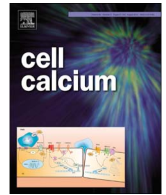
Cell Calcium 71 (2018) 1–14

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca



Coupling of P2Y receptors to Ca^{2+} mobilization in mesenchymal stromal cells from the human adipose tissue

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ARTICLE INFO

Keywords:
Purinoreceptors
Calcium-induced calcium release
IP_3 receptors
Mesenchymal stromal cells
Adipose tissue

ABSTRACT

The purinergic transduction was examined in mesenchymal stromal cells (MSCs) from the human adipose tissue, and several nucleotides, including ATP, UTP, and ADP, were found to mobilize cytosolic Ca^{2+}. Transcripts for multiple purinoreceptors were detected in MSC preparations, including A_1, A_{2A}, A_{2B}, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$, $P2Y_{14}$, $P2X_2$, $P2X_4$, and $P2X_7$. Cellular responses to nucleotides were insignificantly sensitive to bath Ca^{2+}, pointing at a minor contribution of Ca^{2+} entry, and were suppressed by U73122 and 2-APB, implicating the phosphoinositide cascade in coupling P2Y receptors to Ca^{2+} release. While individual cells were sensitive to several P2Y agonists, responsiveness to a given nucleotide varied from cell to cell, suggesting that particular MSCs could employ different sets of purinoreceptors. Caged Ca^{2+} stimulated Ca^{2+}-induced Ca^{2+} release (CICR) that was mediated largely by IP_3 receptors, and resultant Ca^{2+} transients were similar to nucleotide responses by magnitude and kinetics. A variety of findings hinted at CICR to be a universal mechanism that finalizes Ca^{2+} signaling initiated by agonists in MSCs. Individual MSCs responded to nucleotides in an all-or-nothing manner. Presumably just CICR provided invariant Ca^{2+} responses observed in MSCs at different nucleotide concentrations. The effects of isoform specific agonists and antagonists suggested that both $P2Y_1$ and $P2Y_{13}$ were obligatory for ADP responses, while $P2Y_4$ and $P2Y_{11}$ served as primary UTP and ATP receptors, respectively. Extracellular NAD^+ stimulated Ca^{2+} signaling in each ATP-responsive MSC by involving $P2Y_{11}$. The overall data indicate that extracellular nucleotides and NAD^+ can serve as autocrine/paracrine factors regulating MSC functions.

1. Introduction

Cells-to-cell communications and autocrine regulations are mediated by a variety of signaling molecules that are released into and diffuse within the extracellular space to hit on multiple cell surface receptors coupled to intracellular signaling or regulatory processes. Among them, purines (ATP, ADP, β-NAD, ADPR, cADPR, and adenosine) and pyrimidines (UTP and UDP) are released by cells or produced extracellularly by ecto-nucleotidases in virtually all tissues [1–3]. The responsiveness to purines and pyrimidines is widespread among eukaryotic cells, which express numerous purinoreceptors from the P1 and P2 families. The P1 subgroup includes four G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3) recognizing adenosine as an endogenous agonist [4], while the more diverse P2 family is composed of ionotropic P2X and metabotropic P2Y receptors. P2X receptors are cationic channels specifically gated by ATP, while P2Y receptors are activated by multiple purine and pyrimidine nucleotides or by sugar-nucleotides and couple to intracellular second-messenger pathways by heteromeric G proteins [1,5]. In mammals, seven genes encode P2X subunits ($P2X_{1-7}$) that can form homo- and heterotrimeric cation channels with noticeable Ca^{2+} permeability [6,7]. Eight purinergic GPCRs ($P2Y_{1,2,4,6,11,12,13,14}$) have been identified and shown to exhibit distinctive specificities to nucleotides, depending on species [1,5]. For human isoforms, ATP serves as a full agonist for $P2Y_2$ and $P2Y_{11}$ but antagonizes $P2Y_4$, ADP is recognized by $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$, UTP is a full agonist for $P2Y_2$ and $P2Y_4$, UDP effectively stimulates $P2Y_6$ and $P2Y_{14}$ that also detects UDP-glucose and UDP-galactose. The $P2Y_{1,2,4,6,11}$ subtypes are canonically coupled by G_q/G_{11} to the phosphoinositide cascade and Ca^{2+} mobilization, whereas $P2Y_{12,13,14}$ control cAMP production by inhibiting adenylyl cyclase (AC) through G_i/G_o. In addition to G_q/G_{11}, $P2Y_2$ can couple to G_i, liberating βγ-complex that activates phospholipase beta, while the unique capability of $P2Y_{11}$

