 bp
$P2Y_4$	5'-AGCCCAAGTTCTGGAGATGGTG-3' 5'-GGTGGTTCCATTGGCATTGG-3'	492 bp
$P2Y_6$	5'-CACCTGTGATTTGGCAACTG-3' 5'-TCTTGGCAAATGGATGTGAA-3'	336 bp
$P2Y_{12}$	5'-CACCTCAGCCAATACCACCT-3' 5'-AACATGAAGGCCCAGATGAC-3'	453 bp
$P2Y_{13}$	5'-GAAGAGAGGCACATGCAACA-3' 5'-TTACTAATGCCAGGCCAACC-3'	345 bp
$P2Y_{14}$	5'-CAGTGCATGGAGCTCAAAAA-3' 5'-GCAGCCGAGAGTAGCAGAGT-3'	347 bp

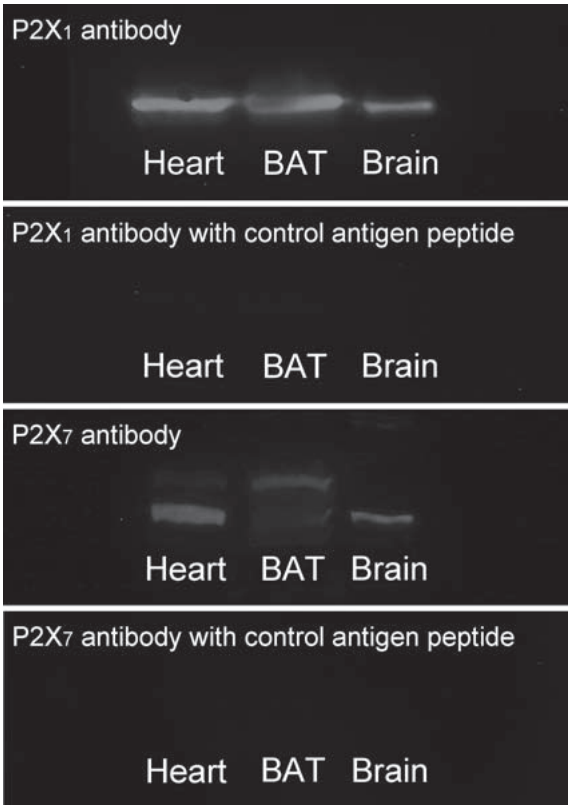

Figure 3

Western blotting of mouse purinergic signaling proteins subtype of $P2X_1$ and $P2X_7$.

Western blotting analysis of $P2X_1$ and $P2X_7$. We detected $P2X_1$ and $P2X_7$ receptor proteins in mouse heart, brown adipocyte tissue (BAT) and brain. Control antigen peptide was used as a negative control.

$P2X_1$ and $P2X_7$ purinergic receptors in mouse brown adipocytes (Fig. 3). To determine these bands are specific or not, blocking experiments using control antigen peptide were performed. Before proceeding with the staining protocol, the antibody specific for $P2X_1$ or $P2X_7$ protein incubated with an excess of control antigen peptide that corresponds to the epitope recognized by the antibody. The antibody that was bound to the control antigen peptide was not available to bind to the epitope present in the protein of $P2X_1$ or $P2X_7$ receptors on the Western blotting. In this study, control antigen peptide inactivate the antibody and the tissue showed no staining. These results suggested that proteins for $P2X_1$ and $P2X_7$ subtypes occurred in mouse brown adipocytes.