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https://doi.org/10.1016/j.ceca.2017.11.001
Received 19 July 2017; Received in revised form 25 October 2017; Accepted 6 November 2017
Available online 07 November 2017
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is to stimulate G_s [1]. P2Y agonists and antagonists are capable of eliciting a large variety of biological effects because apart from ubiquitous coupling to phospholipase C (PLC) and AC, P2Y receptors can also engage such effectors as MAP, PI3, Akt, and PKC kinases, small G-proteins, NO synthase, transactivation of growth factor receptors, and others [8–11].

Mesenchymal stromal cells (MSCs) are described as a heterogeneous cellular pool that includes immature cells responsible for the replenishment of supportive and connective tissues due to their capability to maintain self-renewal and multipotent differentiation [12–14]. The unique biologic properties of these cells attract sufficient interest in the fields of regenerative medicine and immunotherapy [15]. Damaged tissues are an abundant source of extracellular ATP that may be converted by extracellular nucleotidases to ADP and eventually to adenosine [3]. Meanwhile, purinergic agonists acting via multiple purinoreceptors have been documented as an important factor determining MSC fate [16–20]. In particular, ATP serves both as an adipogenic regulator and an osteogenic factor, while its downstream product adenosine switches off adipogenic differentiation and promotes osteogenesis [21,22]. Therefore, MSCs should be exposed to and regulated by nucleotides and adenosine when these cells migrate ***in vivo*** or are transplanted ***ex vivo*** into an injured tissue.

Among MSCs from different sources, adenosine receptors were identified first in human bone marrow-derived MSCs (BM-MSCs) by Evans and co-authors [23], which reported on expression of all four isoforms and posed adenosine as an important regulator of osteoclastogenesis and secretion of the inflammatory cytokine IL-6. Subsequent works implicated adenosine and A_{2B} receptors in regulating differentiation of human and mice BM-MSCs into osteoblasts or adipocytes [24–26]. To maintain spontaneous Ca^{2+} oscillations, BM-MSCs release ATP via hemichannels, providing autocrine stimulation of $P2Y_1$ receptors [27]. Reportedly, human adipose tissue-derived MSCs express most of the 15 P2 receptor subtypes, including $P2X_3$-$P2X_7$ and all P2Y isoforms [28]. Although importance of P2X receptors for MSC physiology is largely undetermined, it has been speculated that $P2X_5$-$P2X_7$ are involved in osteogenesis [28–30] and migration [31]. Multiple studies implicate P2Y receptors in MSC proliferation ($P2Y_1$, $P2Y_{11}$), osteogenesis ($P2Y_2$, $P2Y_{13}$), and adipogenesis ($P2Y_1$, $P2Y_4$, $P2Y_{11}$, $P2Y_{14}$) [21,28,32–34].

Although growing evidence points at the purinergic signaling system as an essential part of a regulatory circuit that controls homeostasis and functionality of MSCs, coupling of P1 and P2 receptors to intracellular signaling pathways in these cells is detailed insufficiently. Being intrinsic to a MSC population [35,36], molecular and functional heterogeneity significantly complicates the analysis of intracellular signaling in MSCs at the level of individual cells. Here we employed the inhibitory analysis, Ca^{2+} imaging, and Ca^{2+} uncaging to examine Ca^{2+} signaling initiated by P2Y agonists in MSCs derived from the human adipose tissue. By using isoform-specific agonists and antagonists, we tried to identify P2Y subtypes coupled to Ca^{2+} mobilization in individual MSCs. While at the level of MSC population, we revealed expression of multiple P2Y isoforms, including $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{13}$, and $P2Y_{14}$, MSC responsiveness to natural and synthetic P2Y agonists markedly varied from cell to cell. Perhaps, an individual MSC employs a specific set of P2Y receptors coupled to Ca^{2+} mobilization.

2. Materials and methods

2.1. Cell isolation and culturing

In this study, all procedures that involved human participants were performed in accordance with the ethical standards approved by the Bioethical Committee of Faculty of Basic Medicine at Lomonosov Moscow State University based on the 1964 Helsinki declaration and its later amendments. The study involved 15 healthy (not suffered from infectious or systemic diseases and malignancies) individuals from 21 to 55 years old, and informed consent was obtained from each participant.

MSCs were isolated from subcutaneous fat tissue of healthy donors of 21–55 years of age using enzymatic digestion as previously described [37]. Briefly, the adipose tissue was extensively washed with 2 volumes of Hank's Balanced Salt Solution (HBSS) containing 5% antibiotic/antimycotic solution (10,000 units of penicillin, 10,000 μg of streptomycin, and 25 μg of Amphotericin B per mL) (HyClone), fragmented, and then digested at 37 °C for 1 h in the presence of collagenase (200 U/ml, Sigma-Aldrich) and dispase (10 U/ml, BD Biosciences). Enzymatic activity was neutralized by adding an equal volume of culture medium (Advance Stem basal medium for human undifferentiated mesenchymal stem cells containing 10% of Advance stem cell growth supplement (CGS), 1% antibiotic/antimycotic solution (HyClone)) and centrifuged at 200 g for 10 min. This led to the sedimentation of diverse cells, including MSCs, macrophages, lymphocytes, and erythrocytes, unlike adipocytes that remained floating. After removal of supernatant, a lysis solution (154 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) was added to a cell pellet to lyse erythrocytes, and cell suspension was centrifuged at 200 g for 10 min. Sedimented cells were re-suspended in the MSC culture medium and filtered through a 100 μm nylon cell strainer (BD Biosciences). As indicated by flow cytometry [38], after isolation and overnight pre-plating, the obtained cell population contained not only MSC cells that basically represented the most abundant subgroup but also admixed macrophages and lymphocytes. The two last cell subgroups were dramatically depleted by culturing for a week in the MSC culture medium and humidified atmosphere (5% CO_2) at 37 °C. The obtained MSC population was maintained at a sub-confluent level (~80% confluency) and passaged using HyQTase (HyClone). By using the methodology described previously [39], cultured cells were demonstrated to differentiate into the osteogenic, chondrogenic and adipogenic directions, the finding confirming their multipotency. In experiments, MSCs of the second to fourth passages were usually used.

2.2. RT-PCR

Total RNA was extracted from a sample containing 10^5-10^6 MSCs by using the RNeasy mini kit (Qiagen). Isolated RNA was treated with DNase I (Ambion) and reverse-transcribed with PrimeScript reverse transcriptase (Takara) and random hexamer primers, following manufacturer's instruction. Obtained cDNA served as a template for PCR with gene-specific primers that were designed to recognize all splice variants of human purinoreceptor genes and four genes encoding cell surface markers for MSCs, including CD73, CD90, CD105, and MCAM (Table 1).

2.3. Preparation of cells for Ca^{2+} imaging

Before assaying with Ca^{2+} imaging, cells were maintained in a 12-socket plate for 12 h in the medium described above but without antibiotics. For isolation, cells cultured in a 1 ml socket were rinsed twice with the Versen solution (Sigma-Aldrich) that was then substituted for 200 μl HyQTase solution (HyClone) for 3–5 min. The enzymatic treatment was terminated by the addition of a 0.8 ml culture medium to a socket. Next, cells were re-suspended, put into a tube, and centrifuged at 50 g for 45 s for sedimentation. Isolated cells were collected by a plastic pipette and plated onto a photometric chamber of nearly 150 μl volume. The last was a disposable coverslip (Menzel-Glaser) with attached ellipsoidal resin wall. The chamber bottom was coated with Cell-Tak (BD Biosciences), enabling strong cell adhesion. Attached cells were then loaded with dyes for 20 min at room temperature (23–25°Ñ) by adding Fluo-4AM (4 μM) or Fluo-4AM (4 μM) + NP-EGTA-AM (4 μM) and Pluronic (0.02%) (all from Molecular Probes) to a bath solution. Loaded cells were rinsed with the bath solution for several times. and kept at 4 °C for 1 hour prior to recordings. Generally, incubation of MSCs at low temperature stabilized intracellular Ca^{2+} and

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