Discussion

The present study shows for the first time that brown adipocytes functionally expressed $P2X_1$ and $P2X_7$ in mice. In Ca^{2+}-imaging study, nominally Ca^{2+}-free Krebs decreased Ca^{2+} responses partially, not completely, elicited by the application of ATP. This result indicates that mouse brown adipocytes expressed both P2X and P2Y receptors. RT-PCR studies suggested the expression of $P2X_3$, $P2X_4$, $P2X_5$, $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{13}$ and $P2Y_{14}$ in addition to $P2X_1$ and $P2X_7$ and Ca^{2+}-imaging studies showed responses due to the application of UTP, a P2Y agonist, and 2MeSATP, a $P2Y_1$ and $P2Y_{11}$ agonist. These results suggests the expression of other subtypes of purinergic receptor proteins, especially P2Y subtype receptor. Further studies are needed to confirm their expressions.

The present RT-PCR experiments showed no expression of $P2Y_4$ receptor subtypes on mouse brown adipocytes. Omatsu-Kanbe et al. reported that rat brown adipocytes expressed mRNA of $P2Y_4$ receptor subtypes in using RT-PCR technique [5]. This discrepancy may result from the differences among rats and mice.

ATP is easy to hydrolyze into adenosine by endogenous ATPase, and adenosine elicits Ca^{2+} responses [13]. However, we believe that ATP, not adenosine, produced the present results, because in the measurement condition, brown adipocytes were directly exposed to running solutions of either the

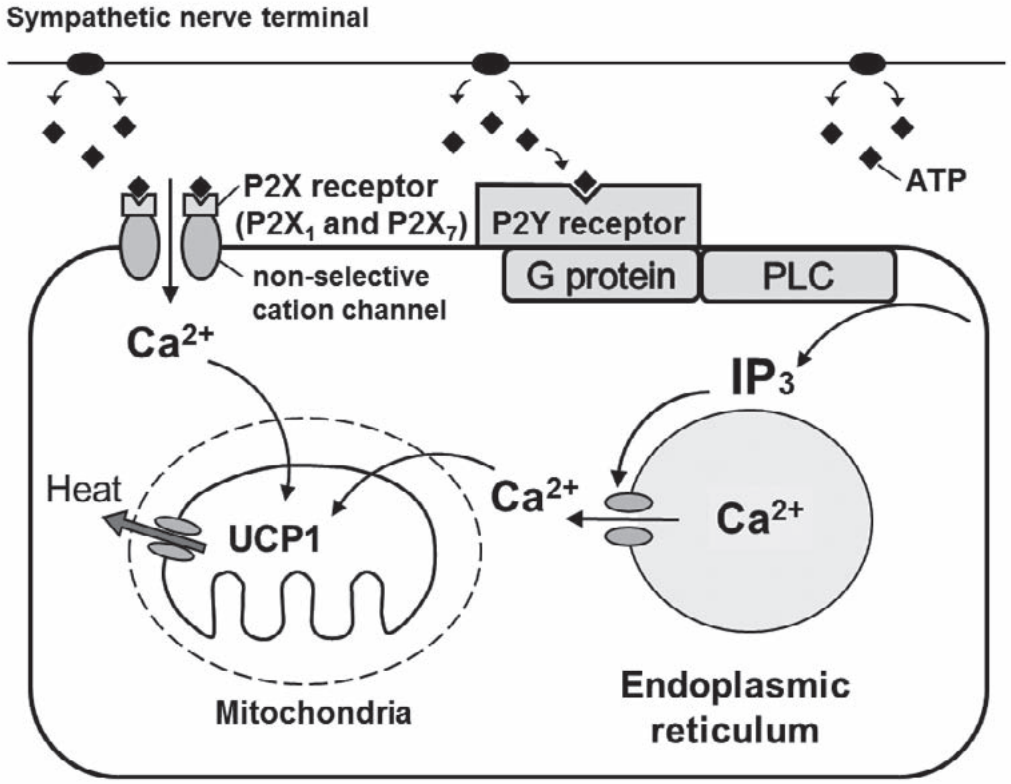

Figure 4. Ca^{2+} signaling pathway by the activation of purinergic receptor.

physiological saline or stimulating ATP solution, which washed away adenosine produced by the hydrolysis and continuously supplied ATP. Also, the present results showed that both ATP and non-hydrolyzed ATP agonists elicited Ca^{2+} responses. We thus conclude that ATP was a primary agonist for the present results, though we do not exclude the functional expression of receptors for adenosine on brown adipocytes.

In response to cold exposure, two types of adrenergic receptor are activated by noradrenaline released from sympathetic nerve. Activation of β_3-adrenoreceptor activates hydrolysis of triglycerides via production of cyclic AMP and activates hormone-dependent lipase in brown adipocytes. This enhances the TCA cycle and the electron transfer chains at the inner mitochondrial membrane, while free fatty acids produced activate a type of uncoupling proteins 1, (UCP1), which uncouples oxidative phosphorylation, leading to heat production. Simultaneously, long-lasting Ca^{2+} rises are occurred [10]. On the other hand, activation of α_{1A}-adrenoceptor elicits a large phasic rise in $[Ca^{2+}]i$ via Ca^{2+} release from the endoplasmic reticulum (ER) through inositol triphosphate (IP_3) receptors. It is known that these intracellular Ca^{2+} enhances thermogenesis and oxygen consumption in brown adipocyte [17]. In this study, we showed Ca^{2+} responses by the application of ATP and ATP analogs. Figure 4 illustrates the likely mechanism for Ca^{2+} responses by the activation of purinergic receptors. Mouse brown adipocytes expressed both P2X and P2Y subtypes of purinergic receptor. P2X receptors are ionotropic receptors and P2Y receptors are G protein-coupled receptors. ATP released from sympathetic nerves activates $P2X_1$ and $P2X_7$ subtypes of purinergic receptor. Activation of $P2X_1$ and $P2X_7$ opens non-selective cation channels which are able to permeate Ca^{2+} and causes passive Ca^{2+} influx from extracellular space. Simultaneously, ATP may activate P2Y receptors. Activation of P2Y receptor leads to production of IP_3 by the activation of phospholipase C (PLC) via the activation of G protein. IP_3 elicits a large phasic rise in $[Ca^{2+}]i$ via Ca^{2+} release from the ER through IP_3 receptors. We assume that these Ca^{2+} elicited by the ATP released from sympathetic nerve terminals also contribute to thermogenesis and oxygen consumption in mouse brown adipocyte.

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《原著》

マウス褐色脂肪細胞における ATP 受容体の発現

早戸亮太郎

要旨

褐色脂肪細胞は熱産生を専門に行う器官である。褐色脂肪細胞は、寒冷曝露により交感神経から放出されたノルアドレナリンを受容し、中性脂肪から遊離脂肪酸を生成する。この遊離脂肪酸がもつエネルギーを熱に変換する事で熱産生を行っている。その際、褐色脂肪細胞内では Ca^{2+} 濃度が上昇する。この Ca^{2+} は褐色脂肪細胞での熱産生量および酸素消費量を増大する事が知られている。交感神経はノルアドレナリンと同時に ATP を神経伝達物質として放出する。これまでの研究により、ラット褐色脂肪細胞ではこの ATP が細胞内 Ca^{2+} 濃度上昇を引き起こす事が報告されている。ラット褐色脂肪細胞に発現する ATP 受容体サブタイプの候補はいくつか挙げられているが、その受容体がどのサブタイプなのか、タンパクレベルで確認された例はない。また、マウス褐色脂肪細胞に発現する ATP 受容体について調べた結果はまだ報告されていない。

今回の研究により、マウス褐色脂肪細胞が $P2X_1$ および $P2X_7$ サブタイプ受容体を機能的に発現する事が示された。これによりマウス褐色脂肪細胞では、交感神経から放出された ATP を $P2X_1$ および $P2X_7$ 受容体を用いて受容する事で細胞内 Ca^{2+} 濃度を上昇させ、熱産生を促進している可能性が考えられる。

キーワード：褐色脂肪細胞、ATP、受容体、細胞内 Ca^{2+} 動態

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